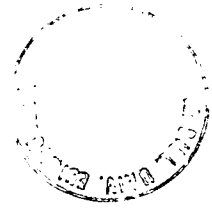
The Role of DEAD box motifs in the restriction of DNA by *Eco*KI

Julie Lynette Webb

PhD
The University of Edinburgh
1998



Abstract

*Eco*KI cuts or modifies DNA according to the methylation states of specific adenine residues in its target recognition site. If these residues are unmethylated the DNA is cut, often thousands of base pairs away from the recognition site. Communication between *Eco*KI bound to the recognition site and the cleavage site occurs by DNA translocation, which is driven by ATP hydrolysis.

The amino acid sequence of the subunit required for restriction contains seven motifs that are conserved in the DEAD box family of proteins. This family is a sub-group of the superfamily of DNA and RNA helicases. Previous studies on DEAD box proteins have found these motifs are involved in the ATPase and helicase activities of these proteins.

To assess the importance of the DEAD box motifs in the restriction of DNA by *Eco*KI, amino acid residues in each of the seven motifs were changed and the effects of these substitutions on restriction phenotype were investigated. Eight proteins, each containing a different amino acid substitution were purified and the DNA binding abilities, nuclease activities and ATPase activities of the proteins were studied.

All changes had an effect on restriction except those changes in motif IV. Motif IV was defined prior to the discovery of a frame-shift in the *hsdR* DNA sequence and these results suggest motif IV has been incorrectly identified. An A619G substitution in motif III slightly impaired restriction, but other substitutions at this position (A619D and A619V) abolished restriction. All other changes prevented any DNA restriction. The nuclease assays with the purified proteins confirmed the *in vivo* results. None of the available evidence indicates that the amino acid substitutions prevent interaction with ATP. The protein with a D577H substitution in motif II showed a reduced DNA binding ability, none of the other changes investigated affected DNA binding. The amino acid substitutions A619G and F730S, which showed some *in vivo* DNA restriction activity, showed wild-type levels of ATPase activity. ATPase activity was not detected with proteins containing changes that abolished restriction, but was observed for the protein with a D502Y substitution in

motif Ia, which showed a reduced but significant level of ATPase activity.

These results show that the sequences in HsdR identified as DEAD box motifs have an important role in restriction by *Eco*KI, particularly in the ATPase activity predicted to be necessary for the translocation of DNA that precedes the endonucleolytic activity of the enzyme.

Declaration

I composed this thesis myself and the work described within is my own unless otherwise stated.

Signed,

Julie Lynette Webb

Acknowledgements

I would like to thank Professor Noreen Murray for her excellent supervision and support throughout my PhD. I would also like to acknowledge the BBSRC for sponsoring me. I thank everyone in the Murray group for their help especially; Lynn Powell, Laurie Cooper and David Dryden for their help and advice with the protein techniques. I would also like to thank Noreen and Lynn for their help and support while I was writing this thesis. I thank Gareth King for his valuable help during the first year of my PhD.

I would like to thank all my mates in Edinburgh, who made the last three years enjoyable and stopped me from being lonely. I particularly want to acknowledge Pete, Ed, Laurie, Jill, Catherine and Steve, who helped me keep sane, if not sober. I thank my parents for buying the computer to write my thesis on and my 'little' brother Irv for buying me lagers. I must also thank Grandma and Grandpa for sending me information on Swindon Town, even though it was usually bad news! Most of all I want to thank Scott for his love, support and tolerance during the last three years.

Contents

Methods

Microbial techniques

DNA techniques

Abbreviations

A	adenosine
AdoMet	*S*-adenosyl methionine
ADP	adenosine diphosphate
AMPS	ammonium persulphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
Ci	curie
dATP	deoxyadenosine triphosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	diaminoethanetetraacetic acid
e.o.p.	efficiency of plating
G	guanine
g	relative centrifugal force
kb	kilobase
KDa	kilodalton
M	molar
mA	milli-amp
μF	micro Faraday
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
Pfu	plaque forming units
PVDF	polyvinylidene difluoride

RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSB	single-strand binding protein
T	thymine
TLC	thin layer chromatography
U	units
UV	ultra violet
V	volts
Δ	deletion
Ω	ohm
°C	degree centigrade

Conventions

1) Bacterial genes are written in italics and their protein products start with capital letters e.g. HsdR is the protein product of *hsdR*.
2) Nomenclature for R/M systems is written according to Smith and Nathans, 1973.
3) Restriction and modification phenotypes are described like $r_k^- m_k^+$. Subscript K indicates the system associated with the phenotype. Superscripts indicate the presence or absence of that activity.
4) The host on which a phage was last propagated is indicated after the phage symbol eg. λ.K means the phage was grown on an *Eco*KI-modifying strain, λ.0 means the phage was grown on a strain that lacks a R/M system.

Amino acids

Amino acids	Three-letter symbol	Symbol
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Genetic code

2nd (across) 1st (down)	U	C	A	G	3rd (down)
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Ochre	Opal	A
	Leu	Ser	Amber	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Introduction

Identification of Restriction/Modification (R/M) systems in bacteria

The existence of R/M systems was first detected while investigating the ability of bacteriophage (phage) to propagate on different bacterial strains (Luria and Human, 1952; Bertani and Weigle, 1953). Phage grown on *E. coli* K-12 (λ.K) were found to grow well on *E. coli* C and *E. coli* K-12, but phage grown on *E. coli* C (λ.C) could only grow well on *E. coli* C. The few λ.C phage that grew on *E. coli* K-12 were modified so that they could grow well on *E. coli* K-12 in subsequent generations. This phenomenon was called host-controlled modification.

It was soon found that the restriction in growth of bacteriophage was associated with the degradation of phage DNA (Lederberg, 1957; Dussoix and Arber, 1962; Arber *et al.*, 1963). The mechanism of host-controlled modification was predicted in the early 1960s (Arber and Dussoix 1962; Dussoix and Arber, 1962). A two-enzyme system was proposed (Arber, 1965a), with one enzyme that could recognise a specific site on DNA and introduce double-strand breaks and one enzyme that recognised the same nucleotide sequence but modified this sequence so the DNA was no longer sensitive to restriction.

The modification enzyme was proposed to be a DNA methylase (Arber, 1965a). This was supported by the observation that when λ phage were grown on methionine auxotrophs in the absence of methionine, the progeny phage lacked any modification (Arber, 1965b). The specific N6 methylation of adenine residues was identified and proposed to protect the DNA from restriction (Smith *et al.*, 1972). This mechanism of protection was soon confirmed (Kühnlein and Arber, 1972; Ravetch *et al.*, 1978; Lautenberger *et al.*, 1978).

The first restriction enzymes were isolated from *E. coli* K-12 (*Eco*KI) and *E. coli B* (*Eco*BI), (Meselson and Yuan, 1968; Linn and Arber, 1968; Roulland-Dussoix and Boyer, 1969; Kühnlein *et al.*, 1969). Smith and Wilcox (1970) isolated a restriction enzyme from *Haemophilus influenzae* (*Hin*dII), which was simpler than *Eco*KI or *Eco*BI. As more restriction enzymes were isolated they were grouped into

two types (I and II) according to enzyme complexity, cofactor requirements, position of DNA cleavage and the symmetry of the target sequence. The ATP-dependent R/M systems including *Eco*KI and *Eco*PI, originally identified as type I, were later subdivided into types I and III according to different cofactor requirements, subunit structures and biochemical activities (Kauc and Piekarowicz, 1978).

Roles for R/M systems

One reason for the presence and maintenance of these systems in bacteria, could be to protect the host from invading foreign DNA (Wood, 1966). However this advantage is soon lost as surviving phages are modified and the host would not be protected in subsequent infections. Levin (1986) suggested that a novel restriction system would be advantageous to a bacterial strain establishing itself in a new phage-infested habitat. R/M systems give the bacteria simultaneous protection against different species of phage. The ends of molecules that result from DNA cutting by a restriction endonuclease can promote recombination with homologous chromosomes, allowing the bacteria to generate variability, and also facilitating the acquisition of foreign DNA (Lederberg, 1973; Price and Bickle, 1986; Naussbaum *et al.*, 1992).

Type I R/M systems

Type I R/M genes in *E. coli* map at 98.5 minutes on the *E. coli* chromosome, close to *serB* (Boyer, 1964; Arber and Wauters-Willems, 1970; Glover, 1970; Bullas *et al.*, 1980; Bachmann and Low, 1980). In *E. coli* K-12 the type I R/M genes are flanked on one side by *mrr* and on the other by *mcrBC* (Raleigh, 1992). *mrr* and *mcrBC* also encode restriction systems, but these are activated, not protected, by methylated bases. This 15kb region of the chromosome is called the Immigration Control Region (ICR).

Three genes are involved in host specificity for DNA (*hsd*) genes (Boyer and

Roulland-Dussoix, 1969; Glover, 1970; Hubacek and Glover, 1970): *hsdR* (Restriction), *hsdM* (Modification) and *hsdS* (Specificity). The *hsd* genes are tightly clustered, in the order *hsdR*, *hsdM* and *hsdS* in *E. coli* K-12. The expression of *hsdR* is controlled from one promoter (P_{res}) upstream of *hsdR* and expression of the *hsdM* and *hsdS* genes is regulated by a separate promoter (P_{mod}) (Sain and Murray, 1980; Loenen *et al.*, 1987). Transcription from both promoters occurs in the same direction (Sain and Murray, 1980).

The three genes encode three polypeptides: HsdR (135kDa), HsdM (62kDa) and HsdS (55kDa) (Sain and Murray, 1980). One HsdS and two HsdM subunits form a methylase (Dryden *et al.*, 1993). Two HsdR subunits combine with the methylase to form a bifunctional methylase/endonuclease (Dryden *et al.*, 1997). The existence of two separate promoters allows synthesis of the methylase independent of the endonuclease. On the transfer of *hsd* genes to a new host the methylase is effective before the endonuclease (Prakash-Cheng and Ryu, 1993), this delay in expression of an *in vivo* restriction activity allows unmodified target sites to be methylated before host DNA is destroyed.

The type I R/M systems have been subdivided into the IA, IB, IC and ID families based on subunit complementation, DNA hybridisation and antibody cross-reactivity (Murray *et al.*, 1982; Price *et al.*, 1987; Barcus *et al.*, 1995; Titheradge *et al.*, 1996). The DNA sequences of *hsdM* and *hsdR* are highly conserved within families (Murray *et al.*, 1993) and subunits of enzymes from the same family are interchangeable; a complex of methylase from *Eco*KI and HsdR polypeptides from *Eco*BI will cut DNA with unmethylated *Eco*KI sites. Antibodies against *Eco*KI (IA) cross-react with *Eco*BI (IA), but do not cross-react with *Eco*AI (IB) (Murray *et al.*, 1982). The majority of type IC R/M systems are found on plasmids, which can be readily transferred between *E. coli* and *Salmonella*.

Table 1 shows different examples of each of the four families of type I R/M systems and the DNA sequence recognised.

Family	R/M system	Recognition site	Reference
IA	*Eco*KI	AAC (N_6) GTGC	Kan *et al.*, 1979
	*Eco*BI	TGC (N_8) TGCT	Lautenberger *et al.*, 1978
	*Sty*LTIII	GAG (N_6) RTAYG	Nagaraja *et al.*, 1985
	*Sty*SPI	AAC(N_6) GTRC	Nagaraja *et al.*, 1985
IB	*Eco*AI	GAG (N_7) GTCA	Suri *et al.*, 1984
	*Eco*EI	GAG (N_7) ATGC	Cowan *et al.*, 1989
	*Sty*SKI	CGAT (N_7) GTTA	Thorpe *et al.*, 1997
IC	*Eco*R124I	GAA (N_6) RTCG	Price *et al.*, 1989
	*Eco*DXXI	TCA (N_7) RTTC	Gubler *et al.*, 1992
	*Eco*prrI	CCA (N_7) RTGC	Tyndall *et al.*, 1994
ID	StySBLI	CGA (N_6) TACC	Titheradge *et al.*, 1996
	*Eco*R9I		Barcus *et al.*, 1995
	*Kpn*AI		Lee *et al.*, 1997

Table I. Examples of the four families of type I R/M systems and their recognition sequences, if known.

HsdS determines the specificity of the enzyme (Boyer and Roulland-Dussoix, 1969; Arber and Linn, 1969; Hubacek and Glover, 1970). The *hsdS* gene contains two regions of variable sequence, each variable region encoding a target recognition domain (TRD) that recognises half of the bipartite target sequence (Gough and Murray, 1983; Fuller-Pace *et al.*, 1985; Nagaraja *et al.*, 1985; Gann *et al.*, 1987; Cowan *et al.*, 1989). UV crosslinking showed that the N-terminal TRD of the *Eco*KI

HsdS subunit interacts with the AAC half of the target sequence (Chen *et al.*, 1995). The two TRDs can be switched between HsdS subunits of enzymes in the same family, to create enzymes with new specificities (Fuller-Pace *et al.*, 1984; Gann *et al.*, 1987; Gubler *et al.*, 1992; Thorpe *et al.*, 1997). In the HsdS amino acid sequence there is some sequence similarity in regions other than those encoding the recognition domains. These regions may encode domains that interact with the HsdM subunits (Gough and Murray, 1983; Taylor *et al.*, 1993; Meister *et al.*, 1993; Abadjieva *et al.*, 1994; Cooper *et al.*, 1994).

Two HsdM subunits combine with one HsdS subunit to form an active methylase (Dryden *et al.*, 1993). *S*-adenosyl-methionine (AdoMet) is the methyl donor in the methylation reaction. The HsdM subunits contain two conserved amino acid elements: (D/E/S)XFXGXG involved in binding of AdoMet and (D/N)PP(F/Y) which has a role in catalysis of DNA methylation (Loenen *et al.*, 1987; Willcock *et al.*, 1994). The target adenine residues are a conserved distance of 10-11 bps apart, with N3 of adenine in the minor groove and N6 in the major groove. The target recognition domains of HsdS may recognise the DNA sequence via the major groove with the HsdS subunit spacer covering the intervening minor groove. UV crosslinking experiments and methylation interference studies agree with this suggestion (Chen *et al.*, 1995; Powell and Murray, 1995). Methylation is predicted to occur via a base flipping mechanism where the adenine base is displaced out of the DNA helix into the catalytic pocket of the HsdM subunit (Klimasauskas *et al.*, 1994; Dryden *et al.*, 1995).

As mentioned earlier, a type I R/M enzyme needs to recognise different methylation states of the target recognition site. ATP has an important role in this recognition. *Eco*KI undergoes a series of conformational changes during DNA recognition (Yuan *et al.*, 1975) as shown in Figure 1. *Eco*KI rapidly binds AdoMet and then undergoes a slow allosteric transition to an activated form. This activated complex will then interact with DNA forming an initial complex with a non-specific site until it reaches a recognition site, when it forms a stable recognition complex. ATP enables the enzyme to distinguish the methylation state of the recognition site.

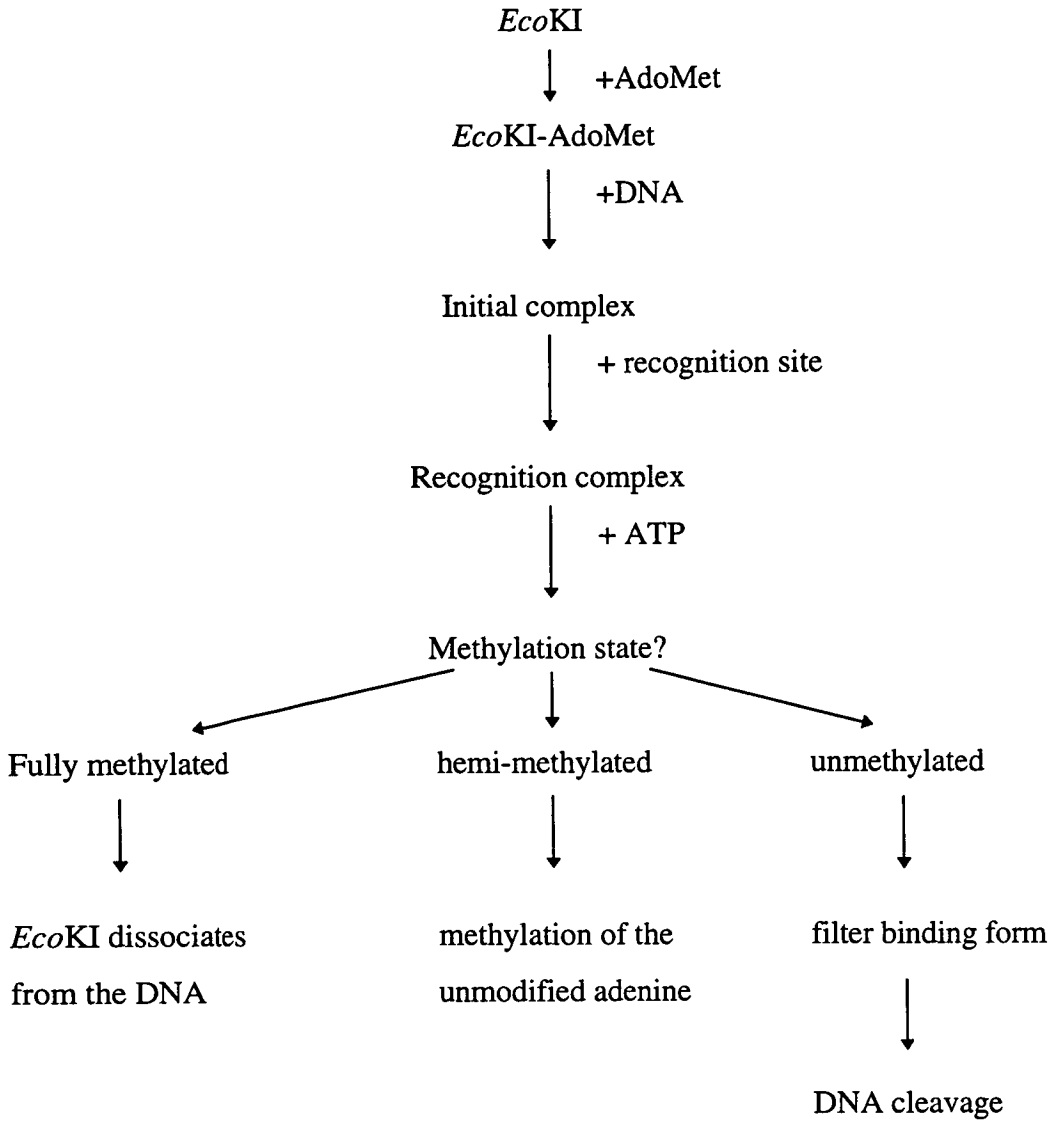


Figure 1. Stages in DNA recognition by *Eco*KI, adapted from Bickle *et al.*, 1978, described in detail in the text.

If both target adenine residues are methylated, ATP reduces the affinity of the enzyme for DNA. If the site is hemi-methylated ATP stimulates the methylation of

the unmodified adenine (Vovis *et al.*, 1974; Burkhardt *et al.*, 1981; Suri *et al.*, 1984). If the site is unmethylated the recognition complex undergoes a transition to a form that can be trapped on filters (Meselson *et al.*, 1972; Bickle *et al.*, 1978). Formation of this complex requires all three cofactors and an unmodified recognition site (Yuan and Meselson, 1970) and always precedes DNA cleavage. Cleavage occurs far from the recognition site, often thousands of base pairs away and is accompanied by large amounts of ATP hydrolysis.

Type II R/M systems

Type II R/M systems consist of two independent enzymes, a methylase and an endonuclease (Roberts and Macelis, 1996; Review: Pingoud and Jeltsch, 1997). The endonuclease requires only Mg^{2+} as a cofactor and the methylase only requires AdoMet. These proteins are encoded by two genes: *res* and *mod*. Expression of each gene is controlled by a separate promoter. Both enzymes recognise the same short palindromic sequence, normally 4-8bp in length. Cleavage occurs symmetrically at a defined position within this site. For this reason they are important as molecular tools and screening has identified over 2500 type II R/M systems.

The methylase, usually a monomer, transfers a methyl group from AdoMet to either a specific adenine or cytosine residue within the recognition sequence. One residue on each DNA strand is methylated to N6-methyladenine, N4-methylcytosine or 5-methylcytosine. The methylase includes one TRD that determines specificity and a catalytic domain that binds AdoMet. DNA methylation by *Hha*I has been shown to involve a base-flipping mechanism where the target residue is flipped out of the DNA helix into the active site of the methylase (Klimasauskas *et al.*, 1994). In the presence of Mg^{2+}, the endonuclease usually a homodimer, will cleave DNA if it contains an unmodified recognition site. *Eco*RV requires cations for specific DNA binding (Taylor *et al.*, 1991), whereas most type II endonucleases only require them for catalysis. A few endonucleases have been crystallized; *Eco*RI-DNA, *Eco*RV and *Eco*RV-DNA, *Pvu*II and *Pvu*II-DNA, *Bam*HI-DNA (Kim, 1990; Winkler, 1993;

Cheng *et al.*, 1994, Athanasiadis *et al.*, 1994; Newman *et al.*, 1995). The cocrystal structures show differences in protein-DNA interaction, which seem to be related to cleavage. *Eco*RI and *Bam*HI approach the DNA from the major groove and produce 5' overhangs on cleavage. *Pvu*II contacts the DNA via the minor groove and produces blunt ends. The scissile phosphoester bonds that are cleaved by *Pvu*II are more accessible from the minor groove. It is likely that enzymes that produce 3' overhangs also approach the DNA from the minor groove (Anderson, 1993). A motif: $PDX_n(D/E)XK$ conserved in the amino acid sequence of many restriction endonucleases is essential for cleavage (Thielking *et al.*, 1991). The acidic residues are thought to chelate Mg^{2+} ions. The two identical subunits of the endonuclease cooperate to ensure that both strands of the DNA are cleaved in a concerted reaction.

On transfer of a type II R/M system to a new host the methylase must modify all the recognition sites to prevent the endonuclease from degrading host DNA. Conversely cleavage of invading phage DNA must occur before the methylase modifies the recognition sites and protects phage DNA from restriction. Therefore it is important to tightly regulate these two enzymes. An additional open reading frame is often found close to the R/M gene cluster, these encode proteins that share homology with helix-turn-helix DNA binding proteins and therefore may act as transcriptional repressors or activators (Tao *et al.*, 1991).

Some type II R/M enzymes have unusual properties. These have been grouped into two separate families: IIs and IIe. Type IIs R/M systems recognise an asymmetrical 4-7 bp sequence. The endonuclease, which acts as a monomer cleaves DNA at a fixed distance of 1 to 20 base pairs away from the recognition site (review: Szybalski, 1991). *Fok*I is a type IIs R/M system, its methylase contains two independent N6 methyltransferase domains, each specific for one strand of the DNA. The *Hga*I methylase consists of two independent 5-meC methylases to ensure both DNA strands are methylated. Type IIe endonucleases only cut the DNA when allosterically activated by the binding of a second recognition site. This site can either be on the same, looped DNA strand, or on a different molecule and does not necessarily have to be cleaved or even be cleavable (Krüger *et al.*, 1988; Conrad and Topal, 1989; review: Krüger *et al.*, 1995). Therefore cleavage depends on the

distance between two adjacent sites, if they are too close to form a loop of DNA or too far apart, the DNA will not be cut. Examples of type IIe enzymes include *Eco*RII and *Nae*I.

Type III R/M systems

Type III R/M systems are similar to type I R/M systems in their requirement for ATP, but have different subunit structures and different systems of recognition. They are the smallest class of R/M systems and only four examples have been identified: *Eco*PI (encoded by the P1 prophage), *Eco*P15 (coded by the *E. coli* plasmid p15B), *Hin*fIII (present in the *Haemophilus influenzae* strain Rf) and *Sty*LTI (in most *Salmonella* strains) (Arber and Dussoix, 1962; Arber and Wauters-Willems, 1970; Piekarowicz and Kalinowska, 1974; Dartois *et al.*, 1993).

Two genes *mod* and *res* (Iida *et al.*, 1983) encode two polypeptides, Mod and Res (75kDa and 106kDa in *Eco*PI respectively) (Hadi *et al.*, 1983). The Mod subunit can recognise a specific DNA site and act as a methylase (Hornby *et al.*, 1987). Mod is the functional equivalent of the HsdS and HsdM subunits that form type I methylases. Both the Mod and Res polypeptides are necessary for the restriction endonuclease function. The endonuclease requires Mg^{2+} and ATP as cofactors and is stimulated by AdoMet. Cleavage occurs on the 3′ side of the recognition sequence 25 to 27bp away. The cleavage distance is fixed for any given site but varies from site to site. Modification is stimulated by Mg^{2+} and ATP and requires AdoMet as a cofactor. In the presence of ATP the methylase competes with the endonuclease, in contrast to type I enzymes which either restrict or modify the DNA, but do not do both simultaneously. Mod recognises a non-symmetrical 5-6 base pair sequence and only methylates one of the DNA strands (De Backer and Colson, 1991a). An adenine residue in the recognition sequence is modified to N6-methyladenine. The 5′ and 3′ ends of the *mod* genes for known type III systems are conserved and are separated by a central unconserved region (Iida *et al.*, 1983). This region controls the specificity of the methylase and the conserved regions may code for the domains of the protein that

interact with the Res subunit. The *mod* and *res* genes are contiguous with the *mod* gene preceding the *res* gene in the DNA sequence. Both genes are transcribed in the same direction (Hümbelin *et al.*, 1988; Dartois *et al.*, 1993). Each gene is transcribed from a separate promoter, but there is evidence for the involvement of at least four promoters in expression (Sharrocks and Hornby, 1991). The expression of type III R/M genes is controlled by different mechanisms; *Eco*PI and *Eco*P15I systems can be transferred from cell to cell, but transfer of the *Sty*LTI system causes cell death from extensive DNA breakdown (De Backer and Colson, 1991b). There is a temporal delay of expression of *Eco*PI restriction activity, methylase activity is detected a few minutes after transfer but restriction activity is not detected for a few hours (Arber, 1974).

*Eco*P15I recognises the sequence CAGCAG and methylates the second adenine (Meisel *et al.*, 1991). This is the only residue that is methylated, therefore when a modified site is replicated one daughter molecule will remain completely modified and one daughter molecule will be completely unmodifed and ought to be subject to restriction (Hadi *et al.*, 1979). It was observed that phage T3 DNA was restricted by *Eco*P15I but T7 DNA was not, although T7 DNA contains 36 recognition sites for *Eco*P15I (Dunn and Studier, 1983). In phage T7 DNA these recognition sites are all in the same orientation; all the CAGCAG sequences are on one strand with the complementary CTGCTG sequence in the other strand (Schroeder *et al.*, 1986). This led to the hypothesis that the restriction endonuclease requires two target sites in inverse orientation as a substrate for restriction (Meisel *et al.*, 1992). This was confirmed by studying restriction on M13 derivatives with different arrangements of *Eco*P15I recognition sites (Meisel *et al.*, 1992). The recognition site for the endonuclease can be regarded as a symmetrical sequence interrupted by a nonspecific spacer of variable length. The maximum and minimum lengths of this spacer has not been determined, but restriction can occur with a spacer as short as 65 bps or as long as 3500 bps. A small amount of ATPase activity occurs during restriction (Reiser and Yuan, 1977; Meisel, *et al.*, 1995; Saha and Rao, 1995).

Type IV R/M systems

Based on the characterisation of the restriction system *Eco*57I from *E. coli* strain RFL57 a new class of R/M systems has been proposed. This group shares characteristics with type IIs R/M systems and others with type III R/M systems (Janulaitis *et al.*, 1992a, b).

One gene encodes an endonuclease (108kDa) and a second the methylase (63kDa); both enzymes act as monomers. The enzymes recognise the DNA sequence CTGAAG. The methylase modifies the second adenine in this site, and the adenine in the complementary strand, to N6-methyladenine. Unusually the endonuclease possesses some methylase activity and can modify one of the adenine residues in the recognition site. The methylase requires AdoMet and is stimulated by Ca^{2+} and Mg^{2+}. The endonuclease requires Mg^{2+} and unlike type II enzymes is stimulated by AdoMet. Unlike type III endonucleases, ATP is not required for restriction. The endonuclease cleaves the DNA 14/16 nucleotides away from the 3' side of the recognition site.

Table 2 illustrates the characteristics that distinguish each class of R/M systems.

Type	Structural features	Requirements for restriction	Cleavage
I	Multi-functional, 3 different subunits	ATP AdoMet Mg^{2+}	Cuts far from recognition site with much ATP hydrolysis
II	Simple, independent of methylase	Mg^{2+}	Cuts at a fixed position within recognition site
IIe	Simple, independent of methylase	Mg^{2+}	Cuts within recognition site when activated by a second site
IIs	Simple, independent of methylase	Mg^{2+}	Cuts 1-20bps from recognition site
III	Multi-functional, 2 different subunits	ATP Mg^{2+} Stimulated by AdoMet	Cuts 25 bps away from one site with some ATP hydrolysis, needs two inversely oriented sites
IV	Simple, posesses methylase activity	Mg^{2+} Stimulated by AdoMet	Cuts 14/16 bp away from site

Table 2. The characteristic features of the endonucleases of different classes of each R/M system.

Mechanism of ATP-dependent restriction

Cleavage by type I endonucleases requires communication between the endonuclease which remains bound to its recognition site and the cleavage site which is often thousands of base pairs from the recognition site. The substrate for type III endonucleases is two inversely orientated recognition sites, also sometimes thousands of base pairs apart. The mechanism of restriction is thought to be similar for both systems.

Restriction by type I enzymes is accompanied by extensive ATP hydrolysis (Eskin and Linn, 1972; Rosamund *et al.*, 1979; Dreier and Bickle, 1996). In contrast only 1% of the level of ATPase activity produced by *Eco*KI is detected during type III restriction (Reiser and Yuan, 1977; Meisel, *et al.*, 1995; Saha and Rao, 1995). This ATP hydrolysis is proposed to drive translocation of DNA past the enzyme which remains bound to its recognition site (Bickle, 1993). Translocation would produce double-stranded loops of DNA (Studier and Bandyopadhyay, 1988), both relaxed and supercoiled loops have been observed under the electron microscope when studying restriction by *Eco*BI and *Eco*KI (Rosamund *et al.*, 1979; Yuan *et al.*, 1980; Endlich and Linn, 1985). Translocation during type I restriction has been proposed to occur in a unidirectional motion with cleavage on one side of the recognition site (Rosamund *et al.*, 1979; Endlich and Linn, 1985), or with a bidirectional motion with cleavage on either side of the recognition site (Yuan *et al.*, 1980).

One model for restriction by type I endonucleases is the Studier model, where the DNA is cut when two adjacent, translocating enzymes meet (Studier and Bandyopadhyay, 1988) see Figure 2. Studier and Bandyopadhyay investigated restriction of phage T7 DNA by *Eco*KI *in vitro*. The sites were saturated with *Eco*KI in the presence of AdoMet and the reaction started by adding ATP. Instead of producing smears of DNA of different sizes, discrete bands were observed when the products of restriction were run on agarose gels. The sizes of these bands indicated that cleavage clustered at the midpoints between adjacent recognition sites.

i)

ii)

iii)

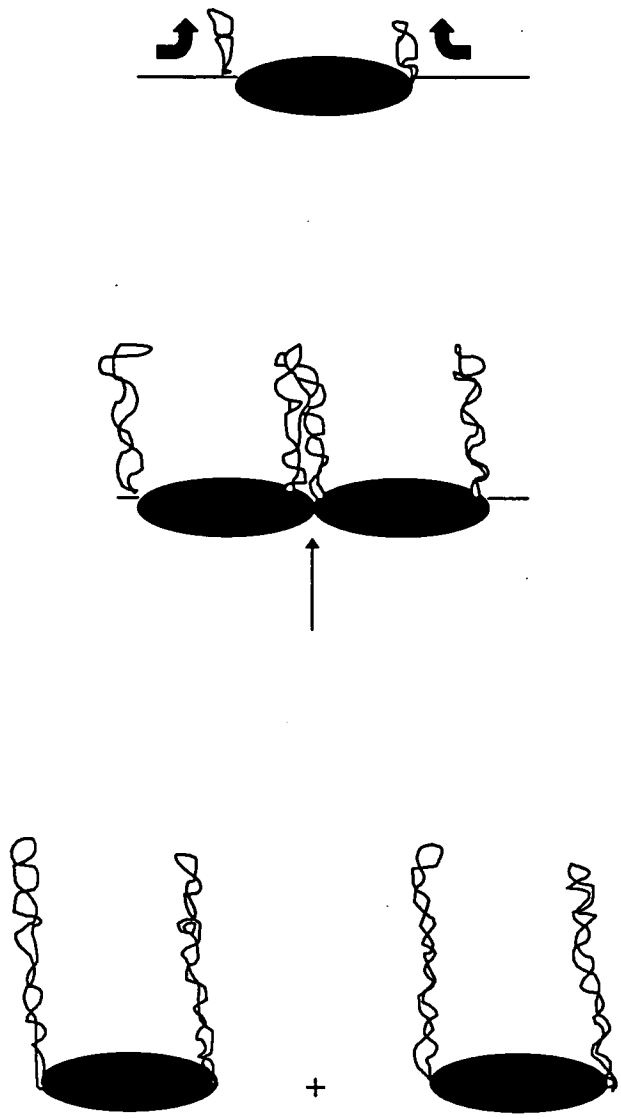


Figure 2. The Studier model for the restriction of DNA by type I endonucleases. In the presence of ATP *Eco*KI translocates DNA past itself simultaneously on both sides producing double-stranded DNA loops (i). The DNA is cut when two neighbouring enzymes meet as a consequence of this translocation, the arrow indicates DNA cleavage which occurs between the two DNA loops (ii). After this primary cleavage event has occurred the endonuclease remains bound, ATP hydrolysis continues and secondary cleavage events take place.

With this information they proposed a model where the endonuclease translocates DNA past itself on both sides simultaneously and the DNA is cut when two adjacent enzymes interact. Restriction occurs between the two adjacent DNA loops.

A similar model has been proposed for restriction by type III endonucleases, with cleavage occurring as a result of communication between two endonucleases which remain bound at their recognition sites (Meisel *et al.*, 1995). In the case of the type III enzymes the DNA is translocated on only one side of the target sequence, the inversely orientated target sequences may impose the directionality on the translocation (Murray *et al.*, 1993). A model requiring translocation is supported by the finding that restriction is blocked if the Lac repressor is bound to its operator sequence in the intervening sequence between two *Eco*P15I sites (Meisel *et al.*, 1995). Cleavage occurs at a site between the target sequence and the loop, 25 base pairs away from the recognition sequence.

One question arising from the Studier model is how substrates containing only one recognition site are cut. Limited cleavage of linear substrates or plasmids containing one site has been demonstrated with type I endonucleases (Murray *et al.*, 1973b; Webb *et al.*, 1996; Janscak *et al.*, 1996). Unbound endonucleases are proposed to cooperate with the bound endonuclease to cut the DNA (Murray, *et al.*, 1973b; Rosamund *et al.*, 1979; Yuan *et al.*, 1980; Studier and Bandyopadhyay, 1988).

The involvement of DNA translocation is supported by studies on restriction by *Eco*R124I, using catenanes as substrates. Cleavage always occurred on the plasmid containing the recognition site and not on the interlinked plasmid, therefore communication between recognition and cleavage sites did not occur by random looping of the DNA (Szczelkun *et al.*, 1996). As DNA is moved past the recognition site it is twisted (Yuan *et al.*, 1980; Endlich and Linn, 1985). When DNA translocation occurs topological barriers may cause the endonuclease to stall. The endonuclease is bound to both the recognition site and non-specific DNA that is being translocated past, this means it is fixed and cannot rotate around the DNA. If the

DNA is twisted to the right hand during translocation this would cause the twist to be reduced behind the complex and increased ahead of the complex (Gellert *et al.*, 1978; Liu and Wang, 1987), eventually creating a barrier against any further translocation. If the endonuclease is stalled it is proposed to nick the DNA in one strand and cleavage occurs when an unbound endonuclease cooperates to cut the second strand (Studier and Bandyopadhyay, 1988; Janscak, *et al.*, 1996).

Szczelkun *et al.*, 1997, found evidence for cleavage by *Eco*R124I in a region close to the recognition site in addition to cleavage mid-way between sites. They propose this represents cleavage occurring between the DNA loop and the recognition site or occurs as a result of nicks that have occurred in this region of the DNA during the initiation of translocation.

After restriction, type I enzymes remain bound to the DNA (Yuan *et al.*, 1975) and electron microscopy results suggest that DNA translocation continues (Endlich and Linn, 1985). In contrast type III endonucleases dissociate after cleavage (Meisel *et al.*, 1995). This could be one reason for the difference in levels of ATP hydrolysis observed.

DEAD box proteins

The HsdR and Res amino acid sequences contain motifs conserved in the DEAD box family of proteins (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993). Named after the single letter amino acid sequence present in one of the conserved motifs (Asp-Glu-Ala-Asp) (Linder *et al.*, 1989). The DEAD box family is a subdivision of the helicase superfamily II. Some of these proteins are known to be helicases, the rest remain putative helicases (Schmid and Linder, 1992). Helicases catalyse the unwinding of DNA or RNA, using energy from ATP hydrolysis to break the hydrogen bonds between DNA strands and to fuel translocation along DNA (reviews: Lohman and Bjornson, 1996; West, 1996). These proteins are found in a wide range of organisms from *E. coli* to *Drosophila* to humans and are involved in a diverse range of important functions such as DNA repair, recombination, splicing and replication.

Conservation of amino acid sequence within the HsdR polypeptide between *Eco*AI and *Eco*EI has high identity in the DEAD box motifs (89%) but has lower overall identity (Murray *et al.*, 1993). This implies that these motifs may have an important role in the restriction mechanism. Figure 3 shows the DEAD box motifs in the HsdR and Res sequences aligned with the sequence of eIF-4A, the prototype of this family. Eukaryotic initiation factor 4A (eIF-4A) is required for mRNA-ribosome binding and plays a central role in translation initiation. It is an RNA-dependent ATPase and exhibits bidirectional RNA unwinding activity *in vitro*, in the presence of eIF-4B and ATP (Grifo *et al.*, 1984; Ray *et al.*, 1985; Rozen *et al.*, 1990).

From the alignment in figure 3 it is clear that the HsdR and Res protein sequences deviate from the eIF-4A sequence. In motif Ia, eIF-4A has the sequence PTRELA, motif II contains DEAD and in motif VI eIF-4A has a histidine residue where the other sequences have a glutamine residue. As more DEAD box proteins were sequenced these deviations were used to subdivide the family into DEAD and DEXH proteins, where X is commonly A or C (Fuller-Pace and Lane, 1992). Type I and III endonucleases are members of the DEXH family. As yet there does not appear to be a relationship with protein function and this subgrouping. Table 3 shows some examples of DEXH and DEAD proteins.

	I	Ia
eIF-4A	YDVIAQAQSGKTAT	ALVLAPTRELAQQI
*Eco*AI	VLLVMATGTGKTYT	ILFLADRNILVDQT
*Eco*KI	ILLAMATGTGKTRT	ILFLVDRRSLGEQA
*Eco*R124I	GYIWHTTGSGKTLT	VFFVVDRKDLDYQT
*Eco*PI	IDVSMETGTGKTYT	FIIIVPTISIKAGT
Consensus	+++ tg GKt s s	++++p r + k

	II	III	IV
eIF-4A	MFVLDEAD	VLLSATM	AVIFINTRRK
*Eco*AI	LIVIDECH	IGLTATP	TIVFCNDIDH
*Eco*KI	CIVVDEAH	IALTATP	TIVFCVTNAH
*Eco*R124I	VFIFDECH	FGFTGTP	AMLAVSSVDA
*Eco*PI	FIIIDEPH	IRYGATFS	TLFFIDDIEG
Consensus	++++DEah	+++tat sgs	+++F t y s

	V	VI
eIF-4A	VLITTDLLARGIDVQ	ENYIHRIGRGGR
*Eco*AI	IATTSELMTTGVDAK	TKFKQIIGRGTG
*Eco*KI	IVVTVDLLTTGVDIP	ILYEQMKGRATR
*Eco*R124I	LLIVVGMFLTGFDAP	HGLMQAFSRTNR
*Eco*PI	FIFSKWTLREGWDNP	TSKLQEVGRGIR
Consensus	++++t + g+ + s	q +GR+gr a

Figure 3. The alignment of DEAD box motifs in HsdR and Res protein sequences (Gorbalenya and Koonin 1991) compared to eIF-4A. With corrected motif III *Eco*KI sequence (Webb *et al.*, 1996) and *Eco*AI sequence (Murray *et al.*, 1993; Titheradge *et al.*, 1996). The consensus residues, derived from proteins in helicase superfamily II, are shown underneath the protein sequences (Gorbalenya *et al.*, 1989). Plus signs represent conserved hydrophobic residues, capital letters indicate conserved amino acids and lower-case letters indicate alternative deviations.

Protein	Motif II	Role	Biochemical Activities	Reference
Rad3, yeast	DEAD	Excision repair of UV damaged DNA	DNA dependent ATPase and DNA helicase activities.	Reynolds and Friedberg, 1981 Sung *et al.*, 1987a Sung *et al.* 1987b Harosh *et al.*, 1989
RecG, *E. coli*	DEXH	Recombination and DNA repair, catalyses branch migration	ATPase activity and DNA helicase activity on branched substrates	Lloyd and Sharples 1993
Prp16, yeast	DEXH	Pre-mRNA splicing	ATPase and RNA helicase activities	Schwer and Guthrie, 1991
SrmB, *E. coli*	DEAD	Ribosomal biogenesis	RNA dependent ATPase and RNA helicase activity	Nishi *et al.*, 1988
p68, mammals	DEAD	Cell growth and division	ATPase and RNA helicase activity	Hirling *et. al.*, 1989
RecQ, *E. coli*	DEXH	Repair of UV damaged DNA	DNA dependent ATPase and DNA helicase activities.	Umezu *et. al.*, 1990

Table 3. Examples of DEAD and DEXH proteins, their roles *in vivo* and biochemical activities of the purified protein.

Roles of DEAD box motifs

Mutagenesis has begun to reveal roles for each motif in the biochemical activities involved in DNA or RNA unwinding.

Motif I and motif II have been previously described as the Walker ATPase A and B motifs respectively, as they are also conserved in ATPases (Walker *et al.*, 1982; Hodgman, 1988a, b). Motif II is an abridged form of the ATPase B motif. The crystal structure of adenylate kinase, an ATPase, revealed that motif I was an ATP binding site (Pai *et al.*, 1977). The lysine residue in this motif is directly involved in binding the γ-phosphate of the nucleotide (Schulz, 1992). Replacement of this residue abolished the ATPase and helicase activities of Rad3, UvrAB complex, eIF-4AB complex, RecB and PriA (Sung *et al.*, 1988; Seeley and Grossman, 1989; Pause and Sonenberg, 1992; Hsieh and Julin, 1992; Zavitz and Marians, 1992).

Motif II (DEAD or DEXH) forms a hydrophobic β-sheet structure. The first aspartic acid residue is in close proximity to motif I and binds Mg^{2+} through a water molecule (Pai *et al.*, 1990; Story and Steitz, 1992). The Mg^{2+} ion is bound to the β and γ phosphates of the nucleotide. In eIF-4A a mutation from DEAD to EEAD, which retains an acidic residue, gave wild type levels of ATPase activity but abolished helicase activity. DEAD to DEAH in eIF-4A showed elevated levels of ATPase activity, but only 10% of RNA helicase activity (Pause and Sonenberg, 1992). A change from DEAD to NEAD or DQAD abolished both ATPase and helicase activities (Pause and Sonenberg, 1992). These results confirm that the first two negatively charged residues in motif II (D and E) are important for Mg^{2+} coordinated ATP hydrolysis. Whilst helicase superfamily II contains DEAD and DEXH box proteins, proteins in helicase superfamily I are DEXX box proteins. This superfamily includes the *E. coli* proteins Rep and UvrD, both of which possess helicase activity, suggesting the highly conserved D and E residues are likely to be essential for DNA or RNA unwinding activity.

The roles of the remaining conserved motifs remain unconfirmed. In eIF-4A mutations in motif III of SAT to AAA enhanced ATPase activity 3-fold but abolished

RNA unwinding, therefore uncoupling the ATPase and helicase activities (Pause and Sonenberg, 1992). In RecG a mutation from TAT to TVT in this motif reduced ATP hydrolysis and stopped branch migration of Holliday junctions (Sharples *et al.*, 1993).

In eIF-4A the histidine residue in motif VI (HRIGRGGR) was changed to QRIGRGGR, the motif conserved in the DEXH family. In addition a double mutant with this change and a change in motif II to DEAH was created. ATPase activity was increased in the double mutant, which retained a small amount of helicase activity. In the single mutant both ATPase and helicase activity were abolished. In some RNA helicases motif VI has been implicated in RNA binding (Gorbalenya *et al.*, 1989; Linder *et al.*, 1989). However mutations in this motif in the vaccinia virus DEXH protein, RNA helicase NPH-II, did not affect RNA binding but reduced and abolished ATPase and helicase activities respectively (Gross and Shuman, 1996).

Two helicases have been crystallised, these proteins are PcrA (*B. stearothermophilus*) complexed with ATP and Rep (*E. coli*) co-crystallised with DNA and ADP (Subramanya *et al.*, 1996; Korolev *et al.*, 1997). Both are members of superfamily 1 so they are not DEAD or DEAH proteins and some of the conserved motifs have very different amino acid sequences from those in DEAD box proteins. Consequently the motifs may have different roles in the mechanism of unwinding. However their structure may provide some clues for the function of the motifs in DEAD or DEAH proteins. Both structures reveal that motifs I and IV are in direct contact with the bound nucleotide. For PcrA, motifs V and VI are at the interface between the nucleotide binding site and the protein domain. The Rep crystal structure shows motifs Ia, III and V are in contact with the bound single-stranded DNA, residues in motif VI are in contact with residues in motifs IV and III and residues in motif IV are in contact with residues in motif V. These contacts indicate a role for some motifs in coordinating activities controlled by other motifs.

Materials and Methods

Materials

Bacterial strains

Strain	Relevant genotypes and restriction phenotype	Reference/Source
NM679	(*hsdRMS*) Δ, ($r_K^-m_K^-$)	King and Murray, 1995
DH5α	*hsdR*, ($r_K^-m_K^+$)	Hanahan, 1983
Ymel	*supF*, ($r_K^+m_K^+$)	Rickenberg and Lester, 1955
NM526	*hsdR*Δ4 derivative of Ymel	King and Murray, 1995
ED8654	*supE*, *supF*, *hsdR*, ($r_K^-m_K^+$)	Borck *et al.*, 1976
AB1157	*rac*$^-$, ($r_K^+m_K^+$)	Low, 1973
NM795	*hsdR* derivative of AB1157, ($r_K^-m_K^+$)	King and Murray, 1995
C600	*supE*, ($r_K^+m_K^+$)	Appleyard, 1954
NM495	*hsdR*Δ4 derivative of C600, ($r_K^-m_K^+$)	N. E. Murray
XL1-blue	*recA*, *hsdR*, F', ($r_K^-m_K^+$)	Bullock *et al.*, 1987

Bacteriophage

λ virulent (laboratory collection), either unmodified (λ*vir*.0) after propagation on NM679 or *Eco*KI-modified (λ*vir*.K) by growth on C600, were used to measure restriction phenotype. Hybrid phages with the left arm of *phi*80 substituted for that of λ, which reduces the number of targets for *Eco*KI (Franklin and Dove, 1969), were also used; these were λNM175 and λNM176 with one *Eco*KI recognition site and λNM105 with two *Eco*KI recognition sites (Murray *et al.*, 1973a).

To transfer mutations to the *E. coli* chromosome the mutations were transferred from pSB2 to a λ vector. The *Eco*RI-*Sma*I fragment containing the mutation in *hsdR* was excised from mutant derivatives of pSB2 and inserted between the left arm of the *Pam* phage λNM1265 (Whittaker *et al.*, 1988) cut with *Sma*I, and the right arm of the *Eam* c1857 phage λNM1347 (Whittaker *et al.*, 1988) cut with *Eco*RI. The recombinants were selected on NM679, a *sup*^{o} *hsd*Δ host.

Plasmids

The Results section details the construction of plasmids made during this study.

Plasmid	Features	Reference
pUC18	ColE1 replicon, carrying *bla*, P_{lac} and multiple cloning sites (mcs)	Yanisch-Perron *et al.*, 1985
pT7-7	ColE1 replicon, carrying P_{T7}, *bla*, mcs and translational initiation region (tir) of T7 gene 10	Tabor, 1990
pSB2	derivative of pT7-7 carrying the *Eco*KI *hsdR* gene	Webb *et al.*, 1996
pRH3	Derivative of pBR322 contains 1.8kb *Hin*dIII fragment from *hsd* region of *E. coli* K-12	Sain and Murray, 1980
p*hsd*$^+$	Derivative of pBR322 with *hsdR*, *M* and *S* genes of *E. coli* K-12	O'Neill *et al.*, 1997
pJW2	*Kpn*I to *Bam*HI fragment of *hsdR* encoding GKT to GIT change in motif I, inserted in pUC18	This thesis
pJW3	As pJW2 with GKT to GRT change	This thesis
pJW4	As pJW2 with GKT to GTT change	This thesis
pJW5	As pJW2 with TAT to TDT change in motif III	This thesis
pJW6	As pJW2 with TAT to TGT change in motif III	This thesis
pJW7	As pJW2 with TAT to TVT change in motif III	This thesis
pJW8-13	*Kpn*I to *Bam*HI fragment with mutation in *hsdR* replacing wild-type *hsdR* sequence in pSB2 (in the same order as pJW2-7)	This thesis
pJW14	pSB2 with change encoding GRAT to GHAT in motif VI	This thesis
pJW15	pSB2 with change encoding GRAT to GLAT in motif VI	This thesis

pJW16	pSB2 with change encoding GRAT to GPAT in motif VI	This thesis
pJW17	p*hsd*$^+$ with change encoding GKT to GRT in motif I	This thesis
pJW18	p*hsd*$^+$ with change encoding FLVD to FLVH in motif Ia	This thesis
pJW19	p*hsd*$^+$ with change encoding FLVD to FLVN in motif Ia	This thesis
pJW20	p*hsd*$^+$ with change encoding FLVD to FLVY in motif Ia	This thesis
pJW21	p*hsd*$^+$ with change encoding DEAH to DEAD in motif II	This thesis
pJW22	p*hsd*$^+$ with change encoding TAT to TGT in motif III	This thesis
pJW23	p*hsd*$^+$ with change encoding FCVT to SCVT in motif IV	This thesis
pJW24	p*hsd*$^+$ with change encoding TTGV to TTCV in motif V	This thesis
pJW25	p*hsd*$^+$ with change encoding TTGV to TTRV in motif V	This thesis
pJW26	p*hsd*$^+$ with change encoding TTGV to TTSV in motif V	This thesis
pJW27	*phsd*$^+$ with change encoding GRAT to GHAT in motif VI	This thesis
p*hsd*$^+$ TVT	p*hsd*$^+$ with change encoding TAT to TVT in motif III	Angela Chen

Oligonucleotides

Oligonucleotides were bought from Oswell DNA service, Medical and Biological Science Building, University of Southampton. Restriction sites and mutation sites are underlined. Degeneracies: B=C, G or T; D=A, G or T; H=A, C or T; V=A, C or G. The use of the oligonucleotide is stated. Some oligonucleotides used as sequencing primers contain degeneracies because they were originally designed to introduce mutations.

Oligonucleotide	5'→3' sequence	Use
M5244	GACCGGTACCGGTABAACCCG	Motif I mutagenesis
M6010	CGCTCTCACCGDCACCCCGG	Motif III mutagenesis top strand
M6011	CCGGGGTGHCGGTGAGAGCG	Motif III mutagenesis bottom strand
V5076	CGAACAGATGAAAGGCCHCGCC ACGCGCTTATGC	Motif VI mutagenesis top strand
V5077	GCATAAGCGCGTGGCGDGGCCT TTCATCTGTTCG	Motif VI mutagenesis bottom strand
M6975	CGACAAGGAAGAGAATGCG	sequencing primer
T9140	CGGGCGCACCACCACCGGACGC ATGG	sequencing primer
T6273	CGCCGACTTTAACCGTGGCC	sequencing primer
G5036	CGTCTCTGDCTACCGTCG	sequencing primer
G5037	CGACGGTAGHCAGAGACG	sequencing primer
G5040	GGCGGATCCTGGTCGATC	PCR / sequencing primer contains *Bam*HI site
G5038	GCGATGGCGACCGGTACCGG	PCR / sequencing primer contains *Kpn*I site

G5039	GCCGCGATHCGCTTCGTC	sequencing primer
1209	TGTCTAGATATCGGCCTAACCA CGTGGTGCGTACGAGCTCAGGC G	Top strand 45mer substrate in ATPase and DNA binding assays
1208	CGCCTGAGCTCGTACGCACCAC GTGGTTAGGCCGATATCTAGAC A	Bottom strand 45mer substrate in ATPase and DNA binding assays
W1827	TGTCTAGATATCGGCCTCCACA CGTGTGTAGTACGAGCTCAGGC G	top strand non-specific 45mer, used in competition binding assay
W1828	CGCCTGAGCTCGTACTACACAC GTGTGGAGGCCGATATCTAGAC A	bottom strand, non-specific 45mer, used in competition binding assay

Media

All media were sterilised by autoclaving at 15lb/in^2 for 15 minutes.

LB broth: 10gl^{-1} NaCl, 10gl^{-1} Difco Bacto tryptone, 5gl $^{-1}$ Difco Bacto yeast extract, pH adjusted to 7.2 with NaOH.

LB agar was made by adding 1.5 gl^{-1} Difco agar to LB.

LB-ampicillin-methicillin agar: 20mg of filter-sterilized ampicillin and 80mg of filter sterilized methicillin were added to 1 litre of molten agar.

NZY + broth: 10 gl^{-1} NZ amine (casein hydrolysate), 5g l^{-1} yeast extract, 5g l^{-1} NaCl and 12.5ml of 1M $MgCl_2$, 12.5ml of 1M $MgSO_4$. 10ml 2M filter-sterilized glucose solution were added after autoclaving.

Baltimore Biological Laboratory (BBL) agar: 5gl $^{-1}$ NaCl, 10gl^{-1} BBL trypticase, 10gl^{-1} Difco agar (6.5gl^{-1} for BBL top agar).

2x TY: 16g Difco Bacto tryptone, 10g Difco Bacto yeast extract, 5g NaCl, made up to 1litre with water and autoclaved.

SOC: 1.8ml 20% glucose, 1ml 1M $MgSO_4$, 1ml 1M $MgCl_2$ was added to 100ml LB.

Solutions

All solutions were made using sterile, deionised water.

20x TBE buffer: 1.78M Tris, 1.78M boric acid, 50mM EDTA (pH8.3).

50x TAE buffer: 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH8.0).

10x TBS buffer: 60.5g Tris, 87.6g NaCl in 1 litre H_20 pH7.5, stored at 4°C.

1x TGS buffer: 6g Tris, 28.8g glycine to 990ml with dH_20 + 10ml 10% SDS.

TE buffer: 10mM Tris (pH7.5), 1mM EDTA (pH8.0).

Phage buffer: 3g KH_2PO_4, 7g Na_2PO_4, 5g NaCl, 1ml of 100mM $MgSO_4.7H_20$, 10ml of 100mM $CaCl_2$, 1ml of 1% w/v gelatine in 1 litre water.

Binding buffer: 20mM Tris pH8.0, 100mM NaCl made up fresh before use.

Western Blotting buffer: 9g tris, 43.2g glycine in 3 litres water.

R buffer: 20mM Tris-HCl, 10mM $MgCl_2$, 7mM β mercaptoethanol, 10% glycerol, 10^{-5} M PMSF and 10^{-5} M benzamidine, pH adjusted to 7.5.

TLC buffer: 0.5M LiCl, 1M formic acid.

Phenol: 250ml water saturated phenol (Rathburn chemicals), 200ml 1M Tris (pH7.8), 14ml Cresol, 500µl β-mercaptoethanol, 0.28g hydroxyquinoline.

Ethidium bromide: stock solution $10mgml^{-1}$ stored at 4°C in dark.

Ampicillin: stock solution $100mgml^{-1}$ used at $100\mu gml^{-1}$ stored at -20°C.

ATP stock solution (0.1M): 60mg ATP was dissolved in 0.8ml H_20 and pH adjusted to 7.0 with 0.1M NaOH and volume corrected to 1ml with H_20. Stored in aliquots at -70°C.

Lysis solution: 25mM Tris.HCl pH 8.0, 10mM EDTA pH 8.0, 1% w/v glucose.

DNA load dye: 20mg bromophenol blue, 2g Ficoll 400 dissolved in 10mls 1xTBE.

SDS load dye: 2.5ml 0.5M Tris, 2ml 20% SDS, 2ml glycerol, 0.1ml 0.1% bromophenol blue, 3.2ml H_20.

Non-denaturing PAGE load dye: 20mg bromophenol blue, 50% glycerol in 10ml

Reagents

DNA sequencing kits, sequenase and radiochemicals were obtained from Amersham International. Type II restriction enzymes were obtained from Boehringer Mannheim and New England BioLabs. ATP was obtained from Boehringer Mannheim. DNA ligase and Vent DNA polymerase were purchased from New England BioLabs and Red Hot DNA polymerase was obtained from Advanced Biotechnologies. *S*-adenosyl methionine was from New England BioLabs. Polynucleotide kinase was a kind gift from Sandra Bruce in Ken Murray's laboratory. AgarAce enzyme and Packaging extracts were obtained from Promega. Ampicillin was from Beecham Pharmaceuticals. The plasmid Flexiprep kit and Sephadex G-50 were bought from Pharmacia Biotech. QuikChange™ Site-Directed Mutagenesis kit was bought from Stratagene. PEI-cellulose TLC plates, low melting point agarose, N,N,N',N'-tetramethylethylenediamine (TEMED), ethidium bromide, DEAE-Sepharose and

Heparin-agarose were bought from Sigma. Caesium chloride was from ICN Biomedicals Inc. Urea and 2-amino-2-(hydroxymethyl)-1,2-propandiol (Tris) were from GibcoBRL. Dialysis tubing was from Medicell International. X-ray film was bought from Dupont (Cronex 4) and Kodak (Biomax MR). Anti-rabbit IgG peroxidase conjugate was bought from Sigma. Agarose, microsep concentrators and Long-Ranger sequencing gel solution were bought from Flowgen. 40% (19:1) and 30% (37.5:1) w/v acrylamide/bis-acrylamide were bought from NBL Gene Sciences Ltd. General laboratory chemicals used were obtained from Fisher, GibcoBRL or Sigma.

Methods

Microbial techniques

Bacterial cultures

5mls of LB was inoculated with a single colony and the culture was grown overnight at 37°C in a 1/2 oz glass bottle and then stored at 4°C. For longer storage the overnight culture was used to inoculate L-agar in a small glass vial, this was incubated overnight at 37°C and stored at room temperature.

Plating cells

A one in 50 dilution of overnight culture in LB was grown to mid-log phase ($O.D._{650}$ 0.6) to about $5x10^8$ cfu ml^{-1} and harvested by centrifugation at 3000g for 5 minutes. The cells were resuspended in 1/2 the original culture volume with 10mM $MgSO_4$ and stored at 4°C.

Plate lysates

A single plaque was picked into 500µl phage buffer containing a drop of chloroform,

200µl of this was mixed with 200µl plating cells. After 15minutes 3ml of BBL-top agar was added and the mixture poured onto fresh BBL agar plates. The plates were incubated for 6-8hours at 37°C. 5mls of LB was added onto the plate and this was kept overnight at 4°C. The broth was decanted from the agar plate, a drop of chloroform added and the solution vortexed. The lysate was clarified by centrifugation to remove debris for 10 minutes at 3000g and titred overnight by making serial dilutions of the phage stock and spotting onto an appropriate bacterial lawn. The lysate was stored at 4°C.

Transferring mutations onto the *E. coli* chromosome

Phage containing a temperature sensitive mutation (λcI857) which prevents repression at temperatures above 37°C and a mutation in their attachment site (*-b*527) were used. The lysate was diluted 10^{-2}, 10^{-3}, 10^{-4} in phage buffer and spotted onto a lawn of AB1157 using a Gilson pipette. The spots were dried and the plate was incubated overnight at 32°C. Turbid plaques were picked using a sterile loop and streaked onto LB plates seeded with c.10^9 λ*c*I$^-$ (λNM63) and h^{80}*imm*λ (λNM220) homoimmune phage, which lyse cells that don't contain a prophage. These plates were incubated overnight at 32°C. Colonies growing on these plates were purified on fresh LB plates overnight at 32°C. The purified colonies were picked into 250µl LB and grown for 7 hours at 32°C. These cultures were spotted onto an AB1157 lawn and incubated overnight at 37°C. The cultures were also streaked onto LB plates in duplicate, one plate incubated at 32°C and one at 42°C. A circle of lysis around the spots on the AB1157 lawn and lysogens that were easily cured at 42°C were indications that the phage had integrated via homologous recombination and not via the defective *att* site on lambda. Once identified the cured colonies were tested for restriction to see if the mutation had been transferred to the chromosome.

Tests for restriction phenotype

Purified colonies were picked into 250µl LB (with 10µgml^{-1} ampicillin if required) and incubated at 37°C (32°C for lysogens) for 6-7 hours, the culture was mixed with 3mls

BBL-agar and then poured onto a BBL plate. Serial dilutions of modified and unmodified phage were spotted onto the lawn. The spots were dried and the plates incubated overnight at 37°C. Once an approximate phenotype was known the test was repeated on whole plates by mixing an appropriate dilution of phage with 200µl cells, incubating at room temperature for 15 minutes, plating onto BBL plates then incubating overnight at 37°C. The number of plaque forming units per ml was calculated.

Transformation of cells with DNA

Electroporation (Heery and Dunican, 1989)

A 1 in 50 dilution of a fresh overnight culture was grown to an $O.D_{650}$ of 0.5 in 2x TY broth. The cells were centrifuged at 3000g at 4°C for 5 minutes and the pellet was washed three times with 10ml water by resuspending and centrifuging at each wash. After removing the water after the final spin, the cells were resuspended in the residual water and 40µl of this cell suspension was mixed with 1µl of the ligation reaction and 5µl water. This was dispensed into a 0.2cm electroporation cuvette and pulsed for 4.8msec with an electric field of $12.5KVcm^{-1}$ using a Gene Pulser electroporator (Biorad Laboratories Ltd), set at 25µF, 2.5 kV and 200Ω. 1ml SOC solution was immediately added to the cells and the solution was incubated at 37°C for 30 minutes, shaking. 10µl, 100µl and 300µl of this culture was spread onto LB-agar plates containing ampicillin at $10ngml^{-1}$ and incubated overnight at 37°C. Colonies were purified overnight by streaking onto another LB-ampicillin plate.

$CaCl_2$ transformation

When supercoiled DNA was introduced into bacterial cells the $CaCl_2$ method for transformation was used, as high efficiencies of transformation were not necessary. Competent cells were prepared by adding 0.5ml of an overnight culture to 25ml LB and growing to an $O.D_{650}$ of 0.5. The cells were harvested by centrifugation at 3000g for 10min and resuspended in 10ml ice cold 0.1M $CaCl_2$, this suspension was left on ice for 10-20 minutes then harvested again as before and resuspended in 800µl of ice cold 0.1M $CaCl_2$ and 200µl of glycerol. Cell solutions were divided into 100µl

aliquots and stored at -70°C.

To transform these cells with DNA, 1µl of plasmid stock solution was mixed with 20µl of thawed competent cells and left on ice for 20 minutes. The cells were then heat shocked at 42°C for 90 seconds. 1ml of LB was added and the cells incubated at 37°C for 30 minutes. This was plated onto an LB-ampicillin plate and incubated overnight at 37°C.

DNA Techniques

Restriction digests

The digest was normally carried out in a 10µl volume with 1-2µg DNA, in the appropriate restriction buffer, using 5 units of enzyme. Most reactions were incubated at 37°C for at least two hours, *Sma*I digests were incubated for at least 8 hours or overnight at 25°C.

Gel electrophoresis of DNA

Horizontal submerged gel electrophoresis tanks were used to separate DNA fragments. Samples were mixed with DNA load dye and loaded onto 0.8%-1.5% agarose gels made up in 1x TBE or 1x TAE buffer. Gels were run at $5Vcm^{-1}$, with $10ngml^{-1}$ ethidium bromide added to the running buffer. Fragments were visualized over a UV-light transilluminator and photographed with a video copy processor (Mitsubishi Electronics Corp).

Extraction of DNA from agarose gels

Electroelution

Dialysis tubing was prepared by boiling for 20 minutes in water containing 2% w/v sodium bicarbonate and 1mM EDTA (pH 8.0). The DNA band was cut from the gel using a scalpel and placed in dialysis tubing containing 1x TBE buffer. The tubing was sealed at both ends and submerged in 1x TBE buffer, the DNA was eluted into the buffer by electrophoresis at $5Vcm^{-1}$ and the direction of electrophoresis reversed

after 30 minutes for 10 seconds, to release DNA from the side of the dialysis tubing. The DNA was precipitated in 2 volumes ethanol and 1/10 volume sodium acetate pH 8.0 at -20°C overnight.

Agarose digestion

The DNA sample was run on 1% low melting point agarose gels, made up in 1x TAE buffer. The desired band was cut out of the gel using a scalpel and placed in a microcentrifuge tube. The agarose was melted by incubation at 70°C for 15 minutes. This solution was transferred to 40°C for 5 minutes before Agar*ACE*™ enzyme (Promega) was added at 1:100 v/v. The tubes were left for an hour at 40°C. The DNA was precipitated by adding 2 volumes of ice-cold ethanol and incubating overnight at -20°C. The DNA was centrifuged at 11000g for 40 minutes and the pellet dried under vacuum for 10 minutes before resuspending in an appropriate volume of H_20.

Ligations

DNA fragments were joined together using 1 unit of T4 DNA ligase (Boehringer Mannheim) in 1x ligase buffer in a 10µl volume and incubated overnight at 16°C.

Packaging phage DNA into a λ vector

Ligations were incubated at 65°C for 10 minutes to denature the ligase. 15µl of packaging extract (Promega) was added to the ligation mix and this was left at room temperature for 2 hours. 500µl of phage buffer was added to the packaging mixture and various dilutions were spotted onto bacterial lawns and incubated overnight at 37°C to calculate the packaging efficiency.

Small-scale preparation of plasmid DNA

Small quantities of DNA were prepared using spin columns (Qiagen). The method is based on adsorption of DNA on silica-gel membranes incorporated in the spin columns. The suggested protocol was slightly modified to increase yields.

A 5ml overnight culture of cells freshly transformed with the plasmid

(normally DH5α cells) was harvested by centrifugation at 11000g for 3 minutes. The cell pellet was resuspended in 500µl buffer P1 (contains RNaseA). 500µl of buffer P2 (NaOH/SDS) was then added to lyse the cells, followed by 500µl of buffer N3, which causes denatured proteins and cellular debris to precipitate. The tubes were centrifuged at 11000g for 10 minutes and the supernatant added to a QIAprep spin column. The columns were centrifuged at 11000g for 45 seconds. The flow-through from the column was discarded and the column was washed with 0.5ml buffer PB (which removes any nucleases) by centrifugation at 11000g for 45 seconds. The flow-through was discarded then the columns washed with 0.75ml buffer PE (which removes salt) by centrifugation at 11000g for 45 seconds. The flow-through was discarded and the columns centrifuged again for 30 seconds, the columns were then placed in clean microcentrifuge tubes and 50µl H_2O was added to elute the DNA. These were left for 10 minutes then centrifuged at 11000g for 5 minutes, the DNA solution was stored at -20°C.

Midi-scale preparation of plasmid DNA

For a midi-scale preparation of plasmid DNA the Flexiprep kit was used (Pharmacia Biotech), which uses a glass matrix (Sephaglas) to purify the plasmid DNA. 50mls of overnight culture were harvested at 7700g for 10 minutes. The cell pellet was then resuspended in 5mls solution I (100mM Tris-HCl (pH7.5), 10mM EDTA, 400µg/ml RNase I) and mixed with 5mls of freshly prepared solution II (1M NaOH, 5.3% (w/v) SDS) then incubated at room temperature for 5 minutes. 5mls of solution III (3M potassium, 5M acetate solution) was then added and the tube placed on ice for 10 minutes. This was centrifuged at 12000g for 15 minutes and the supernatant mixed with 0.7 volume of ambient-temperature isopropanol and incubated at room temperature for 10 minutes to precipitate the DNA. This was centrifuged at 8000g for 20 minutes and the pellet air-dried. 5ml of Sephaglas™ FP suspension was added and the pellet was resuspended by vortexing for 1 minute. The Sephaglas is suspended in a buffered solution of guanidine-HCl, 50mM Tris-HCl (pH7.5) and 10mM CDTA, which promotes binding of DNA to the glass. This is incubated for 10 minutes at room temperature, keeping the Sephaglas in suspension, then pelleted by

centrifugation at 3000g for 2 minutes. The sephaglas-DNA pellet is washed in 5ml wash buffer (20mM Tris-HCl (pH7.5), 2mM EDTA, 200mM NaCl 60% ethanol) followed by 70% ethanol, spinning the pellet down after each wash step. The pellet is air dried for 30 minutes and then resuspended in water (normally 300-500µl depending on the concentration required) and stored at -20°C.

Large-scale preparation of plasmid DNA

Large amounts of DNA were prepared using a method adapted from Clewell and Helsinki (1969). 250ml of cells freshly transformed with the plasmid were harvested by centrifugation at 10000g for 10 minutes. The cell pellet was resuspended in 7mls lysis solution, then 10mg of lysozyme in 0.5ml lysis solution was added and the mixture was left on ice for 5 minutes. 14mls alkaline-SDS (0.2M NaOH, 1%SDS) was then added and this was incubated on ice for 10 more minutes. 3.5mls 3MKAc (pH5.0) was added, tubes were left on ice for a further 5 minutes then centrifuged at 8000g for 10 minutes. The supernatant was separated from the debris by filtering the mixture using glass wool. 15ml of ice-cold isopropanol was added to the supernatant and incubated on ice for 30 minutes. The DNA was precipitated by centrifugation at 8000g for 10 minutes. The pellet was rinsed in 70% ethanol and then air dried, before resuspending in H_20. The volume was corrected to 9.4ml and 9.4g of caesium chloride was added, followed by 0.6ml of ethidium bromide solution ($10mgml^{-1}$). The solution was transferred to a Sorvall Ultracrimp tube (DuPont Ltd) and the tubes were balanced to within 0.02g and heat-sealed. The tubes were centrifuged at 130000g, for 36 hours at 18°C, which formed a density gradient. The DNA bands were visualized using UV-light and the plasmid band (lower band) was removed using a 1ml syringe and a hypodermic needle. Ethidium bromide was extracted from the DNA solution by mixing with TE/NaCl saturated isopropanol, allowing the phases to separate and removing the solvent layer. This was repeated at least four times, or until the ethidium was completely removed. Two volumes of water were then added to the DNA solution followed by twice the new volume with ethanol. The DNA was sedimented by centrifugation at 29000g for 15 minutes. The pellet was rinsed in 70% ethanol and air-dried. It was then resuspended in 500µl TE buffer. Protein was

removed by adding the same volume of phenol, vortexing, then centrifugation at 11000g for 5 minutes and removing the phenol layer. This was repeated, then the plasmid DNA was precipitated by adding two volumes of ice-cold ethanol and incubating at -20°C for at least 30 minutes. The DNA pellet was sedimented by centrifugation at 11000g for 20 minutes. The pellet was rinsed in 70% ethanol and dried under vacuum, then the DNA was resuspended in 500µl TE buffer.

Small-scale preparation of phage λ DNA

100µl of fresh overnight culture was added to 4ml LB plus 40µl 1M $MgSO_4$ and $1x10^8$ phage added. This was grown at 37°C for 3-5 hours or until lysis occurred. A drop of chloroform was added to the phage solution and the mixture was vortexed, then centrifuged at 3000g for 10 minutes to remove cell debris. The supernatant was transferred to a 1/2 ounce bottle and 5µl of $10mgml^{-1}$ RNase and 5µl of $10mgml^{-1}$ DNase was added. This was incubated at 37°C for 30 minutes then transferred to a 15ml corex tube containing 4ml PEG solution (20g Polyethylene glycol, 11.7g NaCl dissolved in 78 mls phage buffer). Phage was precipitated by incubation overnight at 4°C and pelleted by centrifugation at 13000g for 20 minutes. The pellet was dried by inverting the tube for 5 minutes then resuspended in 0.5ml phage buffer, this solution was transferred to a microcentrifuge tube and centrifuged at 11000g for 10 seconds to remove insoluble material. 0.5ml chloroform was added and the mixture vortexed 3 times for a few seconds before centrifugation at 11000g for 1 minute. The chloroform layer was removed then 500µl phenol and 100µl TE buffer was added. This was mixed by inversion then centrifuged at 11000g for two minutes, the phenol layer was removed then 500µl phenol:chloroform (1:1) mixture was added and mixed by inversion. This was centrifuged again at 11000g for 2 minutes to separate the layers. 450µl of the DNA layer was transferred to a fresh microcentrifuge tube and 500µl of chloroform was added and layers were separated by centrifugation at 11000g for 2 minutes. 400µl of phage solution was removed to a clean tube and mixed with 800µl of ethanol. This was incubated on ice for 10 minutes and phage DNA was sedimented by centrifugation at 11000g for 10 minutes. The DNA pellet was rinsed

in 70% ethanol then resuspended in 400µl TE buffer and left for 10 minutes before adding 3M NaAC and 800µl ethanol. After incubation on ice for 10 minutes the DNA was sedimented at 11000g for 10 minutes, air-dried and resuspended in 100µl TE buffer. The phage DNA solution was stored at -20°C.

Measuring DNA concentration

DNA concentrations were calculated by measuring UV-light absorption on a Perkin Elmer Lambda 5 spectrophotometer. An $O.D._{260}$ of 1 = 50µg /ml, for double-stranded DNA solutions.

The Polymerase Chain Reaction

DNA was amplified using the polymerase chain reaction, as described by Saiki *et al.* (1988). Reactions were performed in an OmniGene thermal cycler (Hybaid Ltd). PCR was used to insert specific mutations, screen colonies or plasmids for certain sequences and to provide a clean template for sequencing reactions. When screening colonies for specific mutations or quickly checking certain regions of DNA, Red Hot DNA polymerase (Advanced Biotechnologies) was used. To insert specific mutations Vent® DNA polymerase (New England BioLabs) was used as this possesses 3'→5' proof reading exonuclease activity to ensure additional mutations were not inserted during the reaction.

Primers were designed to be about 24 base pairs long with CG base pairs at both ends. The melting temperature (T_m) was calculated: $T_m \sim 4(C+G) + 2(A+T)$, the annealing temperature used in each reaction was 5 degrees lower than the T_m. Typically the reaction was performed in 100µl, with 1x reaction buffer containing 2mM $MgSO_4$, 2 units of polymerase, 200µM dNTP mix, 0.4µM primers and 10ng of DNA template. Primer concentration was reduced to 0.2µM and only 50µM of dNTP mix was used when amplifying DNA with Red Hot DNA polymerase. A layer of mineral oil was added to cover each reaction mix. A typical reaction cycle was: 96°C for 5 minutes, then 20 cycles of (96°C for 1 minute, 52°C for 40 seconds, 72°C for 40 seconds), then 72°C for 5 minutes. The denaturing temperature was adjusted

to 94°C and cycles increased to 30 when using Red Hot DNA polymerase.

Site specific mutagenesis-Recombinant PCR (Higuchi, 1990)

To insert specific amino acid substitutions, primers containing base substitutions at the desired site of mutation were used (see oligonucleotides listed in materials section). Two separate primary reaction cycles were performed, each reaction used one flanking primer and one of the complementary primers across the site of mutagenesis. After the first round of PCR 10µl of the products was run on an agarose gel to check the fragment had been successfully amplified. The remaining reaction mix was then cleaned to remove the original primers (see PCR purification). 25µl of this DNA mix was then used as a template for a second round of PCR. This amplification cycle used the two flanking primers containing restriction sites to produce the final amplification product containing the desired mutation.

Purification of PCR products

After PCR the products were purified from the original primers using QIAquick purification columns (QIAgen). 500µl buffer PB was added to the whole 100µl PCR solution including the mineral oil layer. This was mixed, applied to a QIAquick column and centrifuged at 11000g for 45 seconds. The flow-through was discarded and the column was washed with 0.75ml buffer PE and centrifuged at 11000g for 45 seconds. The flow-through was discarded and the columns centrifuged for 1 minute at 11000g to ensure all ethanol buffer had been eluted. The PCR product was recovered by elution in 50µl H_20.

QuikChange™ Site-Directed Mutagenesis (Stratagene)

The plasmid pSB2 was used as the substrate for the mutagenesis reaction. Two complementary 35mer oligonucleotides containing the desired base pair substitutions were used to incorporate the changes directly into the *hsdR* gene on pSB2. 10-50ng of plasmid was used as the template in 50µl reactions containing: 1x reaction buffer, 125ng of each primer, 1µl of 10mM dNTP mix (containing 2.5mM of each NTP). 1µl

of *Pfu* DNA polymerase (2.5U/µl) was then added and 30µl of mineral oil was layered on top of this mixture. The reaction cycle consisted of 95°C for 30 seconds and 12 cycles of (95°C for 30 seconds, 55°C for 1 minute and 68°C for 12 minutes). Then 1µl of *Dpn*I endonuclease was added to the reaction and the tubes were incubated at 37°C for 30 minutes (this digests the original template). Epicurian Coli® XL1-Blue cells (Stratagene) were thawed on ice and 50µl of these were mixed with 1µl of DNA and incubated on ice for 30 minutes. These were transferred for 45 seconds to 42°C then placed on ice for a further 2 minutes. 0.5ml of NZY broth, preheated to 42°C, was added to the transformation reactions and this was incubated at 37°C for 1 hour shaking, before plating onto agar-ampicillin plates.

DNA sequencing

DNA sequence was determined by dideoxy chain-termination sequencing (Sanger *et al.*, 1977). Three alternative sequencing methods were used, all using reagents supplied in Sequenase™ kits obtained from Amersham International plc.

i) Standard double-strand DNA sequencing

3-5µg of plasmid DNA was mixed with 1µl of primer, boiled for 4 minutes and snap-freezed on dry ice. This primer/DNA solution was mixed with: 2µl DTT, 0.7µl labelling mix, 2µl reaction buffer, 0.5µl α-^{35}S dATP and 2.5µl diluted sequenase (1:8) and incubated at room temperature for 4 minutes. 4µl of this mixture was added to 2µl of each ddNTP, which had been pre-warmed to 37°C. These were left at 37°C for 3 minutes then 4µl of stop solution was added. Samples were boiled for 2 minutes before loading 4µl onto a 6% polyacrylamide sequencing gel.

ii) Plasmid quick-denature sequencing

This method was essentially the same as i) except using an alternative denaturation method. 2µg of DNA was mixed with 2µl of 1M NaOH and 1µl of primer then incubated for 10 minutes at 37°C, before placing on ice. 2µl of 1M HCl and 2µl of plasmid reaction buffer was added and the tubes placed at 37°C for a further 10 minutes. 5.5µl of reaction mix was added to the denatured DNA/primer. The reaction mix was 1µl DTT, 2µl labelling mix, 0.5µl α-S^{35} dATP and 2µl diluted

enzyme (1:8 v/v in dilution buffer). This was left at room temperature for 3 minutes then 4.5µl of this was added to 2.5µl of each ddNTP, which had been pre-warmed to 37°C. The tubes were incubated at 37°C for 5 minutes and reactions terminated by adding 4µl stop buffer. Samples were boiled for 3 minutes before loading 4µl onto a sequencing gel.

iii) Sequencing directly from PCR amplified DNA

8µl was removed from the PCR tube and mixed with 1µl of alkaline phosphatase and 1µl exonuclease I. This was incubated at 37°C for 15 minutes and then 80°C for 15 minutes. 1µl of primer was added and the tubes were heated at 100°C for 4 minutes, then placed on dry ice. 7.5µl of reaction mix was added to the denatured DNA/primer solution. The reaction mix was: 2µl label mix, 2µl of diluted sequenase (1:1 v/v in dilution buffer), 1µl of DTT, 2µl of reaction buffer and 0.5µl of α-S^{35} dATP. This was incubated for 4 minutes at room temperature before adding 4µl to 4µl of each ddNTP, which had been pre-warmed to 37°C. These reactions were incubated at 37°C for 3 minutes, before adding 4µl of stop solution. 4µl of each sample was loaded on a sequencing gel after heating at 100°C for 3 minutes.

iv) Sequencing gels

The products of sequencing reactions were run on 6% polyacrylamide gels in SequiGen gel apparatus (BioRad). Long Ranger sequencing 50% acrylamide gel solution (Flowgen) was used, which compresses the bands and does not necessitate fixing. Fresh gel mix was prepared by mixing 20g urea, 22ml H_20, 5.6ml 10x TBE and 4.7ml long ranger gel solution. To seal the base of the gel plates, 10ml of gel mix with 100µl 10% ammonium persulphate (AMPS) and 36µl TEMED added, was poured into gel sealer containers and the plates were stood in these containers until the bottom edge was sealed. 100µl 10% AMPS and 36µl TEMED was added to the remaining gel mix and the plates were carefully filled with this solution using a 25ml glass pipette. The gel was left for an hour at room temperature to ensure it had fully polymerised. Once set, the gel plates were positioned in the lower buffer tank which contained 1x TBE buffer and buffer was added to the top tank until the top edge of the gel was covered. The gel was pre-warmed to 55°C before loading the samples.

Gels were run at 37 Watts, occasionally altering the power to keep the gel temperature at 55°C. The gel was dried at 80°C for 90 minutes under vacuum and was visualised by exposing to X-ray film (BioMax) overnight.

5' end-labelling of oligonucleotides

γ-^{32}P ATP (Amersham, 10mCi/ml) was used as a label and T4 polynucleotide kinase was used to label the 5' end of the oligonucleotide (Midgley and Murray, 1985). A 20µl reaction contained 1x PNK buffer (0.5M Tris-HCl pH7.5, 10mM β-mercaptoethanol and 50% glycerol), 2µl γ-^{32}P ATP, 2µl PNK and the desired concentration of oligonucleotide. This was incubated at 37°C for 45 minutes, then 68°C for 10 minutes. Unincorporated label was removed by size-exclusion chromatography through 1ml G25 sepharose spun-columns by centrifugation at 3000g for 3 minutes (Sambrook *et al.*, 1989). The labelled oligonucleotide was annealed to a complementary unlabelled oligonucleotide by heating at 100°C for 5 minutes, 68°C for 10 minutes, 42°C for 10 minutes, 37°C for 10 minutes and finally incubating on ice for 10 minutes. Labelled oligonucleotides were stored at -20°C.

Protein Techniques

Polyacrylamide Gel Electrophoresis (PAGE)

SDS-PAGE (Laemmli, 1970)

To separate proteins on the basis of size samples were run on SDS-PAGE mini-gels (Pharmacia Biotech). Each gel consisted of a stacking gel and a separating gel. The stacking gel was: 250µl 30% acrylamide, 188µl Tris pH6.8, 15µl 10% ammonium persulphate, 5µl TEMED and 1.04ml H_20. The separating gel was: 1.5ml 30% acrylamide, 1.88ml Tris (pH8.8), 50µl 10% ammonium persulphate, 50µl 10% SDS and 7.5µl TEMED. The separating gel mix was poured between two sealed glass plates and a layer of water added to obtain a smooth gel edge. Once set the water was removed, a comb inserted and stacking gel mix was layered on top of the

separating gel. Samples were mixed with SDS load dye before loading and gels were run in 1x TGS buffer at 35mA. High molecular weight markers (Sigma-Aldrich) were used as standards. Gels were stained with Coomassie ($500ml^{-1}$ methanol, $100ml^{-1}$ acetic acid and $1gl^{-1}$ brilliant blue R250) by gently shaking at 37°C for 30 minutes. They were then destained in 10% v/v methanol 10% v/v acetic acid, by gently shaking at room temperature.

Non-denaturing PAGE

To separate protein and DNA samples were run on native PAGE gels. The gel mix was freshly prepared: 8.4ml 30% acrylamide, 38.9ml H_20, 2.5ml 20xTBE, 500µl 10% AMPs, 50µl TEMED. This was poured between two glass plates held together with bulldog clips with a 1mm spacer to seal the gel (ATTO Corp). Gels were run in 1 litre 1x TBE buffer at 35mA. Native load dye was run in one lane to mark the gel front.

Testing for protein expression

A 1:50 dilution of a fresh overnight culture was grown in a 25ml sterile disposable universal for 6-7 hours at 37°C. 0.5ml of this culture was harvested by centrifugation at 11000g for 5 minutes, the cell pellet was resuspended in 40µl SDS load dye and 10µl was loaded onto an SDS-PAGE mini gel. Protein expression was determined by transferring proteins to nylon filters using Western blotting and detecting protein with specific antibody.

Western Blotting

Proteins were transferred to PVDF membranes (Millipore) by wet electrophoretic transfer (Towbin *et al.*, 1979). After electrophoresis the SDS-PAGE gel was sandwiched between 3 layers of blotting paper pre-soaked in blotting buffer and a PVDF membrane which was pre-soaked in methanol. 3 more layers of pre-soaked blotting paper were placed on top of the membrane. This was placed between two foam pads also pre-soaked in blotting buffer and inserted into the blotting apparatus, making certain that the membrane was on the side nearest the anode. The tank was filled with blotting buffer and run for one hour at 40V at 4°C. The membrane was

removed and protein detected using specific antibody.

Detecting proteins with antibody

The POD detection method (Boehringer Mannheim) was used to detect proteins on nylon filters. All solutions were made up with 1x TBS buffer as suggested in the protocol. This method uses secondary antibody labelled with horse radish peroxidase which catalyzes the oxidation of diacylhydrazides (luminol) to give an activated intermediate which decays to ground state and emits light. The membrane was blocked overnight in 1% block solution at 4°C. The block solution was removed and 10µl of primary antibody to *Eco*KI in 0.5% block solution (1:1000 v/v) was added. This was left shaking at room temperature for one hour. The filter was then washed in 20ml TBST (1% tween in 1x TBS buffer) for 10 minutes, shaking. The buffer was removed, replaced with a fresh solution of TBST and shaken for a further 10 minutes. This buffer was removed and the filter was washed in 0.5% blocking solution for 20 minutes, replacing this solution with fresh 0.5 % blocking solution after 10 minutes. This was removed and the filter was probed with secondary antibody. 25µl of antibody was added to 25mls 0.5% blocking solution and this was left shaking for 30 minutes. The filter was washed in TBST buffer for an hour, replacing the buffer every 15 minutes, using about 40ml buffer at each step. The antibody was detected by adding detection solution for 1 minute, then the membrane was wrapped in Saran wrap (Dow Chemical Corp) and immediately exposed to X-ray film (Curix) for 2 seconds. This film was developed and exposure time altered to achieve the best result.

Protein purification

*Eco*KI nuclease was prepared from 5 litres of cells freshly transformed with plasmid, grown in LB plus ampicillin at 37°C for 7-8 hours, shaking at 220rpm in baffled 2 litre flasks to maximise aeration. The cells were harvested at 8000g for 10 minutes and stored at -70°C until required. The cell pellet was weighed and resuspended in 4x w/v R buffer, with fresh phenylmethylsulphonyl fluoride (PMSF) and Benzamidine added. The cells were sonicated for one minute per gram of cells at 4°C and cell debris was

removed by centrifugation for an hour at 12000g. The volume of supernatant was measured, NaCl was added to a final concentration of 0.4M followed by the addition of 10% neutralized polyethylenimine to 0.4% v/v. This was stirred at 4°C for 15 minutes and then centrifuged at 12000g for 30 minutes. Again the volume of supernatant was measured and 70% w/v NH_4SO_4 was added. This was dissolved by stirring at room temperature for 5 minutes and then stirred at 4°C for 15 minutes to precipitate the protein. The suspension was centrifuged at 12000g for 30 minutes and the pellet was resuspended in 50mls R buffer. This solution was dialysed against 2 litres of R buffer for 3 hours at 4°C to remove salt. The protein solution was then loaded onto a DEAE-Sepharose column (20cm x 1.4cm) at 48mls $hour^{-1}$, the column had been pre-equilibrated in R buffer. Once the protein had been loaded onto the column it was washed in R buffer at 48mls $hour^{-1}$ until all unbound protein had eluted. The column was then washed in 500ml 0→0.5M NaCl gradient overnight at 12mls hr^{-1}. 10µl of selected fractions were run on two 9% SDS-PAGE gels to identify fractions containing nuclease. These fractions were pooled and dialysed at 4°C for 3 hours against 2 litres of R buffer. The protein solution was then loaded onto a heparin-agarose column (12 x 1.5cm) at 48mls hr^{-1}, which had been pre-equilibrated in R buffer at 48mls hr^{-1}. Unbound protein was eluted by washing at 48mls hr^{-1} in R buffer and the nuclease was eluted by washing in 500mls 0→1M NaCl gradient at 12mls hr^{-1}. Fractions containing the eluted nuclease were identified, pooled and dialysed against 2 litres of R buffer for 3 hours at 4°C. This was loaded onto a pre-equilibrated heparin-agarose column (1 x 15cm) at 36mls hr^{-1}. The column was then washed in R buffer at 36mls hr^{-1} for 30 minutes. Protein was eluted from the column by washing at 36mls hr^{-1} in 1M NaCl. Fractions containing protein were pooled and the volume measured. The protein sample was usually divided into two; one half of the protein fraction was kept at 4°C overnight, while the other half was loaded onto a Superdex 200 column (60 x 1.5cm) at 48mls hr^{-1}. This column had been pre-washed in 0.2M NaCl at 48mls hr^{-1} for at least 4 hours. To elute the nuclease the column was washed in 0.2M NaCl overnight at 12mls hr^{-1}. The fractions containing nuclease were identified, pooled and stored at 4°C while the second sample of nuclease was run on the superdex column overnight. Both nuclease samples were pooled and the protein

concentration measured on a spectrophotometer. The solution was added into microconcentrators (Microsep) and centrifuged at 8000g until micromolar concentrations of protein were obtained. The volume of protein solution was carefully measured and this was diluted to give a final concentration of 50% glycerol and stored at -20°C.

Measuring protein concentration

The concentration of protein solutions were determined by measuring UV-light absorbance at 280nm and calculating the concentration from the molar extinction coefficient (ε) estimated from the sum of values of the absorbance of tyrosine, tryptophan and phenylalanine residues in the protein (Mihalyi, 1970). *Eco*KI nuclease has a molar extinction coefficient of 371606.

Biochemical assays

Nuclease assay

Nuclease activity was assessed by measuring the digestion of a plasmid over 60 minutes. The plasmid, pRH3, contains two unmodified recognition sites for *Eco*KI (Sain and Murray, 1980). Reactions were performed in 100µl volumes containing 10nM *Eco*KI, 5nM pRH3, 0.1mM *S*-adenosyl-methionine and 50µgml^{-1} BSA. The buffer was 33mM Tris-acetate, 10mM magnesium acetate, 66mM potassium acetate, and 0.5mM dithiothreitol, pH 7.9. The reaction was started with the addition of 2mM ATP. 10µl samples were removed after 0.5, 1,2, 5, 10, 20, 40 and 60 minutes and then heated to 68°C for 10 minutes to stop the reaction. The aliquots were placed on ice until the assay had been completed. 5µl of load dye was added to each sample and these were loaded onto 1% agarose gels. After electrophoresis, the gels were washed in ethidium bromide solution for 30 minutes, washed in water for 5 minutes and visualised under UV light.

DNA binding assay

DNA binding ability and ATP interaction were investigated using gel retardation assays. A 45mer containing one *Eco*KI recognition site (made from complementary synthetic oligonucleotides) was used as DNA substrate:

5′ TGTCTAGATATCGGCCTAACCACGTGGTGCGTACGAGCTCAGGCG 3′

3′ ACAGATCTATAGCCGGATTGGTGCACCACGCATGCTCGAGTCCGC 5′

The *Eco*KI recognition site is underlined. The 5′ end of the bottom strand was labelled with γ-^{32}P using polynucleotide kinase and hybridised to the complementary strand. Increasing concentrations of protein (0→20nM) were mixed with 0.1nM of 45mer in the presence of 100μM SAM, 5% glycerol, with or without 2mM ATP, in freshly prepared 1x binding buffer. This was incubated at room temperature for 10 minutes before running on 5% PAGE gels. Native load dye was loaded into one lane and the gel was run at 35mA until the dye was near to the end of the glass plate. The gels were dried at 80°C for 90 minutes under vacuum and then exposed to photographic film overnight. To measure the binding affinity of the nucleases for DNA the gel was placed in a phosphorimager cassette and the amount of unbound DNA was quantified using Imagequant software.

ATPase assay

ATPase activity was measured by following the release of radiolabelled inorganic phosphate (Pi) from ATP. Two substrates were used in the reaction; either pRH3 or the 45mer oligonucleotide duplex used in the DNA binding assay (without the radioactive label). 100μl reactions contained 10nM nuclease, 5nM pRH3 or 10nM 45mer, 100μM AdoMet, 50μgml^{-1} BSA and 2mM ATP. The buffer was 33mM Tris-acetate, 10mM magnesium acetate, 66mM potassium acetate, and 0.5mM dithiothreitol, pH 7.9. Everything except ATP was mixed and tubes were left for 5 minutes at 37°C before starting the reaction with ATP. The ATP solution contained 0.2μCi γ^{32}P ATP. 10μl samples were removed after 0.5, 1, 2, 5, 10, 15, 20, 40 and 60 minutes and mixed with 2.5μl 0.5M EDTA pH 8.0 to stop the reaction. The release of inorganic phosphate was followed by spotting 1μl of each sample onto a PEI-cellulose TLC plate, spots were placed on top of dried 10mM ATP/10mM ADP

spots used as an internal marker. The spots were dried, then the plate was sandwiched between two glass plates and held in place using bulldog clips. The plate was stood in TLC buffer and left until the solution front had stopped moving up the plate. The plate was dried using a hairdryer, then exposed in a phosphorimager cassette overnight. The spots were scanned using a phosphorimager and analysed using Imagequant software to quantify the amount of inorganic phosphate released. To measure any contaminating ATPase activity a negative control was performed in parallel with each reaction. This was the same reaction mix without AdoMet. Samples were removed at the same time as reaction samples and the background ATPase activity plotted. The equation of this line was calculated and each point from the reaction was corrected by subtracting the amount of background ATPase activity in the reaction mix at that time.

Results

Site-directed mutagenesis of *hsdR* to make changes in DEAD box motifs

A mutational analysis of the DEAD box motifs of the HsdR polypeptide of *Eco*KI was used to investigate the role of these sequences in the restriction of DNA. To introduce mutations for each motif degenerate oligonucleotides were designed, which produced three different amino acids at a chosen position. At least one amino acid was substituted in each of the seven DEAD box motifs. The amino acid residue selected for mutational analysis was one predicted to be relevant, because it was commonly conserved, but generally one for which an alternative residue had been identified in known DEAD box proteins. The results of previous mutational studies in other DEAD box proteins were also considered, particularly for motifs I (GKT), II (DEAH) and III (TAT) which have been extensively investigated. The mutations made and the resulting amino acid substitutions are shown in table 1.

In motif I (GKT) the conserved lysine residue was substituted. From crystallization studies of the ATPases adenylate kinase and recA, the lysine residue of this motif was shown to interact with the β and γ phosphates of the nucleotide (Fry *et al.*, 1986, Story and Steitz, 1992). A change from lysine to asparagine in eIF-4A abolished ATP binding (Rozen *et al.*, 1989) and in Rad3 protein of yeast a change of lysine to arginine did not affect ATP binding, but abolished ATPase and DNA helicase activities (Sung *et al.*, 1988). This result indicates that the positive charge on the amino acid is important for ATP binding. In *Eco*KI the lysine residue was changed to an arginine to retain this positive charge and to two less similar amino acids.

Motif II, the so-called DEAD motif shows variation that is characteristic of the two different subgroups of DEAD box proteins (DEXH and DEAD) (Fuller-Pace and Lane, 1992). In both the HsdR and Res polypeptides of type I and type III type restriction endonucleases respectively, motif II has the sequence DEXH rather than DEAD, where X indicates a variable amino acid at this position.

Motif	Amino acid sequence	Mutation	Amino acid substitutions
I	GTGKT	ata	K477I
		aga	K477R
		aca	K477T
Ia	LFLVDRR	cac	D502H
		aac	D502N
		tac	D502Y
II	IVVDEAH	gat	H577D
		aat	H577N
		tat	H577Y
III	LTATP	gac	A619D
		ggc	A619G
		gtc	A619V
IV	LVFCVT	tgc	F730C
		tcc	F730S
		tac	F730T
V	LLTTGVD	tgc	G799C
		cgc	G799R
		agc	G799S
VI	QMKGRATR	cac	Q822H
		cac	R826H
		ctc	R826L
		ccc	R826P

Table 1. The changes made within each DEAD box motif. The mutated nucleotide within the relevant codon is shown and the resulting amino acid substitution is given. The number defines the position of the amino acid in the HsdR sequence.

A change from DEAD to DEAH in eIF-4A caused loss of activity *in vivo* and gave only 10% of wild-type levels of helicase activity *in vitro* (Schmid and Linder, 1991; Pause and Sonenberg, 1992). In *Eco*KI the substitutions made included the reverse change of DEAH to DEAD.

In RecG a substitution of valine for alanine in motif III (TAT) abolished the ability of RecG to catalyse branch migration of Holliday junctions (Sharples *et al.*, 1994). In *Eco*KI motif III (TAT) the alanine residue was changed to valine, other substitutions also included a change to glycine, the residue present at this position in motif III of the type IC restriction endonuclease *Eco*R124I.

There have been few mutational analyses of the remaining motifs and therefore residues were selected for change entirely on the basis of their conservation in DEAD box proteins. In motif Ia (FLVDR) the conserved aspartic acid residue was substituted. Motif IV (FCVT) is not well conserved in type I restriction enzymes and therefore phenylalanine, the most conserved residue, was targeted. In motif V (LTTGVD) a glycine residue conserved in type I restriction enzymes was changed to serine which is present in other DEAD box proteins at this position. In motif VI (QMKGRATR) the glutamine residue (Q822) was changed for a histidine residue which is conserved in proteins with the DEAD sequence in motif II. When it was discovered that the HsdR polypeptide including this change could not be detected *in vitro*, alternative substitutions were made for an arginine residue (R826). When this arginine is changed to alanine in vaccinia virus RNA helicase NPH-II, ATPase activity is reduced to 10% of wild-type levels (Gross and Shuman, 1996).

To make mutations in *hsdR* encoding these changes, two different procedures were used. PCR was used to introduce changes in motifs I (GKT), Ia (FLVDR), II (DEAH) and III (TAT). The degenerate oligonucleotide used to make the changes in motif I had the sequence: GACC**GGTACC**GGTABAACCCG, where B indicates a C, G or T at this position instead of the wild-type adenine nucleotide. The sequence in bold identifies the *Kpn*I restriction site. The second primer was the complement of a region 551 bp downstream which included a unique *Bam*HI site. The plasmid pSB2, which contains the whole *hsdR* gene was used as a template in the reaction. Motifs Ia (FLVDR), II (DEAH) and III (TAT) were not sufficiently close to either of

these restriction sites to be included in the same primer as the restriction target. Therefore the mutations in these motifs were made using a technique called recombinant PCR (Higuchi, 1990). This method involved two successive rounds of PCR (see figure 1). The first round of PCR involved two separate reactions; each flanking primer was used in combination with one of the complementary oligonucleotides containing the mutation. The two products were pooled and the original primers were removed by purification through Qiagen PCR purification columns. This DNA mix was used as the template for a second round of PCR that used the two flanking primers, one containing the *Kpn*I target and the other the *Bam*HI site. The products of amplification were digested with *Kpn*I and *Bam*HI, cloned in pUC18 and the complete nucleotide sequence of the insert was determined.

The choice of polymerase used in these reactions was critical, when "Red Hot" DNA polymerase (Advanced Biotechnologies) was used extra changes were incorporated during the reaction at a frequency of about 2 errors per 500 base pairs. Therefore Vent® polymerase (New England BioLabs) was chosen as it possesses 3′ to 5′ proof reading exonuclease activity to correct mistakes that may have been incorporated into the product during the reaction.

Once the nucleotide sequence of the fragment was determined to identify the presence of the required mutation and the absence of additional changes, the mutation was introduced into the context of the whole *hsdR* gene by replacing the *Kpn*I to *Bam*HI wild-type sequence in plasmid pSB2 (figure 2a).

Changes in motifs Ia (FLVDR) and II (DEAH) were incorporated using this method by Tanya Prokhorova and Gareth King respectively.

Changes in motifs IV (FCVT), V (LTTGVD) and VI (QMKGRATR) were made directly during the amplification of plasmid pSB2 taking advantage of the Quikchange™ site-directed mutagenesis procedure (Stratagene). This method uses two oligonucleotides containing the mutation, each complementary to opposite strands of pSB2, as primers to amplify the whole plasmid (see figure 2b). *Pfu* DNA polymerase replicates both plasmid strands without displacing the mutant oligonucleotides. On incorporation of the primers a mutated plasmid containing staggered nicks is generated.

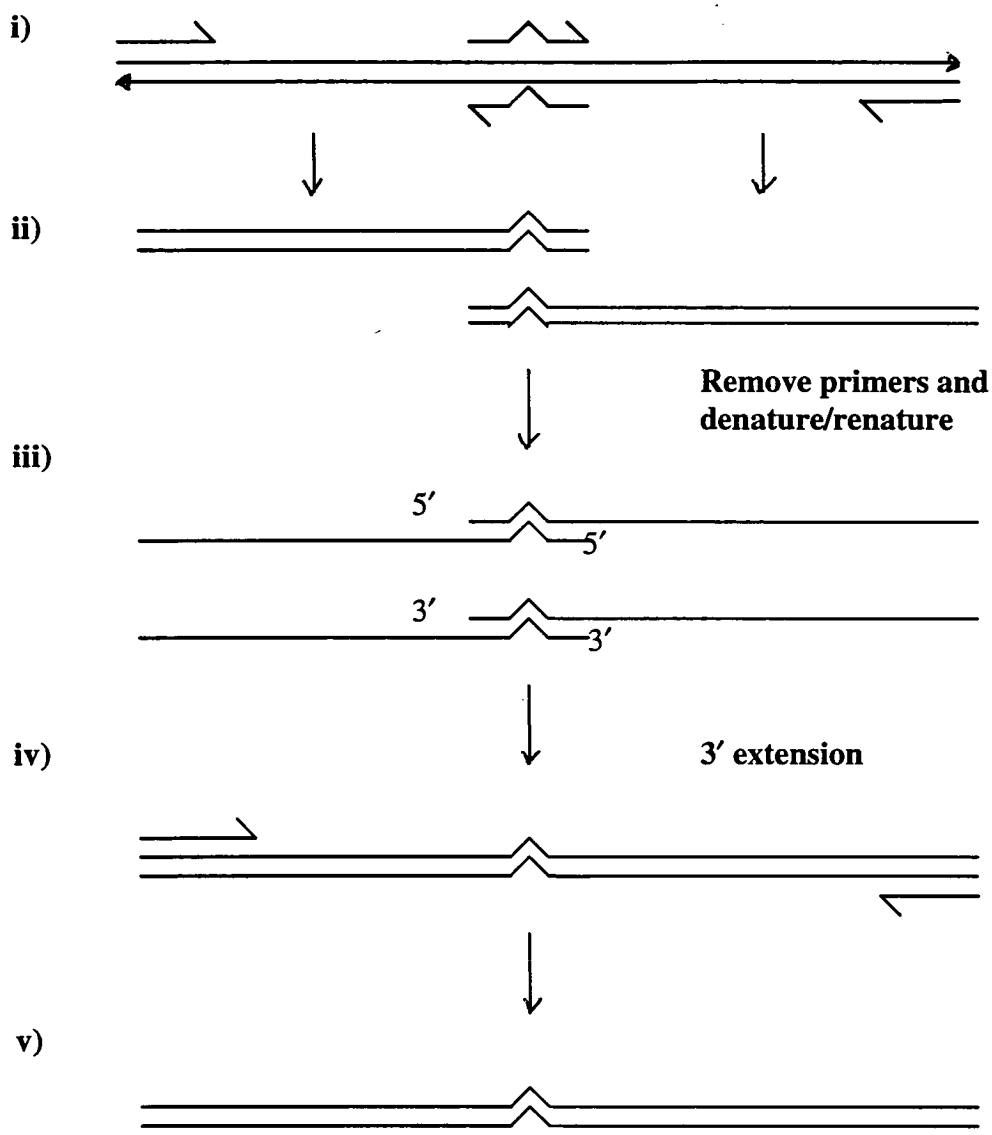


Figure 1. Recombinant PCR (Higuchi, 1990) used to make mutations in motifs Ia (FLVDR), II (DEAH) and III (TAT). i) Lines represent the target DNA sequence, with arrows indicating the directionality of each strand (5′ to 3′). Two PCR products that overlap in sequence are produced, containing the same mutation introduced using the PCR primers (ii). The primary products are denatured and allowed to reanneal which produces two heteroduplex products (iii). The heteroduplexes with 3′ ends are extended by DNA polymerase and the full-length product is produced using the outside primers (v). This PCR product can be digested with *Kpn*I and *Bam*HI and cloned in pUC18.

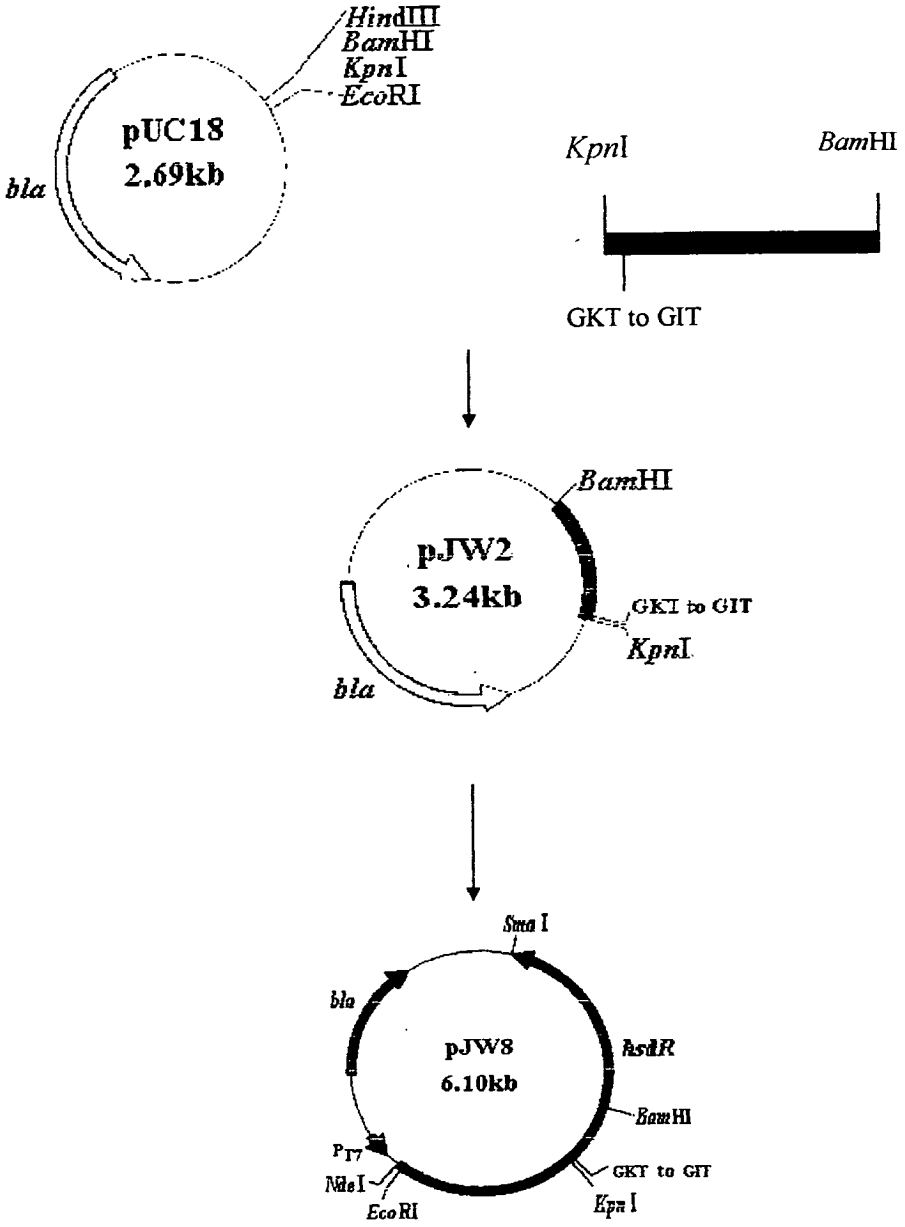
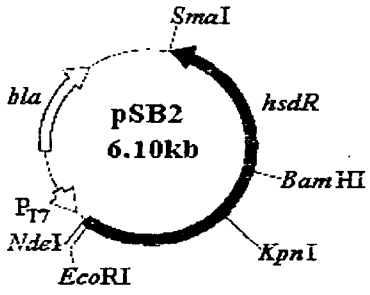
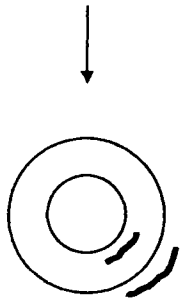


Figure 2a. The steps used to transfer a mutation to the *hsdR* gene. Plasmids pJW2 and pJW8 with a mutation encoding the GIT change in motif I are shown. The PCR product containing the mutation was inserted into pUC18 via *Kpn*I and *Bam*HI restriction sites to give pJW2. The fragment was excised from pJW2 and transferred to the plasmid including *hsdR* (pSB2) to replace the *Kpn*I-*Bam*HI fragment of wild-type sequence, giving pJW8.

i)



ii)



iii)

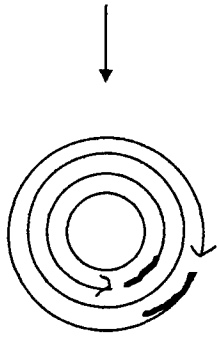


Figure 2b. The Quikchange™ site-directed mutagenesis method used to incorporate mutations directly in *hsdR* in the plasmid pSB2. (i) pSB2 was used as a template in the mutagenesis reaction. Oligonucleotide primers containing the desired mutation were annealed to the denatured template (ii). *Pfu* DNA polymerase extends and incorporates the primers into the *hsdR* sequence, resulting in nicked circular strands (iii). The original template DNA was removed by digestion with *Dpn*I and the new products were isolated after transformation of XLI-Blue supercompetent cells.

The original template was removed by digestion with *Dpn*I, which is specific for methylated or hemi-methylated target sequences. The nicked plasmid products were recovered in Epicurian Coli® XLI-Blue supercompetent cells.

The conditions specified in the Stratagene protocol are intended to decrease the potential for random mutations occurring during the reaction. *Pfu* DNA polymerase has a high fidelity with an average error rate of $1.3x10^{-6}$ mutation frequency/bp/duplication (Cline *et al.*, 1996), so for every twenty plasmids made using this method one may have an extra mutation in the *hsdR* sequence. To confirm that the phenotype produced was a result of the desired mutation, two independent reactions were used to make two plasmids with the same mutation. The presence of the intended mutation was confirmed by sequencing and the restriction phenotypes of both plasmids were assessed. It is extremely unlikely that the r_K^- phenotype resulted from additional mutations in both clones (plasmids), when each clone is of independent origin. It was assumed that the r_K^- phenotype obtained was a result of the intended substitution. All of the six plasmids with substitutions made in motif IV retained an r_K^+ phenotype, consistent with the absence of additional mutations.

One set of mutations in motifs IV, V and the Q to H change in motif VI were made by an undergraduate student under my supervision (Iain Cheeseman), a second set were made by me.

Figure 3 shows the DNA sequence of motifs I (GKT), III (TAT) and VI (QMKGRATR) and the mutations introduced in these motifs.

Figure 3i to 3iii. The DNA sequence of motifs I (GKT), III (TAT) and VI (QMKGRATR) and the mutations inserted in these motifs. The wild-type DNA sequences are shown in the sequencing gels on the left, the other sequencing gels show the three mutations inserted. An arrow indicates the nucleotide changed in each of the three sequences. The amino acid substitutions are shown with the codon change in brackets. The sequencing gels in figure 3i must be read from the top, the gels in figure 3ii must be read from the bottom and the gels in figure 3iii should be read from the top.

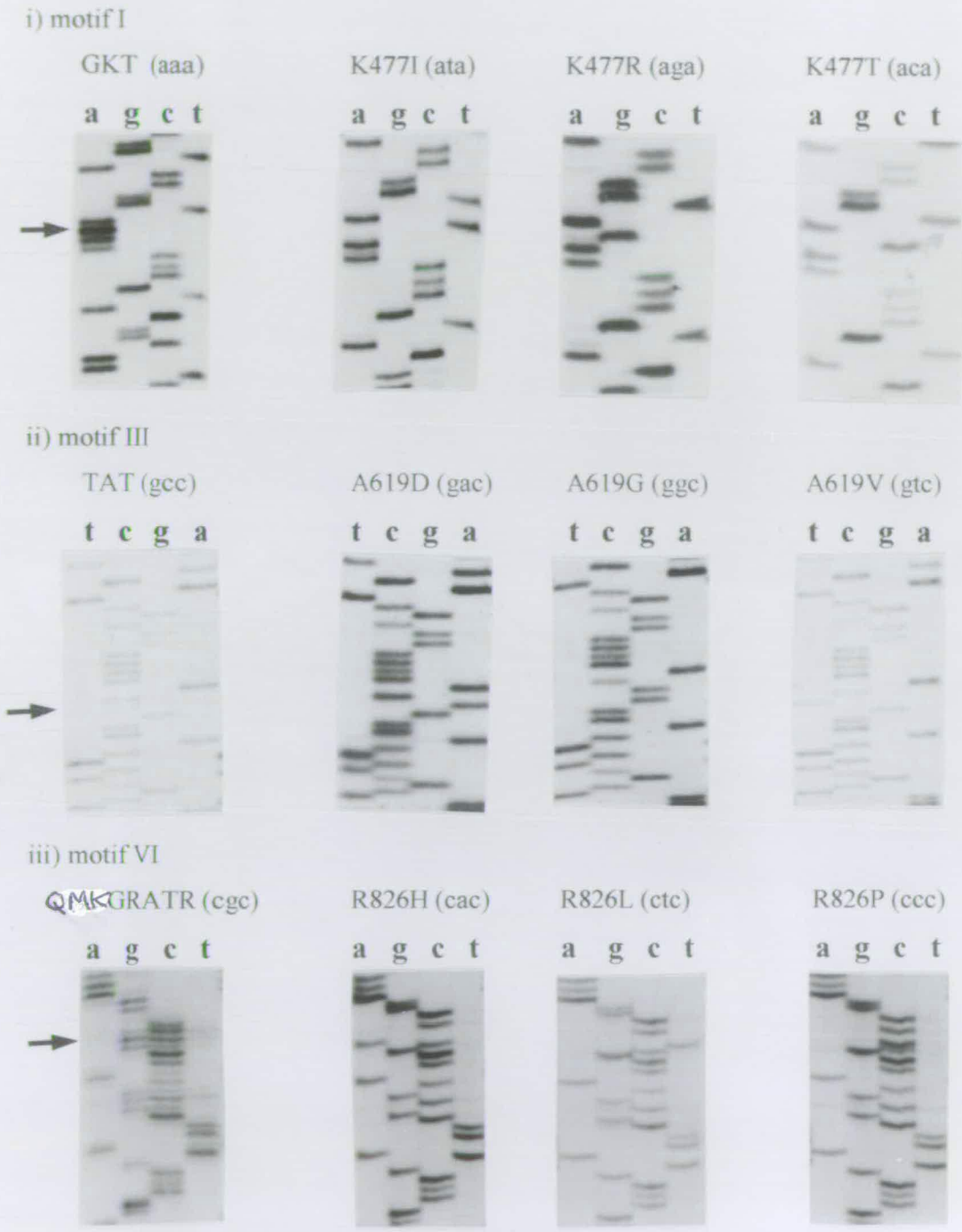


Figure 3 shows the wild-type nucleotide sequence and the mutations inserted in motifs I, III and VI. The amino acid substitutions and the codon change is shown, the arrows indicate the site of mutagenesis.

Effects of mutations on the restriction phenotype of *E. coli* K-12

To assess the effect of changes in the DEAD box motifs restriction phenotypes were determined for the *E. coli* strain AB1157 and its congenic *hsdR*$^-$ derivative NM795 transformed with pSB2 and its mutant derivatives. AB1157 was used because it is *rac*$^-$ and the presence of the Rac prophage has been found to reduce levels of restriction (Loenen and Murray, 1986; King and Murray, 1995).

The restriction phenotype of the transformed cells was determined by assaying the efficiency of plating (e.o.p) of λ*vir*.0. The e.o.p. was calculated relative to the titre on the *hsdR* strain NM795. A low e.o.p. indicates a restriction-proficient phenotype (r_K^+). Table 2 shows the results obtained when the strains carry pSB2, the plasmid encoding wild-type HsdR.

Strain	Efficiency of plating (e.o.p.)
AB1157	3.18×10^{-6} 2.82×10^{-6} 2.67×10^{-6}
NM795 + pSB2	6×10^{-6} 1.45×10^{-5} 3.47×10^{-6}
AB1157 + pSB2	6×10^{-7} 1.45×10^{-6} 1.83×10^{-7}

Table 2. The efficiency of plating of λ*vir*.0 on AB1157 relative to NM795 and the effect on the restriction phenotype when AB1157 and NM795 are transformed with pSB2.

When NM795 is transformed with pSB2, the *hsdR* gene is expressed and the polypeptide combines with host methylase to generate a restriction-proficient strain (r_K^+). When the r_K^+ strain AB1157 is transformed with pSB2, slightly increased levels of restriction are detected in comparison to AB1157 without the plasmid. The relative subunit concentrations normally present in an *hsd*$^+$ cell are [HsdR]<[HsdS]=0.5[HsdM] (Weiserova *et al.*, 1993). This ratio of polypeptides means that there is more methylase than endonuclease in the cell. The presence of pSB2 in AB1157 increases the copy number of *hsdR*. The consequent increase in HsdR polypeptide leads to an increased concentration of *Eco*KI endonuclease.

Table 3 shows the restriction phenotypes when the mutant derivatives of pSB2 were used to transform AB1157 and NM795. When NM795 is transformed with pSB2 containing the mutation in *hsdR*, the effect of the mutation can be assessed by assaying the efficiency of plating of λ*vir*.0 relative to that on NM795 transformed with wild-type pSB2. The effectiveness of this test is absolutely dependent on the cells retaining the plasmid. If the HsdR polypeptide is functional but the cells lose the plasmid, the phage will not be restricted but their progeny will be modified and protected from any subsequent restriction, giving a false r_K^- result. If a mutation that confers a r_K^- phenotype in NM795 is present in AB1157, the high levels of mutant HsdR produced by the plasmid can compete with host HsdR for the methylase and give a r_K^- phenotype. This evidence for an r_K^- phenotype in AB1157 is more reliable than evidence obtained with NM795 because it can only be obtained if the plasmid is retained.

Table 3. Efficiency of plating of λ*vir*.0 on AB1157 and NM795, when the cells carry mutant derivatives of pSB2. The e.o.p. was calculated relative to NM795. The amino acid substitution encoded by the mutation is shown. The number indicates the position of the amino acid in the HsdR polypeptide. The three results shown are from independent tests with the same plasmid, with the exception of the test with the Q822H change which gives results from two plasmids containing the same mutation (see text). *These tests were performed by Diane Ternent and the exact figures were not available, approximate figures are shown.

Motif	Amino acid substitution	e.o.p. on NM795 + plasmid	e.o.p on AB1157 + plasmid
I **GTG<u>K</u>T**	K477I	0.32 0.28 0.26	1.30 1.16 1.05
	K477R	0.72 0.97 0.64	1.28 1.05 1.16
	K477T	0.88 0.90 1.57	1.30 1.02 0.71
Ia **FLV<u>D</u>R**	D502H	0.86 0.68 0.77	0.02 0.08 0.01
	D502N	0.26 0.29 0.20	0.02 0.08 0.01
	D502Y	0.34 0.28 0.21	0.04 0.04 0.01
II **IVVDEA<u>H</u>***	H577D	1	1
	H577N	1	1
	H577Y	1	~$1x10^{-6}$
III **LT<u>A</u>TP**	A619D	0.53 0.43 0.70	1.89 1.30 0.91
	A619G	$2.0x10^{-4}$ $6.9x10^{-4}$ $2.4x10^{-4}$	$1.7x10^{-3}$ $3.3x10^{-3}$ $1.5x10^{-3}$
	A619V	0.29 0.41 0.39	0.50 0.78 0.36

Motif	Amino acid substitution	e.o.p. on NM795 + plasmid	e.o.p. on AB1157 + plasmid
IV **LV<u>F</u>CVT**	F730C	$2.1x10^{-6}$	$8.3x10^{-7}$
		$2.5x10^{-6}$	$1.2x10^{-6}$
		$2.1x10^{-6}$	$1.1x10^{-6}$
	F730T	$2.3x10^{-6}$	$3.4x10^{-8}$
		$2.9x10^{-6}$	$7.4x10^{-8}$
		$6.9x10^{-6}$	$5.1x10^{-8}$
	F730S	$2.5x10^{-7}$	$9.6x10^{-8}$
		$5.0x10^{-7}$	$4.5x10^{-7}$
		$2.8x10^{-6}$	$1.0x10^{-7}$
V **LLTT<u>G</u>VD**	G799C	0.62	0.47
		0.74	0.91
		0.61	0.92
	G799R	0.69	0.75
		1.07	1.25
		0.61	0.91
	G799S	0.84	0.25
		0.47	0.83
		0.74	0.63
VI **<u>Q</u>MKG<u>R</u>ATR**	Q822H	0.22	$3.7x10^{-4}$
		0.21	0.24
		0.57	0.23
	R826H	0.11	0.29
		0.18	0.25
		0.19	0.06
	R826L	0.16	0.15
		0.27	0.16
		0.13	0.12
	R826P	0.11	0.13
		0.11	0.17
		0.06	0.03

The results in table 3 show that most of the mutations in the DEAD box motifs block restriction activity but leave a mutant HsdR polypeptide that can compete with the resident HsdR for host methylase to give a r_K^- phenotype in AB1157. An exception was the H577Y mutation in motif II (DEAH) which gave a r_K^- phenotype in NM795, but did not prevent restriction in AB1157. The substitution of a bulky tyrosine residue may have disrupted correct folding of the HsdR subunit and consequently prevented effective competition with host HsdR for host methylase.

The Q822H substitution in motif VI (QMKGRATR) was made using the Quikchange mutagenesis procedure, therefore two plasmids were made from independent reactions with the same primers. The first plasmid tested gave a r_K^- phenotype in NM795 but did not block restriction in AB1157 (first result in both columns for Q822H in table 3). In contrast, tests with the second plasmid gave a r_K^- phenotype in AB1157. This result suggests the r_K^+ phenotype of AB1157 transformed with the first plasmid made may have been a consequence of the cells losing the plasmid, or the plasmid may have had an extra mutation which disrupted folding and prevented competition with AB1157 HsdR for host methylase. Detectable levels of protein were not observed when the first plasmid made was tested for HsdR production. Production of HsdR polypeptide from the second plasmid made was not tested due to time limitations. A second amino acid residue, arginine 826, was changed in motif VI (QMKGRATR).

Plasmids with changes in motifs Ia (FLVDR), IV (FCVT), V (LTTGVD) and VI (QMKGRATR) were tested for their ability to produce detectable levels of HsdR before selecting a mutation to transfer to the AB1157 chromosome. It was fortunate that the original mutations in motifs I (GKT), II (DEAH) and III (TAT) that were transferred to the chromosome earlier, all produced detectable levels of HsdR polypeptide from the mutant derivatives of pSB2.

Detection of HsdR polypeptide containing changes in the DEAD box motifs

The plasmid pSB2 only encodes HsdR, therefore in order to investigate the effect of the changes on *Eco*KI *in vitro* the mutation had to be transferred to a plasmid encoding all three *hsd* genes (p*hsd*$^+$). A DNA fragment (*Kpn*I to *Bam*HI) was excised from *hsdR* in pSB2 and transferred to the *hsdR* sequence in p*hsd*$^+$ in place of the wild-type fragment. The effects of the amino acid substitutions on the production of nuclease from p*hsd*$^+$ was tested using antibody to the HsdR and HsdM subunits. Some mutations resulted in a loss of detectable levels of HsdR, figure 4 shows a Western blot that illustrates the effect of different amino acid changes on production of HsdR polypeptide detected by antibody to *Eco*KI. Occasionally a subunit smaller than HsdR, but larger than HsdM, was visible on a Western blot, for example lanes 7 and 8 in figure 4. This may indicate that the substitution has disrupted correct folding of HsdR and the protein has been partially degraded by proteases in the cell. The HsdR band in the lane where purified nuclease was loaded (lane 9) has 'burnt out', leaving a space in the middle of the band. This is a consequence of the detection method used and occurs when high concentrations of protein are detected.

If the mutant HsdR showed competition with host HsdR in the AB1157 restriction phenotype tests (table 3), this provided some evidence that the subunit was folding sufficiently well that it would interact with HsdM and HsdS polypeptides. However this result was not necessarily an indication that HsdR would be produced at levels detectable with antibody. Table 4 summarises the effects of the different amino acid substitutions on the detection of HsdR and indicates which mutations were selected for analysis *in vitro*. Expression of the *hsd* genes depended on the *hsd* promoters and the increase in copy number provided by the plasmid vector produced detectable amounts of protein.

Figure 4. The effect of changes in the DEAD box motifs in HsdR on the production of *Eco*KI. Protein samples were run on SDS-PAGE, transferred to PVDF membrane and detected with antibody to the HsdR and HsdM subunits using the POD detection system (Boehringer Mannheim). The arrows indicate the position of HsdR and HsdM on the gel. Lane 1 shows the background from cells without plasmid and lane 2 shows protein detected when these cells are transformed with p*hsd*$^+$. Full length HsdR was detected for polypeptides with the changes H577D (motif II), A619G (motif III) and A619V (motif III). No polypeptides were detected for the change D502H (motif Ia) and fragments of HsdR were seen for the G799S (motif V) and Q822H (motif VI) substitutions.

Lanes: 1) NM679, 2) Wild-type, 3) D502H, 4) H577D, 5) A619G, 6) A619V, 7) G799S, 8) Q822H, 9) Purified *Eco*KI.

Motif	Amino acid substitution	Detection of HsdR	Protein purified
I	K477I	nt	
	K477R	+	K477R
	K477T	nt	
Ia	D502H	-	
	D502N	-	D502Y
	D502Y	+	
II	H577D	+	
	H577N	nt	H577D
	H577Y	nt	
III	A619D	nt	
	A619G	+	A619G and
	A619V	+	A619V
IV	F730C	nt	
	F730S	+	F730S
	F730T	nt	
V	G799C	+	
	G799R	-	G799C
	G799S	f	
VI	Q822H	f	
	R826H	+	R826H
	R826L	nt	
	R826P	nt	

Table 4. The effects of changes in the DEAD box motifs on the detection of HsdR by Western blots. The proteins selected for purification are shown. + indicates full length HsdR was detected, - indicates no HsdR detected and f indicates a fragment of HsdR was produced. nt indicates not tested.

To quantify levels of restriction *in vivo*, one mutation in each motif (two in motif III) was transferred to the AB1157 chromosome, as described in the methods section. Once on the chromosome the mutation is stably maintained and in single copy, under the control of its normal promoter. These results can then be compared with a wild-type *E. coli* strain. These restriction phenotype results are shown in table 5.

The restriction phenotype of the TGT mutant in motif III was studied further by testing the restriction of unmodified phage containing one or two recognition sites for *Eco*KI, rather than the five found in wild-type lambda. Reducing the number if targets increased the sensitivity of the assay. These results are shown in table 6.

Motif and amino acid substitution	**Efficiency of plating**
I GTG**K**T (K477R)	1.01 ± 0.22
Ia LFLV**D**RR (D502Y)	0.92 ± 0.05
II IVVDEA**H** (H577D)	~1*
III LTA**T**P (A619G)	$2.09 \times 10^{-5} \pm 2.22 \times 10^{-5}$
(A619V)	0.97 ± 0.22
IV LV**F**CVT (F730S)	$1.37 \times 10^{-5} \pm 5.91 \times 10^{-6}$
V LLTT**G**VDI (G799C)	1.25 ± 0.21
VI QMKG**R**ATR (R826H)	1.04 ± 0.47

Table 5. The efficiency of plating of λ*vir*.0 on mutant derivatives of AB1157, relative to an r_K^- derivative of AB1157. The amino acid changed in each motif is in bold and the amino acid substitution is indicated in brackets. The mean e.o.p. was calculated from at least four results and the standard deviation is shown. *The H577D mutant was tested by Diane Ternent and the exact e.o.p. is not shown.

Phage (targets for *Eco*KI)	Efficiency of plating		Restriction	
	Wild-type	Mutant (TGT)	Wild-type	Mutant
λNM105 (skλ1 + skλ2)	$2.7x10^{-4}$	$1.1x10^{-2}$	3227 ± 1007	121 ± 51
	$2.9x10^{-4}$	$9.6x10^{-3}$		
	$5.7x10^{-4}$	$1.1x10^{-2}$		
	$2.5x10^{-4}$	$5.1x10^{-3}$		
λNM175 (skλ1)	$1.6x10^{-2}$	$8.6x10^{-2}$	38 ± 23	8.3 ± 2.4
	$1.6x10^{-2}$	$1.2x10^{-1}$		
	$7.7x10^{-2}$	$1.4x10^{-1}$		
	$3.8x10^{-2}$	$1.1x10^{-1}$		
	$4.3x10^{-2}$	$1.9x10^{-1}$		
λNM176 (skλ2)	$2.3x10^{-2}$	$3.1x10^{-1}$	33 ± 10	3.5 ± 1.5
	$2.8x10^{-2}$	$4.8x10^{-1}$		
	$2.9x10^{-2}$	$3.1x10^{-1}$		
	$5.0x10^{-2}$	$1.8x10^{-1}$		

Table 6. The efficiency of plating of phage with one or two recognition sites for *Eco*KI and the corresponding restriction phenotype of the mutant TGT (A619G) and AB1157. Published in Webb *et al.*, 1996. In all experiments a hybrid phage with no targets for *Eco*KI gave an e.o.p. of ~1. The value for restriction was estimated from the inverse of each e.o.p, the means and standard deviations are shown.

The results in table 5 confirm the plasmid phenotype results and show that the DEAD box motifs have an important role in restriction by *Eco*KI. Changes in all motifs affected restriction except changes in motif IV (FCVT), which gave wild-type levels of restriction. The H577D substitution, a conservative change of DEAH to DEAD in motif II, abolished restriction. The A619G substitution in motif III creates the amino acid sequence TGT, which is the sequence present in motif III in *Eco*R124I. When infected with λ*vir*.0 phage, which contains five recognition sites for *Eco*KI, levels of restriction in the A619G mutant appeared much the same as wild-type levels. The sensitivity of the restriction phenotype assay was increased by infecting the cells with phage containing one or two *Eco*KI recognition sites, results are shown in table 6. A noticeable decrease in restriction levels was detected with the A619G mutant compared to wild-type. This was the only mutation that merely impaired restriction, all other changes that had an effect totally abolished restriction.

To gain more insight into how these mutations were affecting restriction, the effects of these changes on different biochemical activities were assessed. The activities tested were those associated with DEAD box motifs such as DNA binding, ATP binding and ATPase activity. Eight proteins with changes representing each of the seven DEAD box motifs, including two proteins with changes in motif III, were purified for *in vitro* analysis. In every case mutations that were transferred to the *E. coli* chromosome encoded the change selected for purification.

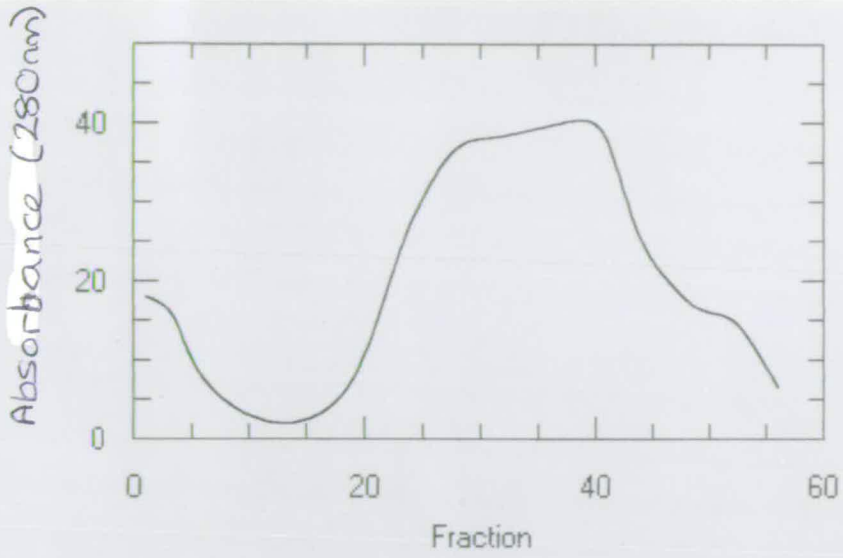
Purification of *Eco*KI with changes in the DEAD box motifs

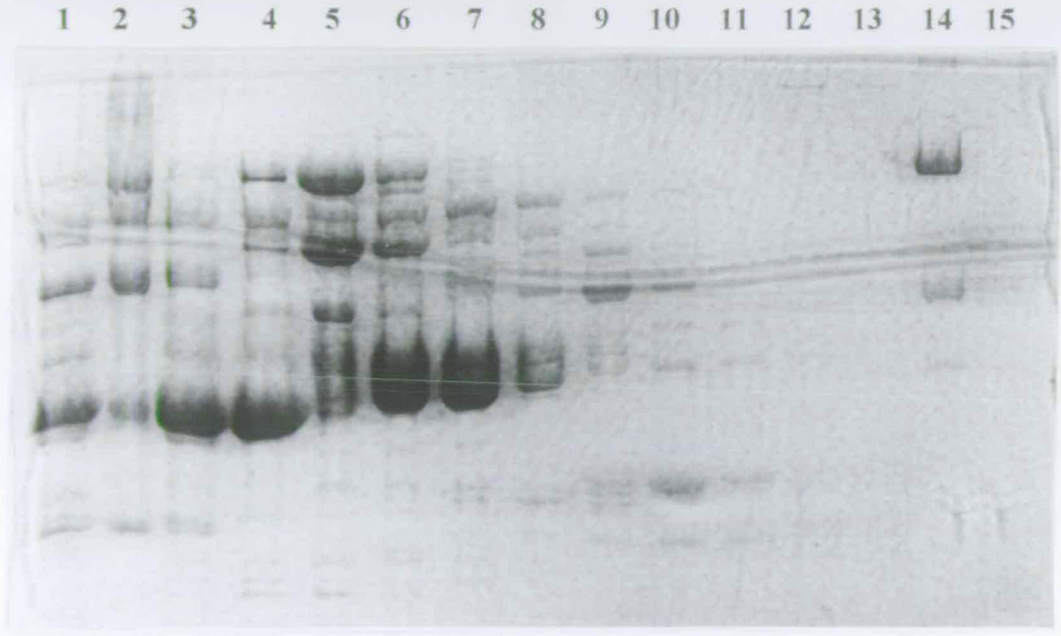
*Eco*KI was harvested from NM679 cells transformed with the derivatives of p*hsd*$^+$ and purified through a series of chromatography columns, as described in detail in the methods section.

Figures 5a-c show the steps involved in purification of *Eco*KI, with the results obtained when purifying *Eco*KI with a D502Y change in motif Ia (FLVDR). Figure 5a shows the protein elution profile from the first column, a DEAE column. The protein mixture was loaded onto this column, the column was washed and bound proteins were eluted using a salt gradient. The elution profile identifies the fractions that contain protein. Samples (of fractions) were loaded on a 9% SDS-PAGE gel to identify fractions containing *Eco*KI. The gel is shown in the lower panel of figure 5a. A large amount of protein binds to the DEAE column and fractions containing *Eco*KI also contain many other proteins. Fractions containing nuclease (32-40) were pooled, the protein solution dialysed and then loaded onto a heparin column (see fig 5b). This column effectively separates many of the contaminating proteins from *Eco*KI, with just a protein smaller in size than HsdS detected in some fractions that contain *Eco*KI. Fractions containing *Eco*KI (41-47) were pooled, the protein solution was dialysed and then loaded onto a smaller heparin column to concentrate the sample. Once concentrated, up to 3mls of the protein solution was loaded onto a Superdex gel filtration column. Figure 5c shows the protein elution profile from this column. The column efficiently removed the small contaminating protein to give apparently pure *Eco*KI. Fractions containing nuclease were pooled and concentrated using microconcentrators (Microsep) until micromolar concentrations of protein were obtained.

Each of the nucleases behaved in the same way as wild-type *Eco*KI, eluting from the columns at similar times. The yields of the purified proteins are compared in table 7.

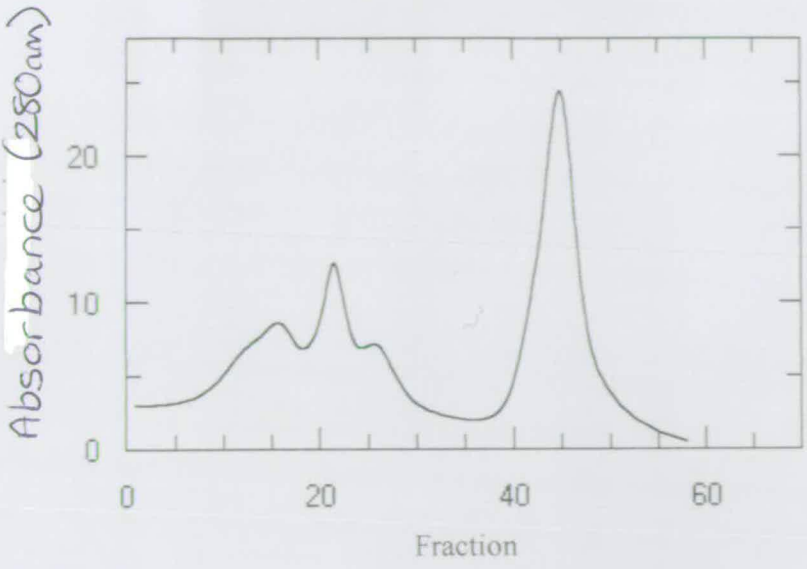
Figure 5a. Purification of *Eco*KI with a D502Y substitution in motif Ia (FLVDR). The upper panel shows the protein elution profile from the DEAE column. The lower panel is a 9% SDS-PAGE gel with fractions from this column, the gel was stained with Coomassie Blue to identify which fractions contain nuclease.

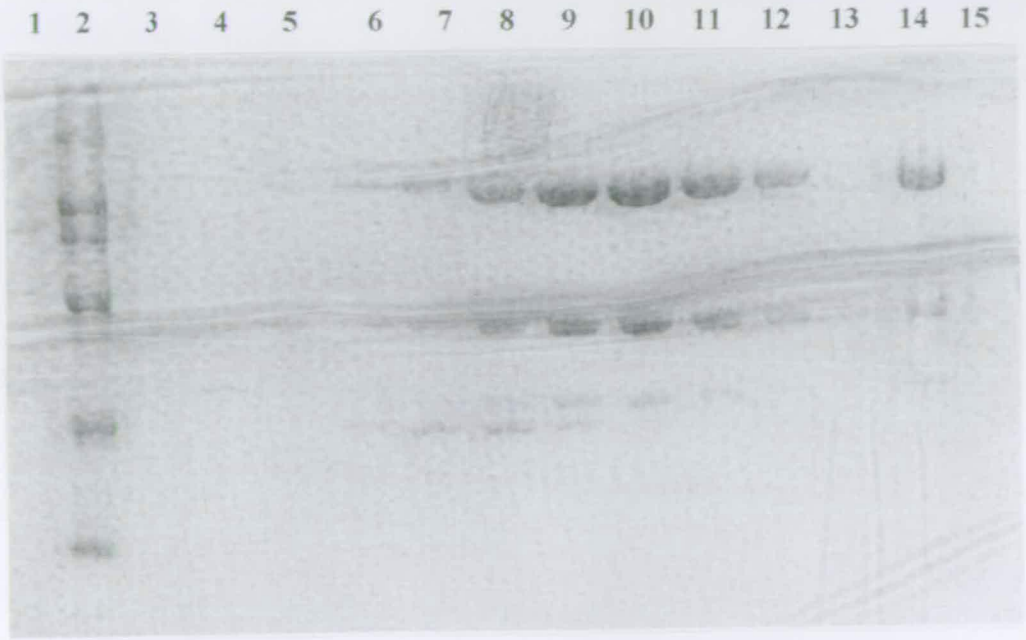




Lanes 1-15: fraction 29, mw marker, fraction 31, 34, 37, 39, 41, 43, 45, 47, 49, 51, 53, purified nuclease, 56.

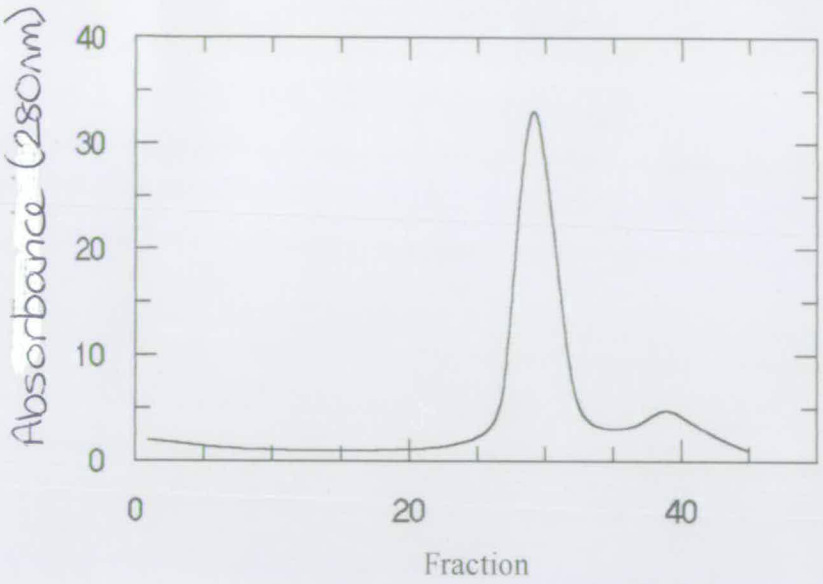
Figure 5b. The purification of *Eco*KI with the amino acid substitution D502Y in motif Ia (FLVDR). The upper panel shows the elution profile of protein from the heparin column. The lower panel is a 9% SDS-PAGE gel with some fractions from this column, stained with Coomassie Blue to show the polypeptides in each fraction.

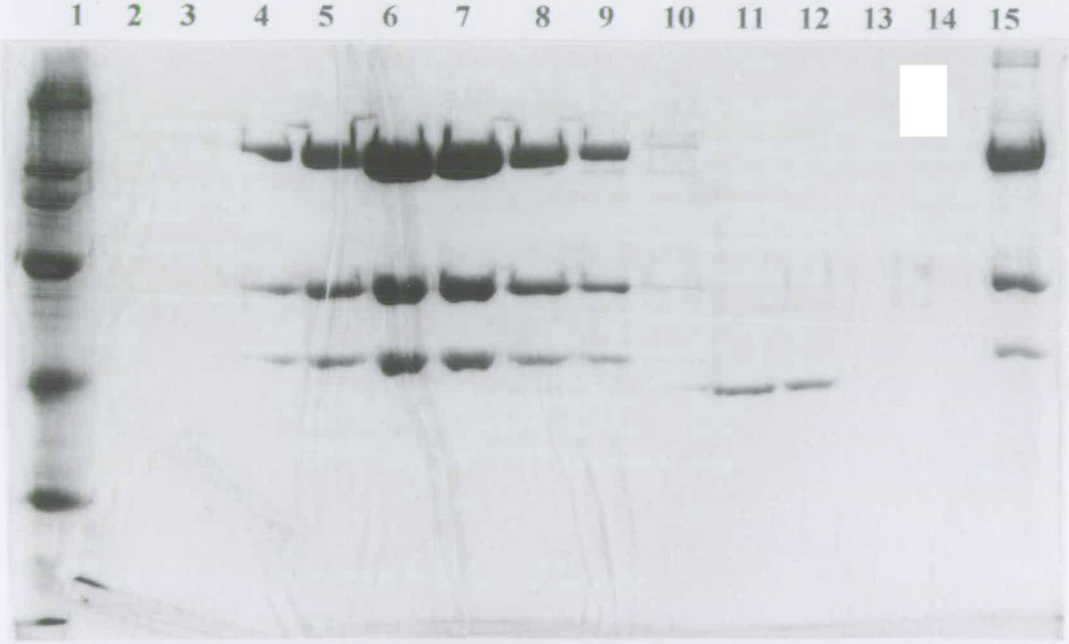




Lanes 1-15: fraction 27, mw marker, fraction 29, 31, 38, 40, 42, 43, 44, 45, 46, 47, 49, purified nuclease, 58.

Figure 5c. The purification of *Eco*KI with a D502Y substitution in DEAD box motif Ia (FLVDR) in HsdR. The upper panel shows the elution profile of protein from the Superdex gel filtration column. The lower panel is a 9% SDS-PAGE gel loaded with some of these fractions and stained with Coomassie Blue to identify fractions that contain *Eco*KI.





Lanes 1-15: mw marker, fraction 7, 14, 27, 28, 29, 30, 31, 32, 33, 39, 41, 52, 62, purified nuclease.

Motif	Amino acid substitution	Amount of protein purified
I GKT	K477R	0.59mg
Ia FLVDR	D502Y	2.09mg
II DEAH	H577D	5.36mg
III TAT	A619G	1.80mg
	A619V	2.76mg
IV FCVT	F730S	3.44mg
V LTTGVD	G799C	0.48mg
VI QMKGRATR	R826H	3.37mg

Table 7. Yields of purified *Eco*KI with changes in the DEAD box motifs of HsdR. All proteins were harvested from 5 litres of cells transformed with derivatives of p*hsd*$^+$ containing the mutation specifying the intended change.

These figures contradict the relative amounts of protein detected in the Western blot in figure 4, page 66, where higher levels of HsdR polypeptide with the A619G change compared to HsdR with the A619V change were detected. In contrast table 7 shows a greater yield of the A619V mutant was achieved after purification. The Westerns were used to determine whether or not the mutations would allow production of detectable amounts of protein and not to compare relative levels of protein production. Protein samples were often difficult to load onto the SDS-PAGE gels and consequently the Western blots could not be used to compare levels of protein production. This difference in sample loading can be seen by comparing the intensity of the background smear that is detected in the Western blot in figure 4. This background in the lane with the A619G mutant (lane 5) is more intense than the background in the lane loaded with the A619V mutant (lane 6), indicating that more of the A619G protein sample was loaded onto the gel.

Nuclease activities of purified proteins

The purified proteins were tested for nuclease activity. The substrate for this assay was the plasmid pRH3, which has two recognition sites for *Eco*KI. This DNA is efficiently cleaved within 20 minutes at 37°C when one molecule of *Eco*KI per recognition site is used (Dryden *et al.*, 1997). In this study, the assays were done to check whether the *in vitro* activity of the enzyme correlated with the *in vivo* restriction phenotypes and not to compare rates of nuclease activity.

The reaction was started with the addition of ATP and samples were removed at different time points from 0.5 to 60 minutes. The samples were heated at 68°C for 10 minutes to stop the reaction and the products analysed on an agarose gel. Experiments were done in parallel with negative and positive controls. The negative control was a sample taken after 60 minutes from an identical reaction without AdoMet. The positive control, used when assaying r_K^- mutants, was an aliquot from a reaction with wild-type *Eco*KI that was stopped after 60 minutes.

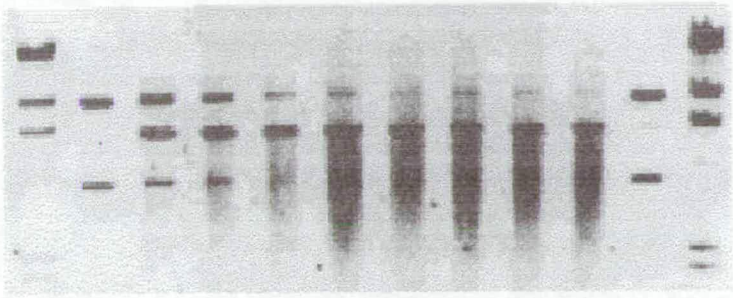
Figures 6a to 6i show the effects of the mutations on nuclease activity. The results were consistent with the corresponding *in vivo* restriction phenotypes of the mutants shown in table 5. Figure 6a shows the nuclease activity of wild-type *Eco*KI, which first linearizes the plasmid and then further degrades the DNA to produce a smear of DNA fragments of different sizes. Figure 6b shows that the protein with a K477R substitution in motif I (GKT) does not possess a nuclease activity. The two bands on the agarose gel represent supercoiled and open-circle (nicked) plasmid DNA. A faint band representing linear DNA can be seen, an indication that the plasmid stock used in the assay included some degraded DNA. A similar result is seen with *Eco*KI with changes in motifs Ia (FLVDR) (fig 6c), motif V (LLTGVD) (fig 6h), motif VI (QMKGRATR) (fig 6i) and the A619V change in motif III (TAT) (fig 6f). The protein with a change in motif II (DEAH) (fig 6d) also does not have any nuclease activity, most of the DNA runs as supercoiled plasmid consistent with the use of a fresh preparation of plasmid DNA in the assay. Unusually, a distinct band can be seen in the positive control lane. However for the purpose of this assay the positive control still shows DNA restriction has occurred and allows a distinction to

be made between a positive and negative result. *Eco*KI with the A619G change in motif III (TAT) (fig 6e) and the F730S (fig 6g) substitution in motif IV both showed nuclease activity similar to wild-type.

Figures 6a to 6i. The effect of the mutations on nuclease activity. Reactions were performed in 100µl volumes containing 10nM *Eco*KI, 5nM pRH3, 0.1mM *S*-adenosyl-methionine, 1x buffer A (Boehringer Mannheim) and 50µgml^{-1} BSA. The reaction was started with the addition of ATP to 2mM. 10µl samples were removed after 0.5, 1, 2, 5, 10, 20, 40 and 60 minutes and then heated to 68°C for 10 minutes to stop the reaction. Three DNA bands can be seen: open circle (nicked) DNA, linearized plasmid and supercoiled plasmid in order of increasing migration. The numbers indicate the time of reaction in minutes. M is the marker, u is uncut pRH3, – ve is a sample taken from the negative control after 60 minutes reaction time and +ve is a sample from a positive control after 60 minutes of reaction.

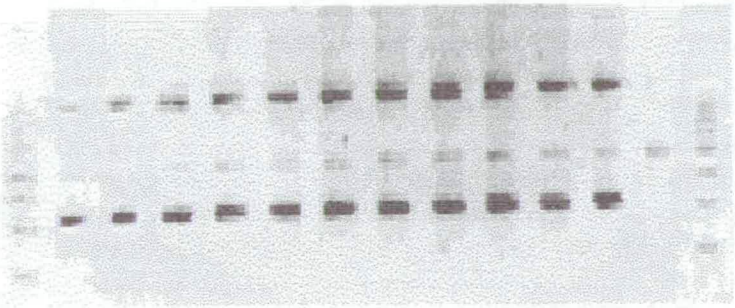
6a. Nuclease activity of wild-type *Eco*KI

M U 0.5 1 2 5 10 20 40 60 –ve M



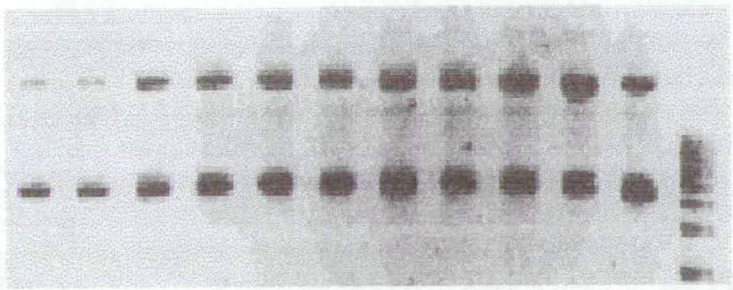
6b. Nuclease activity of K477R (r_K^-)

M U 0.5 1 2 5 10 15 20 40 60 –ve +ve M

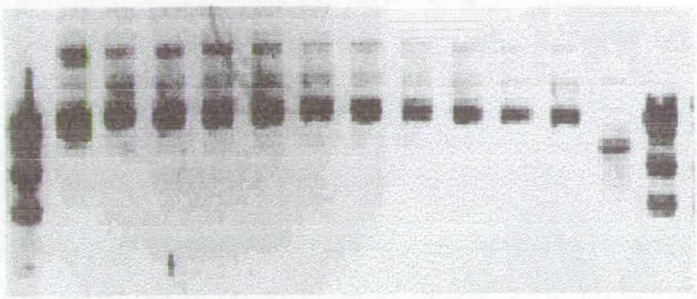


6c. Nuclease activity of D502Y (r_K^-)

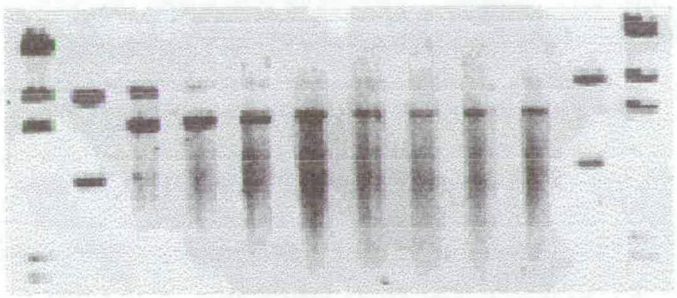
U 0.5 1 2 5 10 15 20 40 60 –ve M



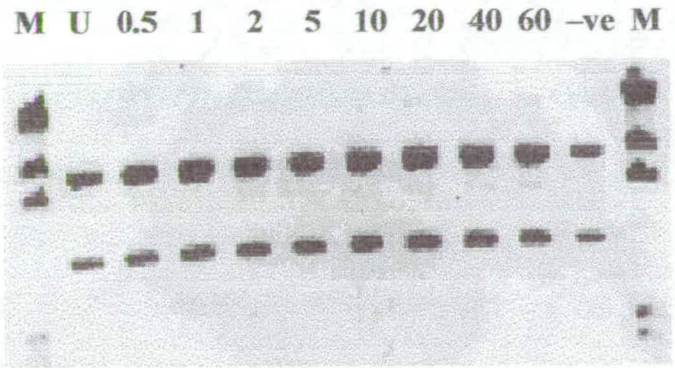
6d. Nuclease activity of H577D (r_K^-)



6e. Nuclease activity of A619G ($r_K^{+/-}$)

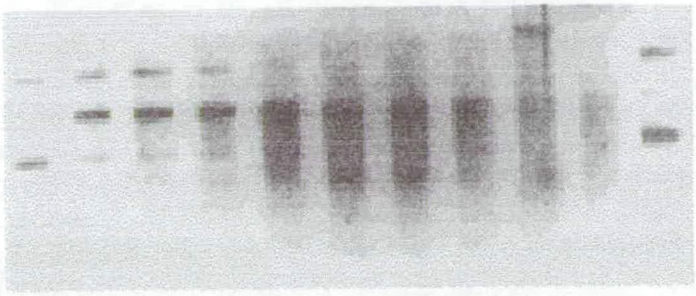


6f. Nuclease activity of A619V (r_K^-)



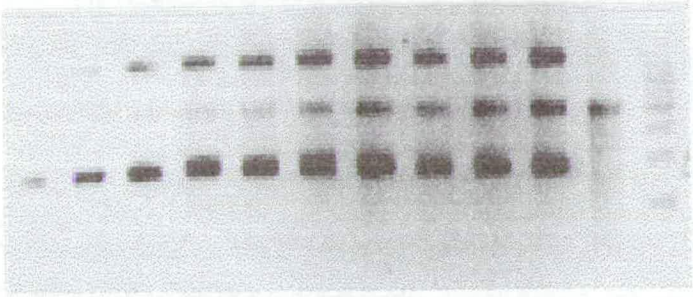
6g. Nuclease activity of F730S (r_K^+)

U 0.5 1 2 5 10 15 20 40 60 –ve



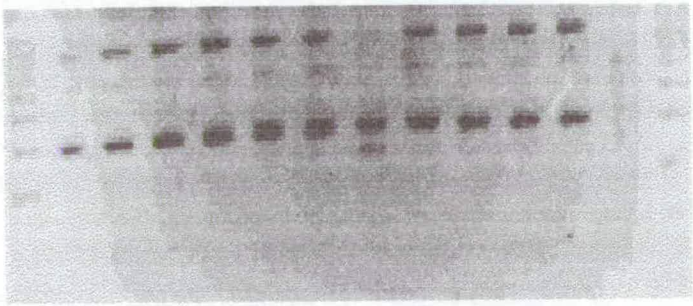
6h. Nuclease activity of G799C (r_K^-)

U 0.5 1 2 5 10 15 20 40 60 +ve M



6i. Nuclease activity of R826H (r_K^-)

M U 0.5 1 2 5 10 15 20 40 60 –ve +ve M



DNA binding and ATP interaction of the purified proteins

To assess the effect of each amino acid substitution on DNA binding, the gel retardation of a 45bp duplex that included one unmodified *Eco*KI recognition site was measured following incubation with increasing concentrations of the relevant protein. DNA (0.1nM) labelled with γ-^{32}P ATP was mixed with protein (0 to 20nM), incubated for 10 minutes and the products were separated on a 5% non-denaturing polyacrylamide gel.

Results are shown in figures 7a to 7r. Figures 7a and 7b show that as wild-type *Eco*KI protein concentration is increased more of the labelled DNA is complexed to protein. Some of the gels show what appears to be labelled single-stranded DNA running below the free DNA, this is most apparent in figures 7m and 7n. This is probably a result of mixing the labelled 45mer with insufficient amounts of unlabelled complementary 45mer during hybridization. The presence of single-stranded DNA does not affect the gel retardation results and a decrease of this single-stranded DNA is not seen over the protein concentration range examined.

Figure 7b shows gel retardation of the 45bp duplex by wild-type *Eco*KI in the presence of ATP. Three different protein-DNA bands are seen; tests using Western blots suggest that these complexes contain the different subunit assemblies of M_2S_1, $R_1M_2S_1$ and $R_2M_2S_1$ in order of decreasing mobility and indeed a complex of purified methylase with DNA migrates at the same position as the band with the highest mobility (Lynn Powell, pers comm). Without ATP the major protein-DNA complex contains $R_2M_2S_1$, with the addition of ATP the major species is $R_1M_2S_1$-DNA. The gel retardation assays using *Eco*KI with amino acid substitutions in HsdR are shown in figures 7c to 7r. All mutant proteins showed an increased tendency to yield the methylase-DNA complex, irrespective of the presence or absence of ATP when compared to wild-type *Eco*KI. As with the wild-type, the addition of ATP increased the dissociation of HsdR subunits from the nuclease-DNA complex for most of the mutant proteins. An ATP-dependent influence on the protein-DNA products detected in the gel is taken as evidence for the interaction of ATP with the *Eco*KI enzyme. However, for *Eco*KI with a H577D change in motif II (DEAH) (figs 7g and 7h) and a

R826H substitution in motif VI (QMKGRATR) (figs 7q and 7r) the retarded DNA ran as DNA-M_2S_1 even in the absence of ATP and consequently no ATP effect was observed for these two proteins. The assays should be performed with higher concentrations of these proteins (30, 40 and 60nM) to try to achieve $R_2M_2S_1$ or $R_1M_2S_1$-DNA complexes.

Direct assays could be used to assess ATP binding. One assay tried followed the elution time of [γ-^{32}P] ATP through a G-25 Sephadex gel filtration column and observed the effect on elution of ATP when *Eco*KI was added before loading onto the column. When wild-type *Eco*KI was added, ATP eluted earlier as a result of the protein binding ATP and preventing ATP molecules from becoming trapped in the pores of the gel matrix. The other proteins tested were *Eco*KI with A619G and A619V substitutions in motif III (TAT). A619G showed some ATP binding but A619V did not show any binding. This assay required high amounts of nuclease and once some gel retardation assays had been done, it was evident that both of these proteins interacted with ATP. The ATP-*Eco*KI complex for the A619V substitution was apparently not sufficiently stable to allow coelution of ATP and nuclease. An alternative way of measuring ATP-binding would be to study UV cross-linking of [α-^{32}P] labelled ATP to the proteins.

To check that retardation of the 45bp duplex was the result of a specific protein-DNA interaction, competition experiments were performed using a similar 45bp duplex without the *Eco*KI recognition site. 100-fold excess cold non-specific DNA or cold specific DNA was mixed with the protein in addition to the labelled specific 45bp duplex. This result is shown in figure 8. When excess cold specific DNA was added it competed with the radiolabelled DNA for protein, so no complex was detected by autoradiography. When excess cold non-specific DNA was added, it did not compete with the radiolabelled DNA for protein binding and a retarded protein-DNA complex was detected. When excess non-specific DNA was added, the species observed were as seen at this protein concentration in the absence of competitor (fig 7b, d, f, h, j, l, n, p, r, 8nM protein track), except for TVT. For this mutant no $R_2M_2S_1$ or $R_1M_2S_1$ complexes were observed in the presence of competitor in contrast to fig 7l track 8. 8nM protein was used in this assay as some free DNA

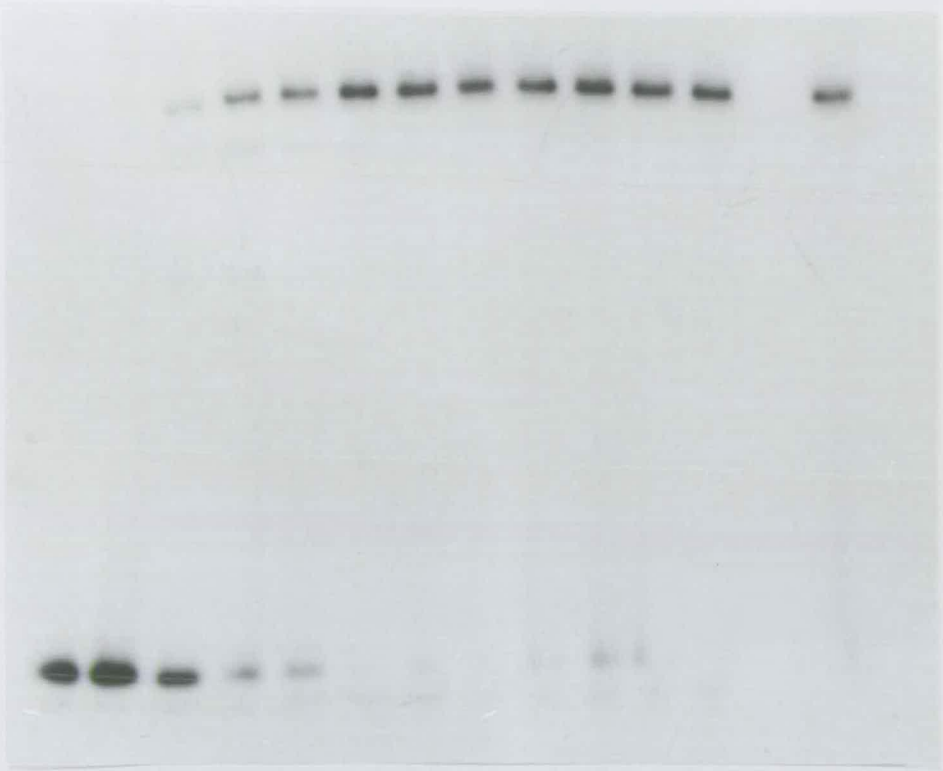
can still be seen on the gels at this concentration (figures 7a to 7r). This assay proved that the bound complexes observed represent a specific interaction of the protein with the *Eco*KI recognition site.

Dissociation constant (K_d) values were estimated by calculating the concentration of protein needed to bind half of the DNA under conditions where the protein was in excess over the DNA (Fried, 1989). These values were determined by plotting log protein concentration against % DNA bound from assays in the presence of ATP and AdoMet (see fig 7 for examples). The proportion of bound DNA was calculated after quantifying the amount of free DNA (using the phosphorimager) for each protein concentration, because this is more accurate than measuring the amount of protein bound DNA (Revzin, 1989). Free DNA does not change once it enters the gel but complexed DNA can dissociate producing a smear which is difficult to quantify. The K_d estimates for the wild-type and mutant proteins are shown in table 8. The accuracy of the K_d estimates is limited by the dissociation of the protein, as the amount of protein in the $R_2M_2S_1$ form changes at different protein concentrations.

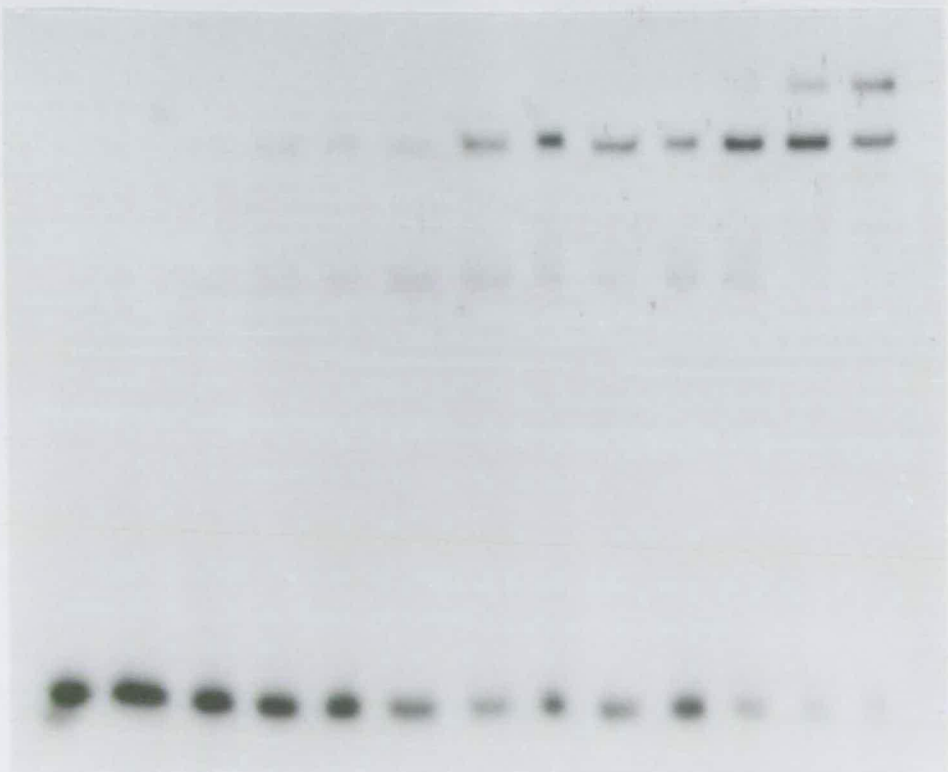
Figures 7a and 7b show that the addition of ATP reduces the binding affinity of wild-type *Eco*KI for DNA. In contrast the K477R and H577D mutants in figures 7c and 7d, 7g and 7h respectively, show that the addition of ATP has the opposite effect and increases the binding affinity of the protein for DNA. The other mutant proteins do not show significant differences but most show a slight increase in DNA binding affinity in the presence of ATP. It would be informative to measure binding affinities of the proteins without ATP and compare then to the figures calculated in the presence of ATP shown in table 8, p108. The addition of ATP changes the conformation of *Eco*KI to give a different footprint (Lynn Powell, pers comm) in wild-type *Eco*KI this conformational change decreases DNA binding affinity. It is possible that the amino acid substitutions in the mutant proteins have affected this change in conformation in such a way as to stimulate DNA binding.

Figures 7a and 7b. Gel retardation by wild-type *Eco*KI endonuclease. A 45bp duplex including one recognition site for *Eco*KI was used as a substrate. Protein (at concentrations ranging from 0 to 20nM) was mixed with DNA (0.1nM) in the presence of AdoMet (100μM), glycerol (5%), ATP (0 or 2mM), in binding buffer. Samples were incubated at room temperature for 10 minutes before running on 5% PAGE gels. The reactions in a) were done in the absence of ATP, those in b) in the presence of ATP. Numbers indicate concentration of protein in nM.

7a

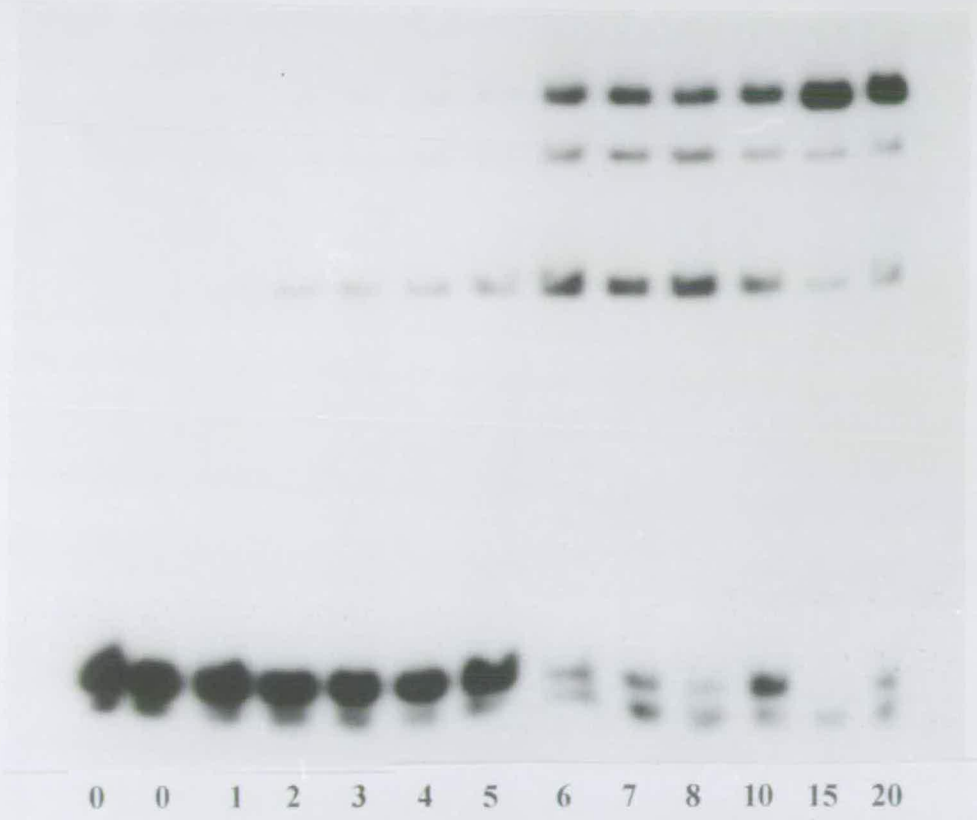


7b

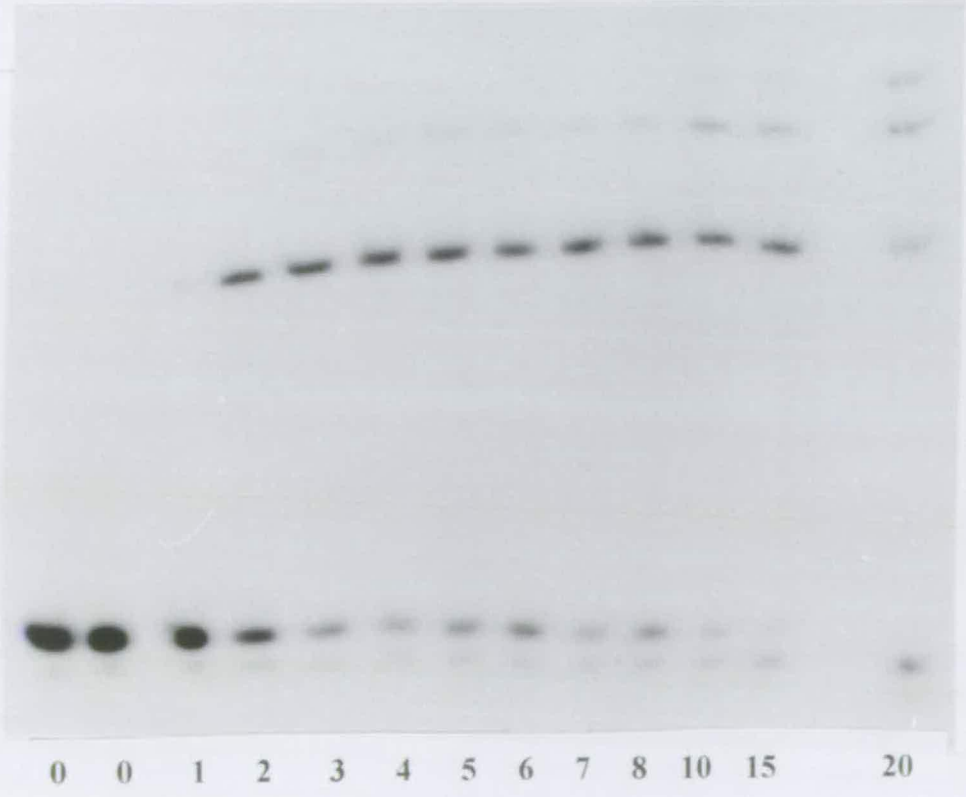


Figures 7c and 7d. Gel retardation by *Eco*KI with a K477R substitution in motif I (GKT) (r_K^-). Conditions for 7c and 7d were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7c

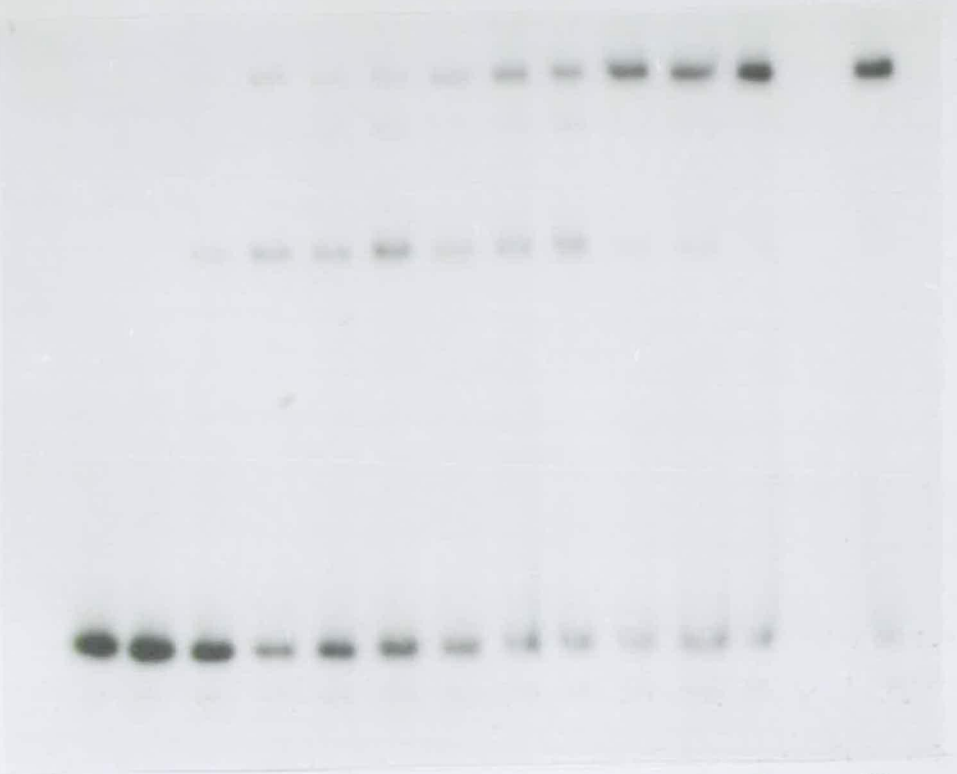


7d



Figures 7e and 7f. Gel retardation by *Eco*KI with a D502Y substitution in motif Ia (FLVDR) (r_K^-). Conditions for 7e and 7f were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

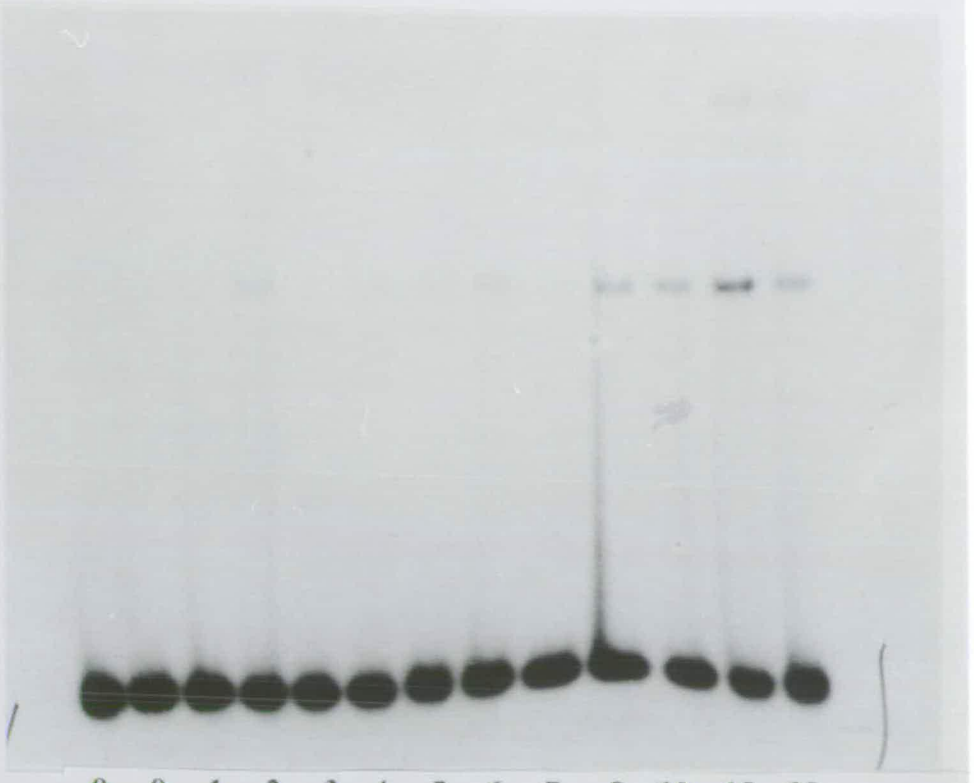
7e



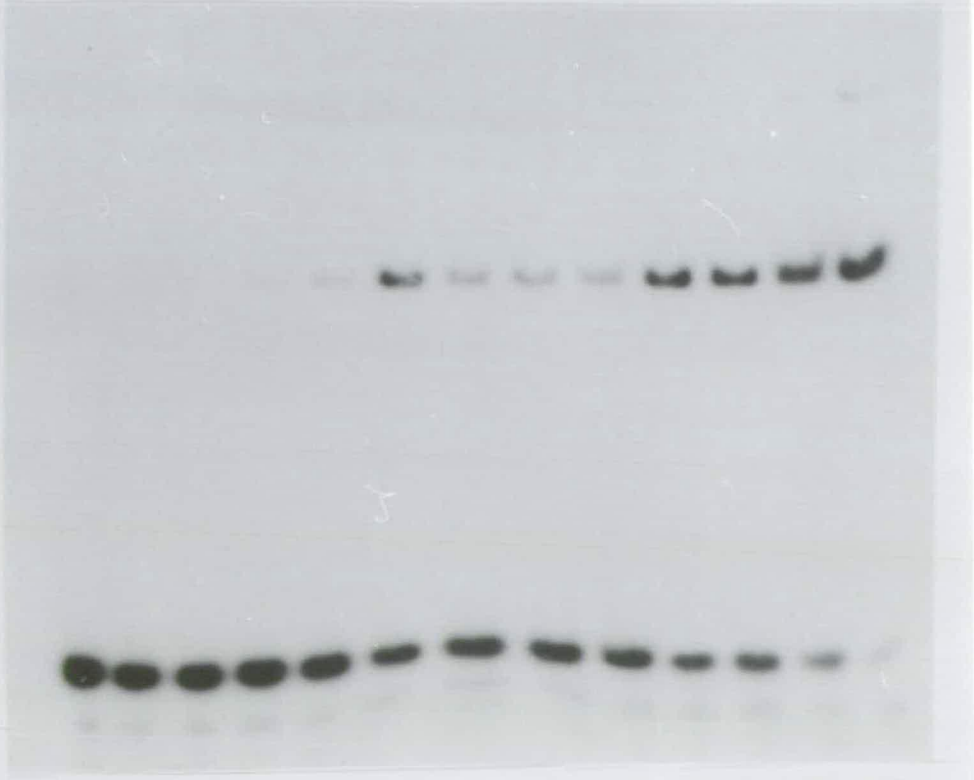
7f

Figures 7g and 7h. Gel retardation by *Eco*KI with a H577D substitution in motif II (DEAH) (r_K^-). Conditions for 7g and 7h were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7g

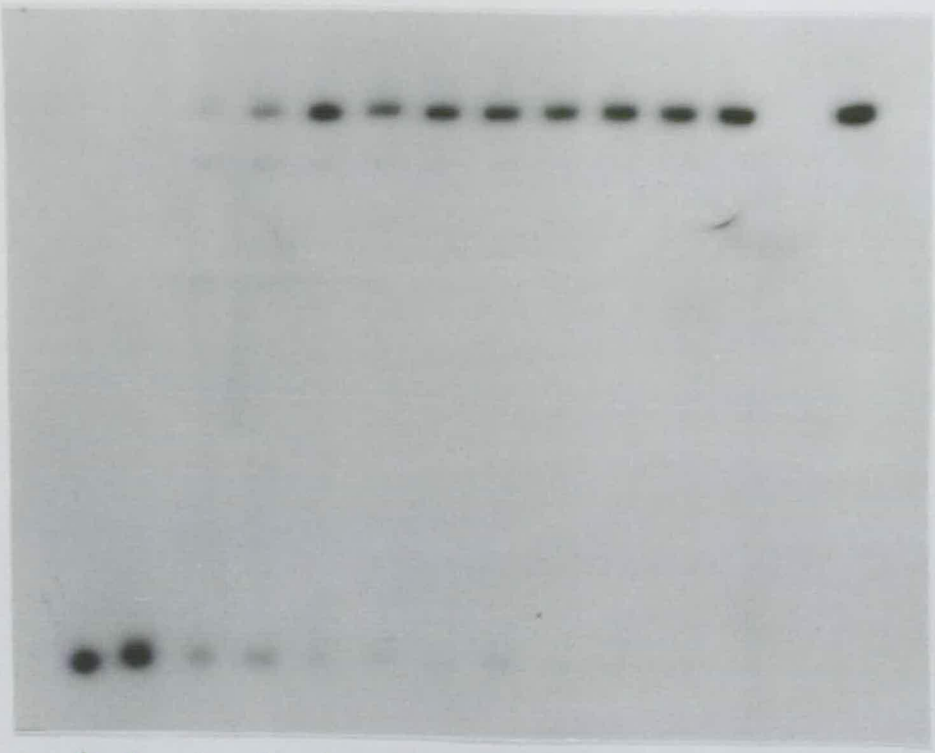


7h

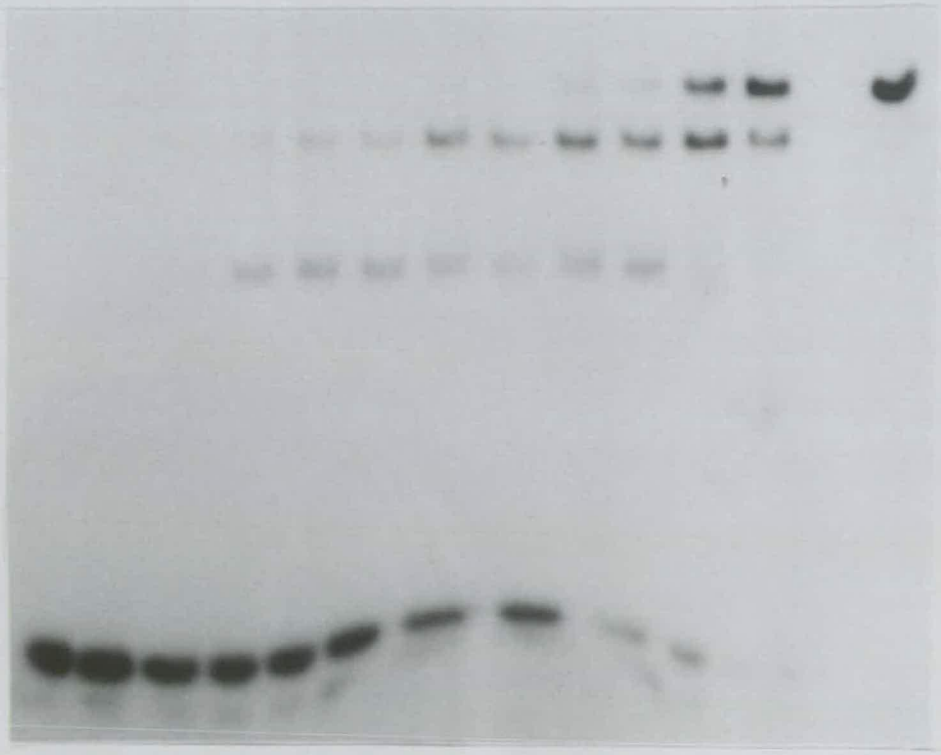


Figures 7i and 7j. Gel retardation by *Eco*KI with a A619G substitution in motif III (TAT) ($r_K^{+/-}$). Conditions for 7i and 7j were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7i

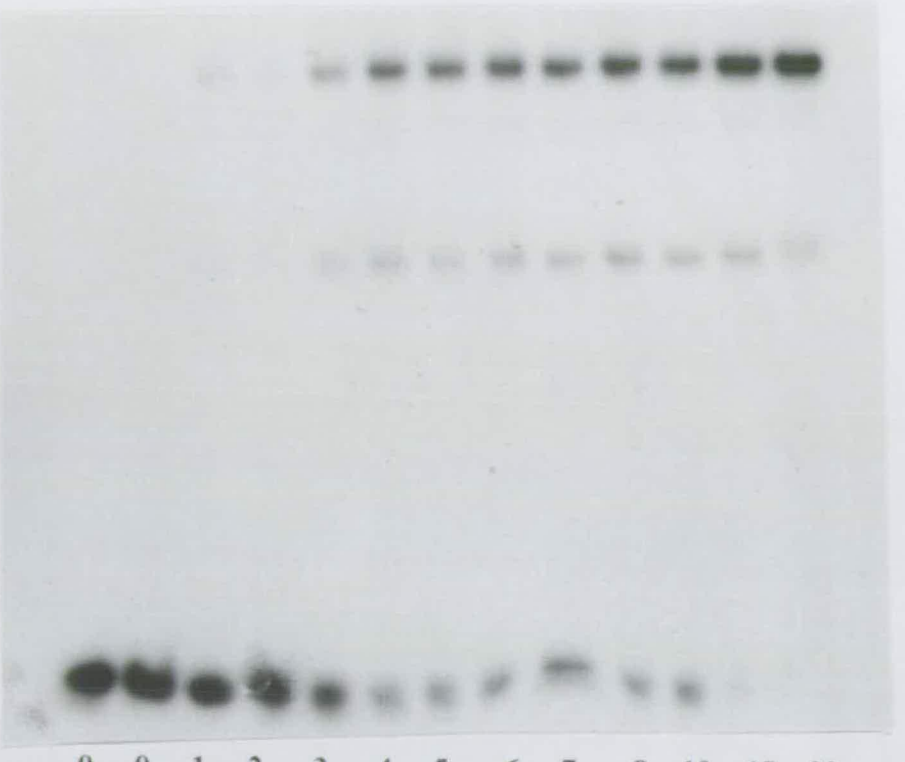


7j

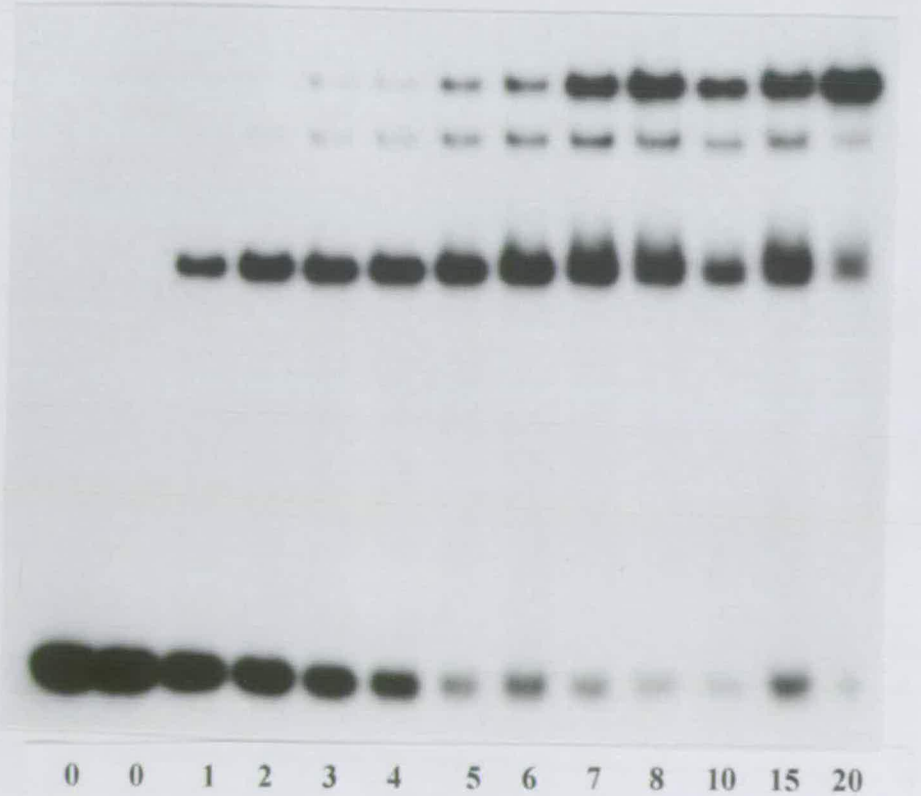


Figures 7k and 7l. Gel retardation by *Eco*KI with a A619V substitution in motif III (TAT) (r_K^-). Conditions for 7k and 7l were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7k

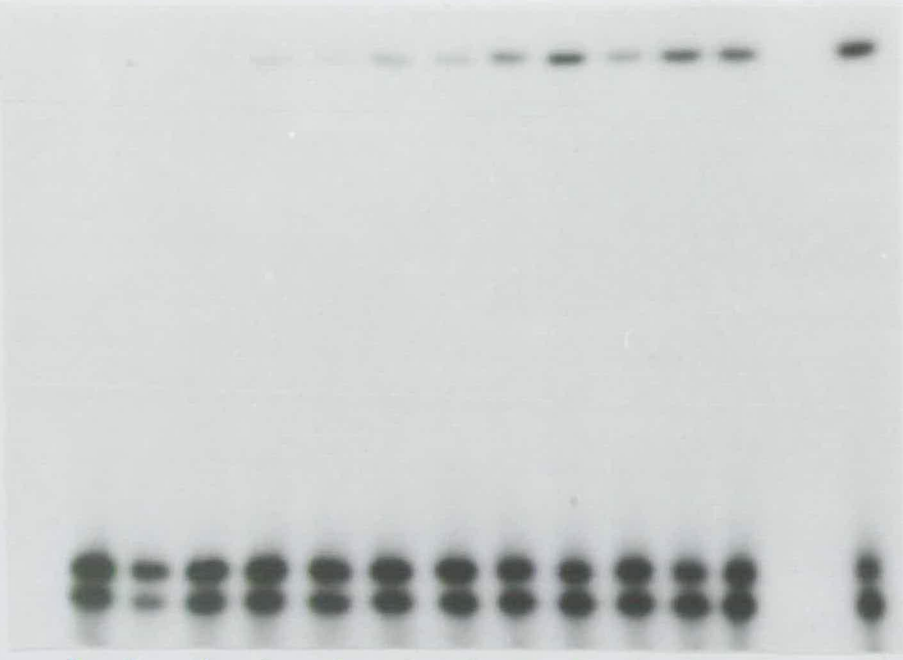


7l

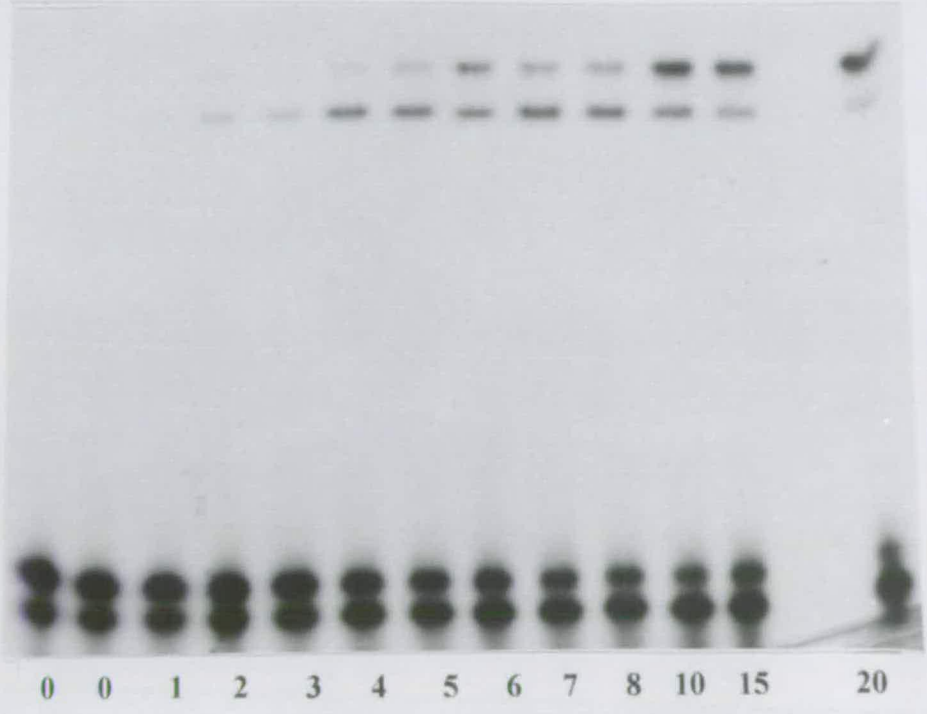


Figures 7m and 7n. Gel retardation by *Eco*KI with a F730S substitution in motif IV (FCVT) (r_K^+). Conditions for 7m and 7n were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7m

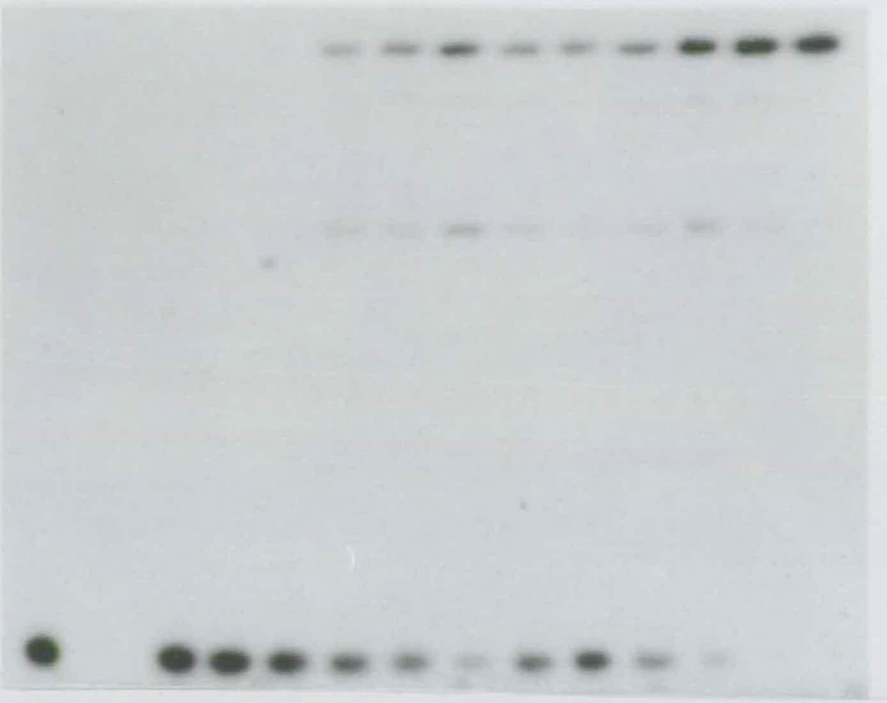


7n



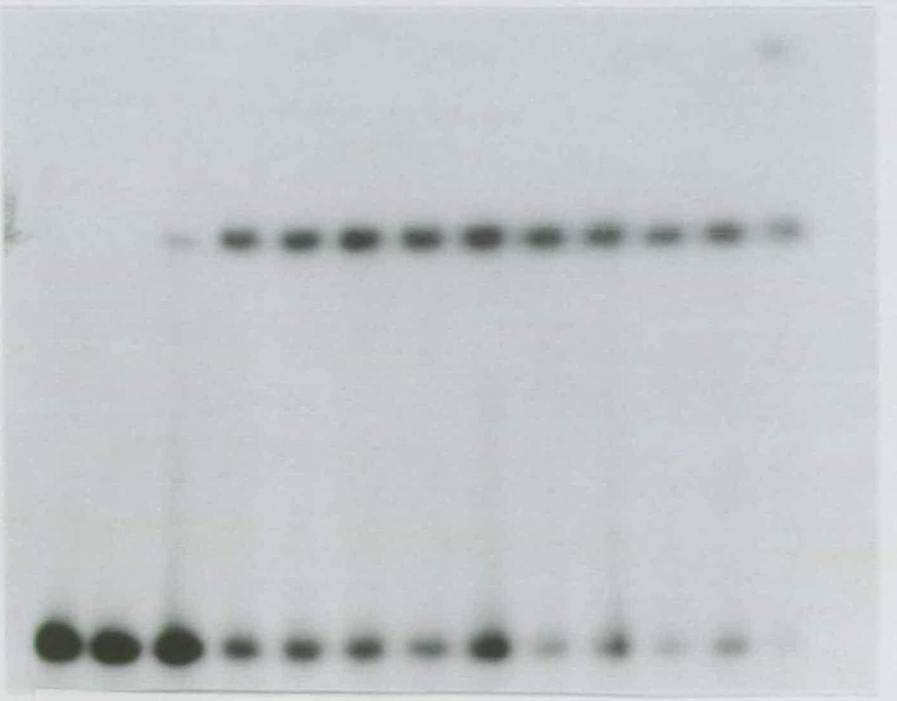
Figures 7o and 7p. Gel retardation by *Eco*KI with a G799C substitution in motif V (LTTGVD) (r_K^-). Conditions for 7o and 7p were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7o



0 0 1 2 3 4 5 6 7 8 10 15 20

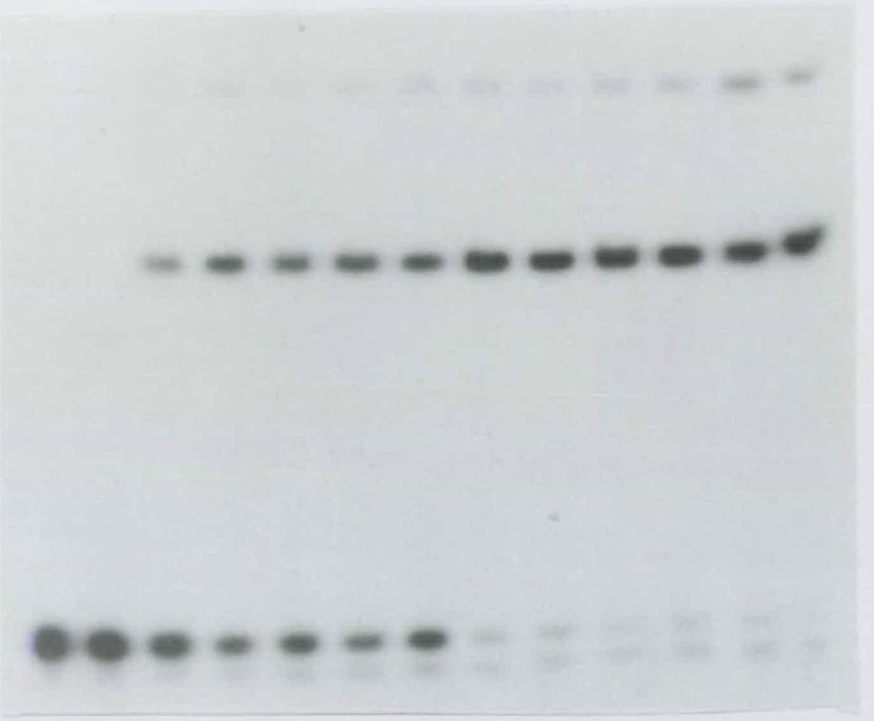
7p



0 0 1 2 3 4 5 6 7 8 10 15 20

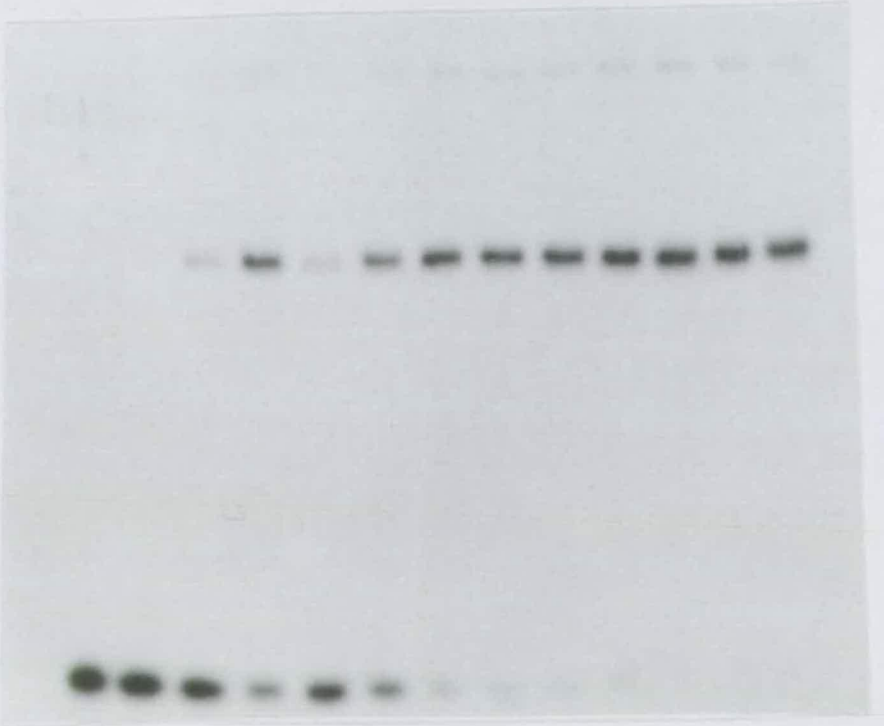
Figures 7q and 7r. Gel retardation by *Eco*KI with a R826H substitution in motif VI (QMKGRATR) (r_K^-). Conditions for 7q and 7r were as described for 7a and 7b respectively. Numbers indicate concentration of protein in nM.

7q



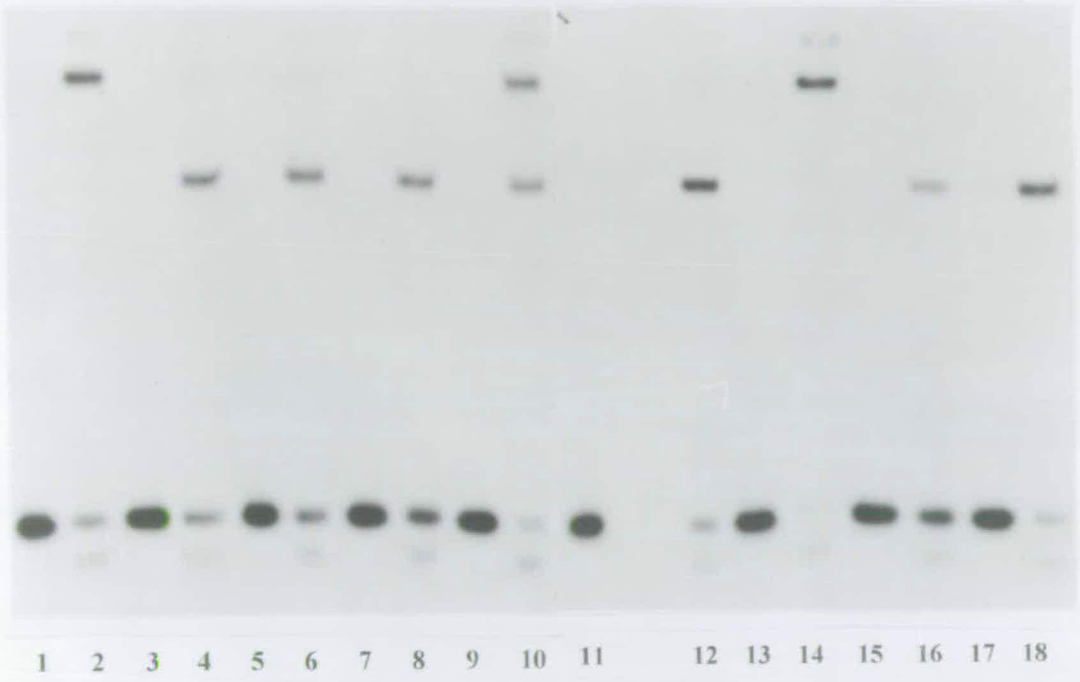
0 0 1 2 3 4 5 6 7 8 10 15 20

7r



0 0 1 2 3 4 5 6 7 8 10 15 20

Figure 8. Gel retardation of a radiolabelled specific 45bp duplex (0.1nM) by 8nM *Eco*KI with 100x excess of cold specific or cold non-specific 45bp duplex DNA included in the reaction in the presence of AdoMet (100μM) and ATP (2mM). Lanes 1 and 2 are wild-type *Eco*KI. Lanes 3 and 4: *Eco*KI with a K477R substitution in motif I (GKT). Lanes 5 and 6: *Eco*KI with a D502Y substitution in motif Ia (FLVDR). Lanes 7 and 8: *Eco*KI with a H577D substitution in motif II (DEAH). Lanes 9 and 10 are *Eco*KI with a A619G change in motif III (TAT). Lanes 11 and 12 are *Eco*KI with a A619V change in motif III (TAT). Lanes 13 and 14 are *Eco*KI with a F730S change in motif IV (FCVT). Lanes 15 and 16 are *Eco*KI with a G799C change in motif V (LTTGVD). Lanes 17 and 18 are *Eco*KI with a R826H change in motif VI (QMKGRATR). The first lane of each protein has excess specific 45bp duplex, the second lane excess non-specific 45bp duplex in the reaction.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Motif	Amino acid substitution	K_d (nM)
Wild-type		2.22 ± 1.13
I	K477R	3.92
Ia	D502Y	3.48
II	H577D	6.03 ± 1.81
III	A619G	2.30 ± 1.22
	A619V	2.12 ± 0.37
IV	F730S	2.94
V	G799C	2.87
VI	R826H	2.61

Table 8. The effects of the amino acid substitutions on the binding affinity of *Eco*KI for DNA. The values were determined by gel retardation for binding of a 45bp duplex containing one unmodified *Eco*KI recognition site, in the presence of ATP and AdoMet. For results performed in duplicate or triplicate, mean values and standard deviations are given.

Other than the H577D substitution in motif II (DEAH) the amino acid changes do not affect the binding affinity of *Eco*KI endonuclease for DNA. Calculated binding affinities were in the range 2-3nM, the same K_d for DNA as *Eco*KI methylase (Powell *et al.*, 1993). The H577D protein has a K_d of about 6nM. At this concentration, indeed up to 20nM, the protein-DNA complex observed with H577D is the M_2S_1-DNA complex (see fig 7h). *Eco*KI with the R826H substitution in motif VI (QMKGRATR) also readily loses HsdR subunits (see fig 7r). The difference in K_d is clear when comparing the gel retardation results from these two proteins. The H577D protein requires concentrations of 4 or 5nM before a retarded DNA band is apparent, whereas the R826H mutant gives a visible band at 1nM. The K_d for the F730S mutant was 2.94nM, this is not consistent with the gels in figures 7m and 7n which indicate that the mutant does not readily bind DNA. This gel photo was included to show the apparent single-stranded DNA and a different gel without this extra DNA band were used to calculate the K_d for this mutant.

The gel retardation results for wild-type *Eco*KI show that at 10nM protein concentration most of the bound DNA is in the $R_1M_2S_1$ form, which is not active nuclease. 10nM is the concentration of *Eco*KI used in the nuclease assay and this suggests that either the complexes observed on the gel are not representative of those in solution or the small amount of $R_2M_2S_1$ present at 10nM wild-type *Eco*KI is responsible for activity. To test if the apparent dissociation of HsdR subunits from the methylase was affecting results in other *in vitro* assays, the nuclease assay described earlier was repeated using *Eco*KI containing the A619V substitution in motif III (TAT) at higher concentrations of protein (40nM as opposed to 10nM). At 40nM, in the presence of ATP, the DNA-protein complex runs as the active nuclease form ($R_2M_2S_1$). A619V, which showed no nuclease activity at 10nM, gave the same result in the nuclease assay at 40nM, indicating that the increased dissociation of the mutant proteins observed in the binding assays is not responsible for the negative results obtained in the nuclease assays. It is likely that the relative proportions of the complexes in solution are different from those in the gel due to dilution upon loading.

In summary, the gel retardation results show that the substitutions in the DEAD box motifs do not generally prevent ATP interaction, as the addition of ATP

to the binding reactions for most of the mutant proteins affected the pattern of protein-DNA complexes. These results do not show if the changes alter affinities for ATP as excess ATP was used. Only the H577D substitution in motif II (DEAH) affected DNA binding, causing a reduction in affinity. The binding observed was tested for specificity and shown to indicate specific interaction of the proteins with the *Eco*KI recognition sequence.

Effect of changes in DEAD box motifs on ATPase activity

Restriction of DNA is accompanied and followed by extensive ATP hydrolysis (Eskin and Linn, 1972; Rosamund *et al.*, 1979; Dreier and Bickle, 1996). This activity is necessary for DNA cleavage (Eskin and Linn, 1972). Previous studies on DEAD box proteins have shown some of the conserved motifs to be essential to ATPase activity (Pause and Sonenberg, 1992; Sharples *et al.*, 1994; Gross and Shuman, 1996). Motif I and motif II have been previously described as the Walker ATPase A and B motifs respectively and are also conserved in ATPases (Walker *et al.*, 1982; Hodgman, 1988a+b).

pRH3, the plasmid with two *Eco*KI recognition sites that was used in the nuclease assay, was used as a substrate in ATPase assays. In addition, any proteins that showed some activity using pRH3 as a substrate were tested for their activity on the 45bp duplex containing one *Eco*KI site, used previously in the DNA binding assay. Both substrates were used in an attempt to identify whether separable ATPase activities exist; activities preceding, during and after cleavage. In contrast to the plasmid, the 45bp duplex is not degraded by *Eco*KI (Lynn Powell, pers comm) and if separate activities existed this substrate would only support ATP hydrolysis prior to the cleavage step. To allow comparison of the results, pRH3 was used at half the molar concentration of the 45bp duplex so the same amount of *Eco*KI was used in each assay, with one molecule of *Eco*KI added for every recognition site.

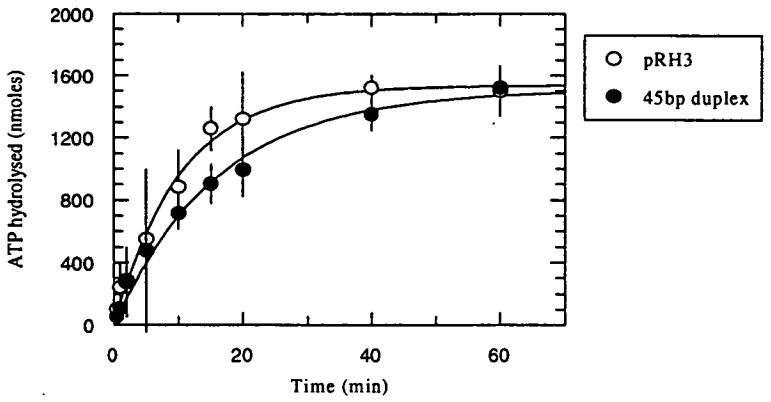
ATPase activity was measured by following the release of radiolabelled inorganic phosphate from ATP. Aliquots were removed from the reaction and mixed with EDTA to stop ATPase activity. The products of the reaction were applied to PEI-cellulose TLC plates and ATP was separated from inorganic phosphate by chromatography. The amount of inorganic phosphate released was measured on a phosphorimager. The results are plotted in figures 9a to 9i and they show that the DEAD box motifs in HsdR are important for the ATPase activity involved in restriction of DNA by *Eco*KI. Results were corrected for any background ATPase activity as described in the Methods section, page 48. Levels of background activity were low, usually up to 2% ATP hydrolysis after 60 minutes. Any ATPase activity remaining after correcting for background levels indicates that the mutant can hydrolyase a certain amount of ATP (see figures 9b, 9d and 9f). Results where no ATPase activity is plotted represent mutant proteins that produced similar levels of ATP hydrolysis as the negative control without AdoMet (see figures 9h and 9i).

The ATPase activity of wild-type *Eco*KI is shown in figure 9a. The plasmid and the 45bp duplex support similar levels of ATP hydrolysis with slightly more activity detected with the plasmid, although this difference does not appear significant as the error bars overlap.

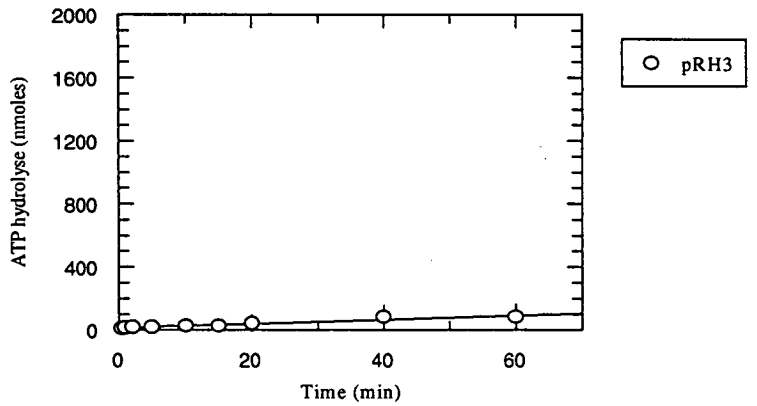
Figures 9b to 9i show that most of the amino acid changes that conferred an r_K^- phenotype abolished or severely impaired ATPase activity, this is consistent with ATPase activity being essential for restriction. *Eco*KI with a change in motif Ia (FLVDR) is r_K^- but shows a reduced, but significant level of ATPase activity in the assay with pRH3 as a substrate (fig 9c). Little, if any ATPase activity was detected with the 45bp duplex as a DNA substrate. *Eco*KI with the substitution in motif IV (FCVT) showed levels of ATPase activity similar to wild-type *Eco*KI, which is consistent with previous results that suggest the phenotype of this mutant is as wild-type. The protein with a A619G substitution in motif III (TAT) shows similar levels of ATPase activity with the plasmid substrate, but a slightly decreased rate of reaction with the 45bp duplex. This decrease may be related to the slightly impaired levels of restriction detected *in vivo*, for this protein.

Figures 9a to 9i. The effect of changes in the DEAD box motifs on the ATPase activity of *Eco*KI. 100µl reactions contained 10nM nuclease, 5nM pRH3 or 10nM 45bp duplex, 100µM AdoMet, 50µgml^{-1} BSA, 1x buffer A (Boehringer Mannheim) and 2mM ATP. The ATP solution contained 0.2µCi γ^{32}P ATP. 10µl samples were removed after 0.5, 1, 2, 5, 10, 15, 20, 40 and 60 minutes and mixed with 2.5µl 0.5M EDTA pH 8.0 to stop the reaction. Mean figures are shown calculated from at least three results, with standard deviations illustrated using error bars.

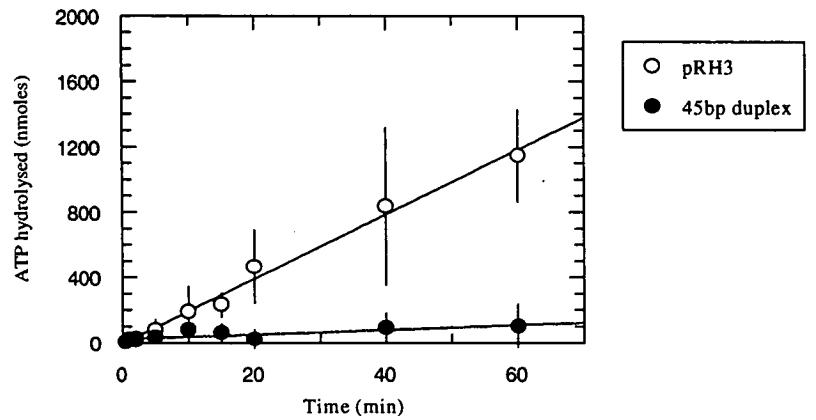
9a. Wild-type *Eco*KI



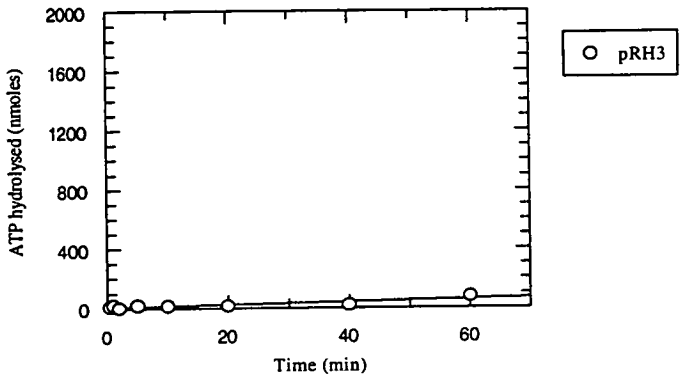
9b. *Eco*KI with a K477R substitution in motif I GKT), (r_K^-).



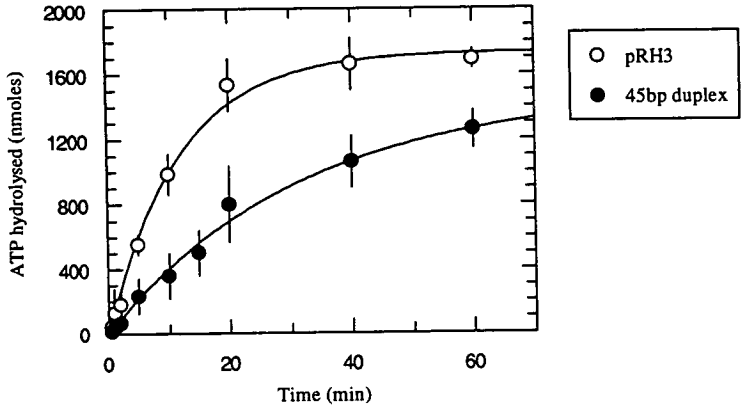
9c. *Eco*KI with a D502Y substitution in motif Ia (FLVDR), (r_K^-).



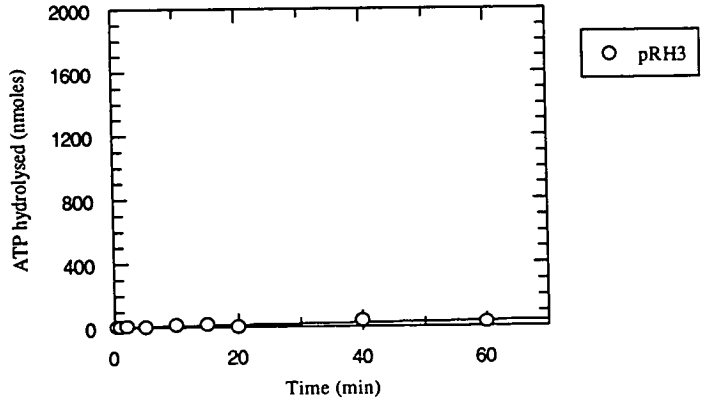
9d. *Eco*KI with a H577D substitution in motif II (DEAH), (r_K^-).



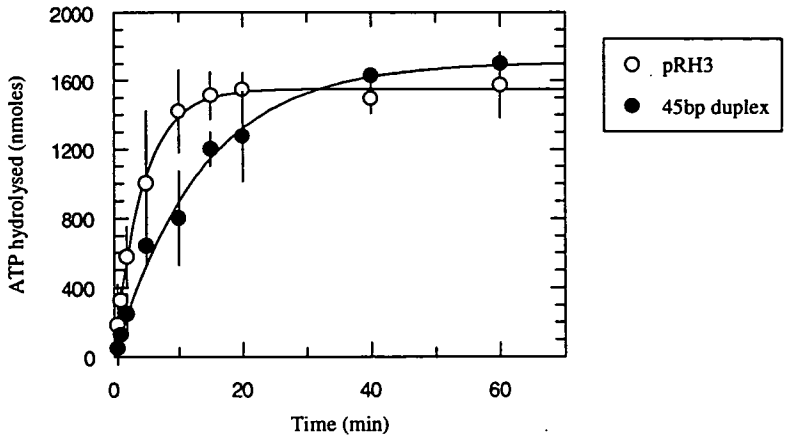
9e. *Eco*KI with a A619G substitution in motif III (TAT) ($r_K^{+/-}$).



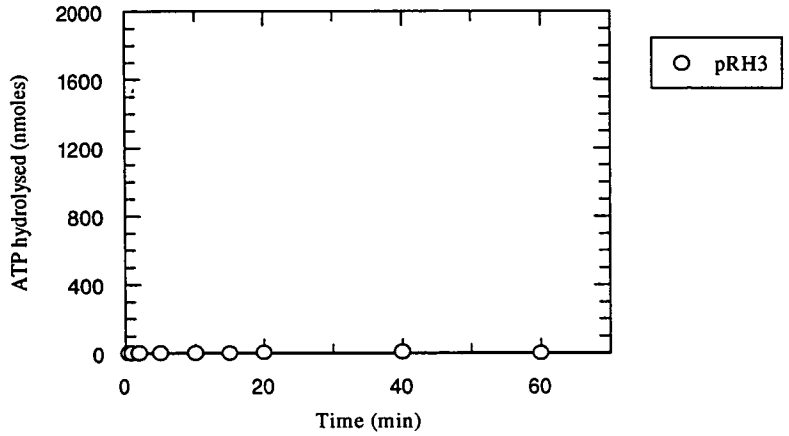
9f. *Eco*KI with a A619V substitution in motif III (TAT) (r_K^-).



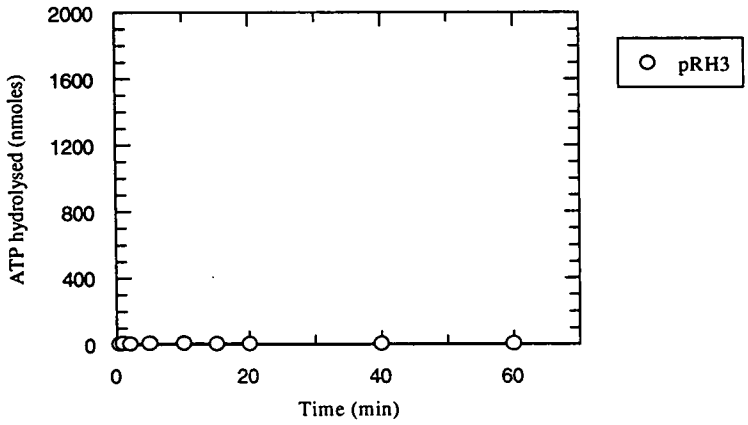
9g. *Eco*KI with a F730S substitution in motif IV (FCVT) (r_K^+).



9h. *Eco*KI with a G799C substitution in motif V (LTTGVD) (r_K^-).



9i. *Eco*KI with a R826H substitution in motif VI (QMKGRATR) (r_K^-).



General discussion

To investigate the role of DEAD box motifs in restriction of DNA by *Eco*KI, amino acid residues in these motifs were targeted for mutational analysis. Many proteins with these motifs are known to be helicases (Schmid and Linder, 1992). In *Eco*KI the DEAD box motifs may have a role in the DNA translocation that is proposed to occur during restriction (Murray *et al.*, 1993). Motifs I and II are also conserved in ATPases and are known as the Walker ATPase A and B motifs (Hodgman, 1988a+b; Walker *et al.*, 1993). Restriction by type I enzymes is accompanied by extensive ATP hydrolysis (Eskin and Linn, 1972; Rosamund *et al.*, 1979; Dreier and Bickle, 1996), that is proposed to drive DNA translocation (Yuan *et al.*, 1980).

To study the role of the DEAD box motifs in *Eco*KI, an amino acid residue in each of the seven motifs (I, Ia, II to VI) was changed by site-directed mutagenesis. Representative mutations were transferred to the chromosome of *E. coli*. The effect of these mutations on the ability of *Eco*KI to cleave DNA was investigated by measuring the restriction phenotype of the mutant bacteria, and proteins containing the same changes were purified. None of the changes affected the elution profile of the proteins from the columns used to purify them. The proteins were tested for nuclease activity, DNA binding and ATPase activity.

The results show that six of the seven sequences identified as DEAD box motifs have an essential role in restriction. Only changes within the sequence originally proposed to be motif IV had no effect on restriction. The properties of the protein with a F730S substitution in motif IV were indistinguishable from wild-type *Eco*KI. These results support the suggestion (Titheradge *et al.*, 1996) that motif IV was incorrectly identified in the *Eco*KI HsdR sequence (Gorbalenya and Koonin, 1991). Gorbalenya and Koonin (1991) identified the DEAD box motifs in the sequences of type I (*Eco*KI and *Eco*R124I) and III (*Eco*PI) restriction endonucleases. They found motif III in *Eco*KI and motif IV in *Eco*R124I difficult to identify. A frame-shift discovered in the HsdR sequence (Burland *et al.*, 1995), together with additional sequences of type I endonucleases (Titheradge *et al.*, 1996), allowed the easy identification of motif III in *Eco*KI (Webb *et al.*, 1996) and also indicated a

region of similarity located between motifs III and V, which is an alternative candidate for motif IV (Titheradge *et al.*, 1996). Mutations in this alternative region, designated region Y, confer a restriction-deficient phenotype (Graham Davies pers comm). Purified *Eco*KI protein with a F629Y change in this region (FGEPVYRY) lacked both ATPase and nuclease activities (my results). In the crystal structure of PcrA, a helicase isolated from *B. stearothermophilus*, a tyrosine residue in motif IV was shown to be involved in stacking the bound nucleotide (Subramanya *et al.*, 1996). Region Y contains a tyrosine residue which is strictly conserved in type I restriction endonucleases.

On the assumption that region Y identifies motif IV, mutations in all seven DEAD box motifs affected DNA restriction; all changes prevented DNA restriction, except an A619G change in motif III, which only impaired DNA restriction. This difference was only detected when the sensitivity of the test for the restriction phenotype of the mutant bacteria was increased, by using phage with only one or two *Eco*KI recognition sites. The purified protein containing this change showed apparently wild-type levels of nuclease and ATPase activity. This amino acid substitution changed the sequence in motif III from TAT to TGT, the amino acid sequence in motif III of the type IC restriction/modification system *Eco*R124I and therefore functional in the context of *Eco*R124I. The two other changes at this position, A619V and A619D, abolished restriction.

The conservative change of DEAH to DEAD (H577D) in motif II prevented DNA restriction. *In vitro*, *Eco*KI with the H577D change had neither ATPase nor nuclease activity. In eIF-4A the converse change of DEAD to DEAH showed elevated levels of ATPase activity and only 10% of RNA helicase activity (Pause and Sonenberg, 1992). A conservative change of Q822H in motif VI (QMKGRATR) abolished both the ATPase and nuclease activities of *Eco*KI. The opposite change of H to Q in motif VI in eIF-4A (HRIGRGGR) prevented ATPase and helicase activities. A double mutant that also had the change of DEAD to DEAH in motif II in eIF-4A showed increased levels of ATP hydrolysis and retained some helicase activity (Pause and Sonenberg, 1992). It would be interesting to see the effects of creating a double mutant with both H577D and Q822H changes in *Eco*KI, as this might be

predicted to have some ATPase activity and perhaps show a small amount of restriction.

Mutations in motif I (GKT) abolished DNA restriction activity *in vivo*. Motif I is directly involved in ATP binding (Pai *et al.*, 1977); the 3-D structures of ATPases adenylate kinase and RecA show that the lysine residue of this motif interacts with the β and γ phosphates of the nucleotide (Fry *et al.*, 1986; Story and Steitz, 1992). A change of lysine to asparagine in eIF-4A abolished ATP binding (Rozen *et al.*, 1989). *Eco*KI in which the lysine of this motif was changed to arginine and therefore retained a positively charged amino acid at this position, interacted with ATP but showed no ATPase or nuclease activities. The same change in Rad3 abolished ATPase and helicase activities but retained ATP binding activity (Sung *et al.*, 1988). It would be appropriate to purify *Eco*KI containing one of the other changes introduced at this position to check the prediction that ATP interaction would be abolished.

Changes in motifs V (LTTGVD) and VI (QMKGRATR) conferred r_K^- phenotypes. Purified proteins with these changes showed no ATPase activity. The r_K^- phenotypes resulting from mutations in the DEAD box motifs correlate with deficiencies in ATPase activity. Yuan *et al.* (1980) found that the DNA loops produced from DNA translocation were not observed in the absence of ATP hydrolysis and suggested that this hydrolysis drives DNA translocation. Meisel *et al.* (1995) proposed a model for restriction by the type III endonuclease *Eco*P15I, which involved DNA translocation driven by ATP hydrolysis. It is interesting that only 1% of the level of ATP hydrolysis with type I endonucleases occurs with restriction by type III enzymes. This implies that a reduction in ATP hydrolysis in *Eco*KI may not prevent DNA translocation. Type III enzymes dissociate after cleavage, but type I enzymes continue to translocate DNA after the initial cut, which may be one reason for the difference in ATP hydrolysis.

Results from assays with the 45bp duplex show that ATP hydrolysis can be supported by a non-cleavable substrate. Studies on restriction by *Eco*R124I found non-cleavable substrates supported ATPase activity (Dreier and Bickle, 1996). ATPase activity of *Eco*KI was also tested using a 25bp duplex with one recognition site as a substrate. *Eco*KI methylase specifically binds to this 25mer and the *Eco*KI

endonuclease gives the same footprint as the methylase which suggests it will also bind the 25bp duplex (Lynn Powell, pers comm). However the shorter oligonucleotide did not support any detectable ATPase activity. The *Eco*KI endonuclease would require most if not all of the 25 base pairs for specific DNA binding and would not be able to support any DNA translocation as there would be insufficient nucleotides outside the bound complex. This result could imply that ATP is not hydrolysed if DNA translocation cannot occur, this suggestion is supported by results that show the addition of DNaseI stops ATP hydrolysis during restriction by *Eco*R124I (Dreier and Bickle, 1996). The protein D502Y with a change in motif Ia (FLVDR) lacked nuclease activity, but possessed a reduced level of ATPase activity. Either the level of ATP hydrolysis did not allow sufficient DNA translocation or DNA translocation occurred but the mutation prevented cleavage.

All the mutant proteins except the protein with the H577D change in motif II (DEAH) had the same binding affinity for DNA as wild-type (K_d = 2-3nM). The substitution in motif II may have slightly distorted folding of the polypeptide to interfere with specific binding resulting in the higher K_d (6nM). It is likely that one or more of these motifs are involved in the non-specific DNA binding that would be necessary to initiate DNA translocation. The crystal structure of *E. coli* Rep helicase bound to single-stranded DNA shows that amino acid residues of motifs Ia, III and V are in contact with the DNA (Korolev *et al.*, 1997). This information, and the lack of ATPase activity found for proteins with changes in motifs III and V, suggests that these motifs may participate in the initiation of translocation or some other essential aspect of the translocation process.

The only previous investigation of the role of the DEAD box motifs in restriction endonucleases has been in *Eco*PI (Saha and Rao, 1997). They studied the effects of changes in motifs I and II (DEPH in *Eco*PI), the changes reduced or abolished ATPase activity and prevented DNA restriction. It would be interesting to see the results of changes in the remaining DEAD box motifs and compare them to the results of this study.

It is now possible to test the effects of these mutations on DNA translocation. An assay has been adapted from one that measures the rate of phage T7 entry into the

cell (Garcia and Molineux, 1995). This assay uses bacteria containing a Dam-overproducing plasmid to provide methylation of GATC sequences within the cell. The transfer of the phage genome into the cell can be monitored by digesting DNA with *Dpn*I, as methylation makes the DNA sensitive to *Dpn*I. Fragments of T7 DNA within the cell can be identified using labelled DNA as a probe. Normally T7 ejects 850bps of its DNA into the cell, *E. coli* RNA polymerase transcribes the early genes on this DNA and once T7 RNA polymerase is produced it takes over transcription of the phage genome. The DNA is pulled into the cell as it is transcribed. To measure the rate of translocation by *Eco*KI rifampicin is added to the cell to inhibit *E. coli* RNA polymerase and hence transcription of the gene encoding T7 RNA polymerase. The phage genome has been altered so the leading end that is first ejected into the cell contains one *Eco*KI recognition site, whilst all other *Eco*KI sites in T7 have been removed. *Eco*KI binds to the recognition site and begins to translocate the DNA, as it does so it pulls the phage DNA into the *E. coli* cell. In this assay wild-type *Eco*KI translocated DNA at an average rate of 120bp per second at 30°C (Ian Molineux, pers comm). Initial studies found that the K477R, H577D and A619V changes in motifs I (GKT), II (DEAH) and III (TAT) respectively, prevented DNA translocation and the A619G change in motif III shows apparently wild-type levels of DNA translocation (Ian Molineux pers comm). These experiments were done using mutant derivatives of AB1157. Unfortunately, AB1157 was not an ideal recipient in these experiments, and the experiments with all the mutations must be done using derivatives of the strain described by Garcia and Molineux (1995). It would be interesting to see whether the D502Y change in motif Ia (FLVDR), which confers an ATPase positive and restriction-negative phenotype, will allow DNA translocation.

None of the mutant proteins has been tested to see if they remain capable of interaction with DNA to form a filter-binding complex. As described earlier *Eco*KI binds AdoMet and then undergoes a transition to an activated form. When this activated complex interacts with an unmethylated recognition site the complex undergoes a transition to a form that can be trapped on filters (Meselson *et al.*, 1972; Bickle *et al.*, 1978). Formation of this complex requires ATP binding but is

independent of ATP hydrolysis. Results from filter-binding assays would show if the mutations disrupt activation of the endonuclease, an initial step crucial for restriction activity.

Many DEAD box proteins investigated have been shown to be helicases (Schmid and Linder, 1992). Therefore it is important to ascertain if *Eco*KI endonuclease possesses a helicase activity. The presence of DEAD box motifs do not prove helicase activity, indeed a few proteins with these motifs do not possess helicase activity. These include *E. coli* transcription repair coupling factor and Rad5 in yeast (Selby and Sancar, 1993; Johnson *et al.*, 1994). The *E. coli* transcription repair coupling factor is encoded by the *mfd* gene and is necessary for strand-specific repair. The protein recognizes a stalled RNA polymerase enzyme and recruits the excision repair enzyme, (A)BC excinuclease. The amino acid sequence contains all seven DEAD box motifs and, like *Eco*KI, is a member of the DEXH subgroup. Biochemical studies found the protein possesses ATPase activity but not helicase activity (Selby and Sancar, 1993). Rad5 functions in postreplication repair of UV-damaged DNA. Purified Rad5 protein was found to have a single-stranded DNA dependent ATPase activity but no helicase activity (Johnson *et al.*, 1994). If *Eco*KI does possess a DNA unwinding activity it could be involved in DNA translocation (Gorbalenya and Koonin, 1991; Murray *et al.*, 1993; Webb *et al.*, 1996) or DNA unwinding at the cleavage site (Gorbalenya and Koonin, 1991; Dartois *et al.*, 1993).

The standard biochemical test for unwinding activity measures the displacement of a short radiolabelled oligonucleotide from a single-stranded DNA circle (an example can be found in Tsaneva *et al.*, 1993). This assay would not be appropriate for measuring DNA unwinding by *Eco*KI as the enzyme remains bound to DNA at one point and does not move along the DNA unwinding it like conventional helicases. Consequently the oligonucleotide would not be displaced, as it would be fixed to the single-stranded circle by *Eco*KI. One alternative helicase assay was attempted during this study. This measured the intrinsic fluorescence of SSB protein when included in the *Eco*KI nuclease reaction and looked for a quenching of this fluorescence which would indicate the SSB protein had bound to single-stranded DNA (Roman and Kowalczykowski, 1989). A drop in fluorescence was not detected,

which may have indicated a lack of unwinding. It is possible that the large SSB protein did not have sufficient access to bind to single-stranded DNA, if unwinding occurred close to *Eco*KI

To date a helicase activity has not been shown for any restriction endonucleases. Saha and Rao (1997) failed to find any unwinding activity with the type III restriction endonuclease *Eco*PI. However they used the previously described method of measuring the displacement of a labelled oligonucleotide and the lack of activity observed could be a result of problems with this assay. If unwinding occurs during translocation, the extent of unwinding may be small with reannealing of the DNA following soon after unwinding. Unwinding may act to relieve the steric problems proposed to occur during translocation. Alternatively the co-operation of a topoisomerase during translocation could overcome steric problems (Graham Davies and Noreen Murray, pers comm).

This study shows the DEAD box motifs are important for the nuclease and ATPase activities of *Eco*KI. Further assays for DNA translocation and DNA binding, as observed by the filter-binding assay, may provide additional information on the roles of these sequences in the pathway that progresses from DNA-binding to the eventual cutting of the DNA. The relevance of the DEAD box motifs to either a helicase or an alternative activity is critical to the understanding of the mechanism of restriction of type I R/M enzymes. The crystal structure of *Eco*KI, or domains of this enzyme, in the presence and absence of cofactors and substrate, is an ultimate but difficult goal. At present comparisons with proteins of known structure may provide additional insight as they have done for domains of the methyltransferase component of *Eco*KI (Dryden *et al.*, 1995).

References

Abedjieva, A., Webb, M., Patel, J., Zinkevich, V. and Firman, K. 1994. Deletions within the DNA recognition subunit of M.*Eco*R124I that identify a region involved in protein-protein interactions between HsdS and HsdM. *J. Mol. Biol.* 241: 35-43.

Anderson, J. E. 1993. Restriction endonucleases and modification methylases. *Curr. Opin. Struct. Biol.* 3: 24-30.

Appleyard, R. K. 1954. Segregation of new lysogenic types during growth of a doubly lysogenic strain derived from *Escherichia coli* K-12. *Genetics.* 39: 440-452.

Arber, W. 1965a. Host controlled modification of bacteriophage. *Ann. Rev. Microbiol.* 19: 365-378.

Arber, W. 1965b. Host specificity of DNA produced by *Escherichia coli* V. The role of methionine in the production of host specificity. *J. Mol. Biol.* 11: 247-256.

Arber, W. 1974. DNA modification and restriction. *Prog. Nucleic Acids Res. Mol. Biol.* 14: 1-37.

Arber, W. and Dussoix, D. 1962. Host specificity of DNA produced by *Escherichia coli.* I. Host controlled modification of bacteriophage λ. *J. Mol. Biol.* 5: 18-36.

Arber, W., Hattman, S. and Dussoix, D. 1963. On the host-controlled modification of bacteriophage λ. *Virology.* 21: 30-35.

Arber, W. and Linn, S. 1969. DNA modification and Restriction. *Ann. Rev. Biochem.* 38: 467-500.

Arber, W. and Wauters-Willems, D. 1970. Host specificity of DNA produced by *Escherichia coli.* XII. The two restriction and modification systems of strain 15T-. *Mol. Gen. Genet.* 108: 203-217.

Athanasiadis, A., Vlassi, M., Kotsifaki, D., Tucker, P. A., Wilson, K. S. and Kokkinidis, M. 1994. Crystal structure of *Pvu*II endonuclease reveals extensive structural homologies to *Eco*RV. *Nature Struct. Biol.* 1: 469-475.

Bachmann, B. J. and Low, K. B. 1980. Linkage map of *Escherichia coli* K-12, Edition 6. *Microbiol. Rev.* 44: 1-56.

Barcus, V. A., Titheradge, A. J. B. and Murray, N. E. 1995. The diversity of alleles at the *hsd* locus in natural populations of *Escherichia coli. Genetics.* 140: 1187-1197.

Bertani, G. and Weigle, J. J. 1953. Host controlled variation in bacterial viruses. *J. Bacteriol.* 65: 113-121.

Bickle, T. A. 1993. The ATP-dependent restriction enzymes. In Linn, S. M., Lloyd, R. S. and Roberts, E. J. (eds), *Nucleases.* 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 89-109.

Bickle, T. A., Brack, C. and Yuan, R. 1978. ATP-induced conformational changes in the restriction endonuclease from *Escherichia coli* K12. *Proc. Natl. Acad. Sci. USA.* 75: 3099-3103.

Borck, K., Beggs, J. D., Brammar, W. J., Hopkins, A. S. and Murray, N. E. 1976. The construction *in vitro* of transducing derivatives of phage lambda. *Mol. Gen. Genet.* 146: 199-207.

Boyer, H. W. 1964. Genetic control of restriction and modification in *Escherichia Coli. J. Bacteriol.* 88: 1652-1660.

Boyer, H. W., and Roulland-Dussoix, D. 1969. A complementation annalysis of the restriction and modification of DNA in *Escherichia coli. J. Mol. Biol.* 41: 459-472.

Bullas, L. R., Colson, C. and Neufield, B. 1980. Deoxyribonucleic acid restriction and modification systems in *Salmonella*: Chromosomally located systems of different serotypes. *J. Bacteriol.* 141: 275-292.

Bullock, W. O., Fernandez, J. M. and Short, J. M. 1987. XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *BioTechniques.* 5: 376-378.

Burckhardt, J., Wiesemann, J., Hamilton, D. L. and Yuan, R. 1981. Complexes formed between the restriction endonucleases *Eco*K and heteroduplex DNA. *J. Mol. Biol.* 153: 425-440.

Burland, V., Plunkett, III, G., Sofia, H. J., Daniels, D. L. and Blattner, F. R. 1995. Analysis of the *Escherichia coli* genome VI: DNA sequence of the region from 92.8 through 100 min. *Nucl. Acids. Res.* 23: 2105-2119.

Chen, A., Powell, L. M., Dryden, D. T. F., Murray, N. E. and Brown, T. 1995. Tyrosine 27 of the specific polypeptide of *Eco*K can be uv crosslinked to a bromodeoxyuridine-substituted DNA target sequence. *Nucleic Acids Res.* 23: 1177-1183.

Cheng, X., Balendiran, K., Schildkraut, I. and Anderson, J. E. 1994. Structure of *Pvu*II endonuclease with cognate DNA. *EMBO J.* 13: 3927-3935.

Clewell, D. B. and Helinski, D. R. 1969. Supercoiled circular DNA-protein complex in *E. coli*: purification and induced conversion to an open circular DNA form. *Proc. Natl. Acad. Sci. USA.* 62: 1159-1166.

Cline, J., Braman, J. C. and Hogrefe, H. H. 1996. PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res.* 24: 3546-3551.

Conrad, M. and Topal, M. D. 1989. DNA and spermidine provide a switch mechanism to regulate the activity of restriction enzyme *Nae*I. *Proc. Natl. Acad. Sci. USA.* 86: 9707-9711.

Cooper, L. P. and Dryden, D. T. F. 1994. The domains of a type I DNA methyltransferase. Interactions and role in recognition of DNA methylation. *J. Mol. Biol.* 236: 1011-1012.

Cowan, G. M., Gann, A. A. F. and Murray, N. E. 1989. Conservation of complex DNA recognition domains between families of restriction enzymes. *Cell.* 56: 103-109.

Dartois, V., De Backer, O. and Colson, C. 1993. Sequence of the *Salmonella typhimurium Sty*LT1 restriction-modification genes. Homologies with *Eco*P1 and *Eco*P151 type III R-M systems and presence of helicase domains. *Gene.* 127: 105-110.

De Backer, O. and Colson, C. 1991a. Identification of the recognition sequence for the M.*Sty*LTI methyltransferase of *Salmonella typhimurium* LT7: an asymmetric site typical of type-III enzymes. *Gene.* 97: 103-107.

De Backer, O. and Colson, C. 1991b. Transfer of the genes for the *Sty*LTI restriction-modification system of *Salmonella typhimurium* to strains lacking modification ability results in death of recipient cells and degradation of their DNA. *J. Bacteriol.* 173: 1328-1330.

Dreier, J. and Bickle, T. A. 1996. ATPase activity of the type IC restriction modification system *Eco*R124II. *J. Mol. Biol.* 257: 960-969.

Dryden, D. T. F., Cooper, L. P. and Murray, N. E. 1993. Purification and characterisation of the methyltransferase from the type I restriction and modification system of *Escherichia coli* K12. *J. Biol. Chem.* 268: 13228-13236.

Dryden, D. T. F., Sturrock, S. and Winter, M. 1995. Structural modelling of a type I DNA methyltransferase. *Nature Structural Biology.* 2: 632-635.

Dryden, D. T. F., Cooper, L. P., Thorpe, P. H. and Byron, O. 1997. The *in vitro* assembly of the *Eco*KI type I DNA restriction/modification enzyme and its *in vivo* implications. *Biochemistry.* 36: 1065-1076.

Dunn, J. J. and Studier, F. W. 1983. The complete nucleotide sequence of bacteriophage T7 DNA, and the locations of T7 genetic elements. *J. Mol. Biol.* 166: 477-535.

Dussoix, D. and Arber, W. 1962. Host specificity of DNA produced by *Escherichia coli*. II. Control over acceptance of DNA from infecting phage lambda. *J. Mol. Biol.* 5: 37-49.

Endlich, B. and Linn, S. 1985. The DNA restriction endonuclease of *Escherichia coli* B. I: Studies of the DNA translocation and the ATPase activities. *J. Biol. Chem.* 260: 5720-5728.

Eskin, B. and Linn, S. 1972. The deoxyribonucleic acid modification and restriction enzymes of *Escherichia coli* B. II: Purification, subunit structure and catalytic properties of the restriction endonuclease. *J. Biol. Chem.* 247: 6183-6191.

Franklin, N. C. and Dove, W. F. 1969. Genetic evidence for restriction targets in the DNA of phages λ and ϕ80. *Genet. Res.* 14: 151-157.

Fried, M. G. 1989. Measurement of protein-DNA interaction parameters by electrophoresis mobility shift assay. *Electrophoresis.* 10: 366-376.

Fry, D. C., Kuby, S. A. and Mildran, A. S. 1986. ATP-binding site of adenylate kinase: mechanistic implications of its homolog with *ras*-encoded p21, F_1-ATPase, and other nucleotide-binding proteins. *Proc. Natl. Acad. Sci. USA.* 83: 907-911.

Fuller-Pace, F. V., Bullas, L. R., Delius, H. and Murray, N. E. 1984. Genetic recombination can generate altered restriction specificity. *Proc. Natl. Acad. Sci. USA.* 81: 6095-6099.

Fuller-Pace, F. V., V., Cowan, G. M. and Murray, N. E. 1985. *Eco*A and *Eco*E; alternatives to the *Eco*K family of type I restriction and modification systems of *E. coli*. *J. Mol. Biol.* 185: 65-75.

Fuller-Pace, F. V. and Lane, D. P. 1992. In Eckstein, F. and Lilley, D. M. J. (eds). *Nucleic Acids and Molecular Biology.* 6: 159-173.

Gann, A. A. F., Campbell, A. J. B., Collins, J. F., Coulson, A. F. W. and Murray, N. E. 1987. Reassortment of DNA recognition domains and the evolution of new specificities. *Mol. Microbiol.* 1: 13-22.

García, L. R. and Molineux, I. J. 1995. Rate of translocation of bacteriophage T7 DNA across the membranes of *Escherichia coli*. *J. of Bacteriol.* 177: 4066-4076.

Gellert, M., Mizuuchi, K., O'Dea, M. H. Okmon, H. and Tomizawa, J. 1978. DNA gyrase and DNA supercoiling. *Cold Spring Harbor Symp. Quant. Biol.* 43: 35-40.

Glover, S. W. 1970. Functional analysis of host-specificity mutants in *Escherichia coli*. *Genet. Res.* 15: 237-250.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. Blinov, V. M. 1989. Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic Acids Res.* 17: 4713-4730.

Gorbalenya, A. E. and Koonin, E. V. 1991. Endonuclease (R) subunits of type I and type III restriction-modification enzymes contain a helicase like domain. *FEBS Letts.* 291: 277-281.

Gough, J. A. and Murray, N. E. 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. *J. Mol. Biol.* 166: 1-19.

Grifo, J. A., Abramson, R. D., Satler, C. A. and Merrick, W. C. 1984. RNA-stimulated ATPase activity of eukaryotic initiation factors. *J. Biol. Chem.* 259: 8648-8654.

Gross, C. H. and Shuman, S. 1996. The QRxGRxGRxxxG motif of the Vaccinia Virus DExH box RNA helicase NPH-II is required for ATP hydrolysis and RNA unwinding but not for RNA binding. *J. of Virology.* 70: 1706-1713.

Gubler, M., Braguglia, D., Meyer, J., Piekarowicz, A. and Bickle, T. A. 1992. Recombination of constant and variable modules alters DNA sequence recognition by type IC restriction-modification enzymes. *EMBO J.* 11: 233-240.

Hadi, S. M., Bächi, B., Shepherd, J. C. W., Yuan, R. and Bickle, T. A. 1979. DNA recognition and cleavage by the *Eco*P15 restriction endonuclease. *J. Mol. Biol.* 134: 655-666.

Hadi, S. M., Bächi, B., Iida, S. and Bickle, T. A. 1983. DNA restriction-modification enzymes of phage P1 and plasmid p15B. Subunit functions and structural homologies. *J. Mol. Biol.* 165: 19-34.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166: 557-580.

Harosh, I., Naumovski, L. and Friedberg, E. C. 1989. Purification and characterization of Rad3 ATPase/DNA helicase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* 264:L 29532.

Heery, D. M. and Dunican, L. K. 1989. Improved efficiency M13 cloning using electroporation. *Nucleic Acids Research.* 17: 8006.

Higuchi, R. 1990. In Innis, M. A., Gelfand, D. H., Sninsky, J. J. and White, T. J. (eds), *PCR Protocols: A Guide to Methods and Applications.* Academic Press, Inc., San Diego, CA, pp177-183.

Hirling, H., Scheffner, M., Restle, T. and Stahl, H. 1989. RNA helicase activity associated with the human p68 protein. *Nature.* 339: 562-564.

Hodgman, T. C. 1988a. A new superfamily of replicative proteins. *Nature.* 333: 22-23.

Hodgman, T. C. 1988b. A new superfamily of replicative proteins. Erratum. *Nature.* 333: 578.

Hornby, D. P., Muller, M. and Bickle, T. A. 1987. High level expression of the *Eco*PI modification methylase gene and characterization of the gene product. *Gene.* 54: 239-245.

Hsieh, S. and Julin, D. A. 1992. Alteration by site-specific mutagenesis of the conserved lysine residue in the consensus ATP-binding sequence of the RecB protein of *Escherichia coli. Nucleic Acids Res.* 20: 5647-5653.

Hubacek, J. and Glover, S. W. 1970. Complementation analysis of temperature sensitive host specificity mutations in *Escherichia coli. J. Mol. Biol.* 50: 111-127.

Hümbelin, M., Suri, B., Rao, D. N., Hornby, D. P. Eberle, H. Pripfl,T., Kenel, S. and Bickle, T. A. 1988. Type III DNA restriction and modification systems *Eco*PI and *Eco*P15. Nucleotide sequence of the *Eco*PI operon, the *Eco*P15 *mod* gene and some *Eco*PI *mod* mutants. *J. Mol. Biol.* 200: 23-29.

Iida, S., Meyer, J., Bächi, B., Stålhammar-Carlemalm, M., Schrickel, S., Bickle, T. A. and Arber, W. 1983a. DNA restriction-modification genes of phage P1 and plasmid p15B. Structure and *in vitro* transcription. *J. Mol. Biol.* 165: 1-18.

Janscak, P., Abadjieva, A. and Firman, K. 1996. The type I restriction endonuclease R.*Eco*R124I: Over-production and biochemical properties. *J. Mol. Biol.* 257: 977-991.

Janulaitis, A., Petrusyte, M., Maneliene, Z., Klimasauskas, S. and Butkus, V. 1992a. Purification and properties of the *Eco*57I restriction endonuclease and methylase - prototypes of a new class (type IV). *Nucleic Acids Res.* 20: 6043-6049.

Janulaitis, A., Vaisvila, R., Timinskas, A., Klimasauskas, S. and Butkus, V. 1992b. Cloning and sequence analysis of the genes coding for *Eco*57I type IV restriction-modification enzymes. *Nucleic Acids Res.* 20: 6051-6056.

Johnson, R. E., Prakash, S. and Prakash, L. 1994. Yeast DNA repair protein RAD5 that promotes instability of simple repetitive sequences is a DNA-dependent ATPase. *J. Biol. Chem.* 269: 28259-28262.

Kan, N. C., Lautenberger, J. A., Edgell, M. H. and Hutschison III, C. A. 1979. The nucleotide sequence recognized by the *Escherichia coli* K12 restriction and modification enzymes. *J. Mol. Biol.* 130: 191-209.

Kauc, L. and Piekarowicz, A. 1978. Purification and properties of a new restriction endonuclease from *Haemophilus influenzae* Rf. *Eur. J. Biochem.* 92: 417-426.

Kim, Y., Grable, J. C. and Love, R. 1990. Refinement of *Eco*RI endonuclease crystal structure: A revised protein chain tracing. *Science.* 249: 1307-1309.

King, G. and Murray, N. E. 1995. Restriction alleviation and modification enhancement by the Rac prophage of *Escherichia coli* K-12. *Mol. Microbiol.* 16: 769-777.

Klimasauskus, S., Kumar, S., Roberts, R. J. and Cheng, X. 1994. *Hha*I methyltransferase flips its target base out of the DNA helix. *Cell.* 76: 357-369.

Korolev, S., Hsieh, J., Gauss, G. H., Lohman, T. M. and Waksman, .G. 1997. Major domain swiveling revealed by the crystal structures of complexes of *E. coli* Rep helicase bound to single-stranded DNA and ADP. *Cell.* 90: 635-647.

Krüger, D. H., Barcak, G. J., Reuter, M. and Smith, H. O. 1988. *Eco*RII can be activated to cleave refractory DNA recognition sites. *Nucleic Acids Res.* 16: 3997-4008.

Krüger, D. H. Kupper, D., Meisel, A., Reuter, M. and Schroeder, C. 1995. The significance of distance and orientation of restriction endonuclease recognition sites in viral DNA genomes. *FEMS Microbiol. Rev.* 17: 177-184.

Kühnlein, U. Linn S. and Arber, W. 1969. Host specificity of DNA produced by *Escherichia coli.* XI. *In vitro* modification of phage fd replicative form. *Proc. Natl. Acad. Sci. USA.* 63: 556-567.

Kühnlein, U. and Arber, W. 1972. Host specificity of DNA produced by *Escherichia coli.* XV. The role of nucleotide methylation in *in vitro* B-specific modification. *J. Mol. Biol.* 63: 9-19.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lautenberger, J. A., Kan, N. C., Lackey, D., Linn, S., Edgell, M. H. and Hutchinson III, C. A. 1978. Recognition site of *Escherichia coli* B restriction enzyme on ϕXsB1 and simian virus 40 DNAs: An interrupted sequence. *Proc. Natl. Acad. Sci. USA*. 75: 2271-2275.

Lederberg, S. 1957. Suppression of the multiplication of heterologous bacteriophages in lysogenic bacteria. *Virology*. 3: 496-513.

Lederberg, S. 1973. cited by Radding, E. M. In Molecular mechanisms of genetic recombination. *Annu. Rev. Genet*. 7: 87.

Lee, N. S., Rutebuka, O., Arakawa, T., Bickle, T. A. and Ryu, J. 1997. *Kpn*AI, a new type I restriction-modification system in *Klebsiella pneumoniae*. *J. Mol. Biol.* 271: 342-348.

Levin, B. 1986. Restriction modification immunity and the maintenance of genetic diversity in bacterial populations. In *Evolutionary Processes and Theory*. Karlin, S. and Nero, E. (eds). New York: Academic Press pp669-688.

Linder, P., Lasko, P. F., Ashburner, M., Leeroy, P., Nielson, P. J., Nishi, K., Schnier, J. and Slonimski, P. P. 1989. Birth of the DEAD box. *Nature*. 337: 121-122.

Linn, S. and Arber, W. 1968. Host specificity of DNA produced by *Escherichia coli*, X. *In vitro* restriction of phage fd replicative form. *Proc. Natl. Acad. Sci. USA*. 59: 1300-1306.

Liu, L. F. and Wang, J. C. 1987. Supercoiling of the DNA template during transcription. *Proc. Natl. Acad. Sci. USA*. 84: 7024-7027.

Lloyd, R. G. and Sharples, G. J. 1993. Processing of recombination intermediates by the RecG and RuvAB proteins of *Escherichia coli*. *Nucleic Acids Res*. 21: 1719-1725.

Loenen, W. A. M. and Murray, N. E. 1986. Modification enhancement by restriction alleviation protein (Ral) of bacteriophage lambda. *J. Mol. Biol.* 190: 11-22.

Loenen, W. A. M., Daniel, A. S., Braymer, H. D. and Murray, N. E. 1987. Organization and sequence of the *hsd* genes of *Escherichia coli* K12. *J. Mol. Biol.* 198: 159-170.

Lohman, T. M. and Bjornson, K. P. 1996. Mechanisms of helicase-catalysed DNA unwinding. *Annu. Rev. Biochem*. 65: 169-214.

Low, B. 1973. Rapid mapping of conditional and auxotrophic mutations in *Escherichia coli* K-12. *J. Bacteriol.* 113: 798-812.

Luria, S. E. and Human, M. L. 1952. A non-hereditary, host-induced variation of bacterial viruses. *J. Bacteriol.* 64: 557-569.

Meisel, A., Krüger, D. H. and Bickle, T. A. 1991. M.*Eco*P15 methylates the second adenine in its recognition sequence. *Nucleic Acids Res.* 19: 3997.

Meisel, A., Bickle, T. A., Krüger, D. H. and schroeder, C. 1992. Type III restriction enzymes need two inversely orientated recognition sites for DNA cleavage. *Nature (London).* 355: 467-469.

Meisel, A., Mackeldanz, P., Bickle, T. A., Krüger, D. H. and Schroeder, C. 1995. Type III restriction endonucleases translocate DNA in a reaction driven by recognition site-specific ATP hydrolysis. *EMBO J.* 14: 2958-2966.

Meister, J., MacWilliams, M., Hübner, P., Jütte, H., Skrzypek, E., Piekarowicz, A. and Bickle, T. A. 1993. Macroevolution by transposition: drastic modification of DNA recognition by a type I restriction enzyme following Tn5 transposition. *EMBO J.* 12: 4585-4591.

Meselson, M. and Yuan, R. 1968. DNA restriction enzyme from *E. coli*. *Nature.* 217: 1110-1114.

Meselson, M., Yuan, R., Heywood, J. 1972. Restriction and modification of DNA. *Ann. Rev. Biochem.* 41: 447-466.

Midgley, C. A. and Murray, N. E. 1985. T4 polynucleotide kinase; cloning of the gene (*pse*T) and amplification of its product. *EMBO J.* 4: 2695-2703.

Milhayi, E. 1970. Numeric values of the absorbances of the aromatic amino acids in acid, neutral and alkaline solutions. *In Handbook of Biochemistry.* Sober, H. A. (ed). CRC Press, Ohio. pp. B75-B77.

Murray, N. E., Manduca de Ritis, P. and Foster, L. A. 1973a. DNA targets for the *Escherichia coli* K restriction system analyzed genetically in recombinants between phages ϕ80 and lambda. *Mol. Gen. Genet.* 120: 261-281.

Murray, N. E., Batten, P. L. and Murray, K. 1973b. Restriction of bacteriophage λ by *Escherichia coli* K. *J. Mol. Biol.* 81: 395-407.

Murray, N. E., Gough, J. A., Suri, B. and Bickle, T. A. 1982. Structural homologies among type I restriction-modification systems. *EMBO J.* 1: 535-539.

Murray, N. E., Daniel, A. S., Cowan, G. M. and Sharp, P. M. 1993. Conservation of motifs within the unusually variable polypeptide sequences of type I restriction and modification enzymes. *Mol. Microbiol.* 9: 133-143.

Nagaraja, V., Sheperd, J. C. W. and Bickle, T. A. 1985. A hybrid recognition sequence in a recombinant restriction enzyme and the evolution of DNA sequence specificity. *Nature*. 316: 371-372.

Naussbaum, A., Shalit, M. and Cohen, A. 1992. Restriction-stimulated homologous recombination by plasmids by the recE pathway of *Escherichia coli*. *Genetics*. 130: 37-49.

Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I. And Aggarwal, A. K. 1995. Structure of *Bam*HI endonuclease bound to DNA: partial folding and unfolding on DNA binding. *Science*. 269: 656-663.

Nishi, K., Morel-Deville, F., Hershey, J. W. B., Leighton, T. and Schnier, J. 1988. An eIF-4A-like protein is a suppressor of an *Escherichia coli* mutant defective in SOS ribosomal subunit assembly. *Nature*. 336: 496-498.

O'Neill, M., Chen, A. and Murray, N. E. 1997. The restriction-modification genes of *Escherichia coli* K-12 may not be selfish; they do not resist loss and are readily replaced by alleles conferring different specificities. *Proc. Natl. Acad. Sci.* 94: 14596-14601.

Pai, E. G., Sachsenheimer, W., Schirmer, R. H. and Schultz, G. E. 1977. Substrate positions and induced fit in crystalline adenylate kinase. *J. Mol. Biol.* 114: 37-45.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W. and Wittinghofer, A. 1990. Refined crystal structure of the triphosphate conformation of H-*ras* p21 at 1.35Å resolution: implications for the mechanism of GTP hydrolysis. *EMBO J.* 9: 2351-2359.

Pause, A. and Sonenburg, N. 1992. Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. *EMBO J.* 7: 2643-2654

Piekarowicz, A. and Kalinowska, J. 1974. Host specificity of DNA in *Haemophilus influenzae*: Similarity between host-specificity types of *Haemophilus influenzae* Re and R*f*. *J. Gen. Microbiol.* 81: 405-411.

Pingoud, A. and Jeltsch, A. 1997. Recognition and cleavage of DNA by type-II restriction endonucleases. *Eur. J. Biochem.* 246: 1-22.

Powell, L. M., Dryden, D. T. F., Willcock, D. F., Pain, R. H. and Murray, N. E. 1993. DNA recognition by the *Eco*K methyltransferase. The influence of DNA methylation and the cofactor *S*-adenosyl-L-methionine. *J. Mol. Biol.* 234: 60-71.

Powell, L. M. and Murray, N. E. 1995. *S*-adenosyl methionine alters the DNA contacts of the *Eco*KI methyltransferase. *Nucleic Acids Res.* 23: 967-974.

Prakash-Cheng, A. and Ryu, J. 1993. Delayed expression of in vivo restriction acitivity following conjugal transfer of *Escherichia coli hsd*K (restriction-modification) genes. *J. Bacteriol.* 175: 4905-4906.

Price, C. and Bickle, T. A. 1986. A possible role for restriction in bacterial evolution. *Micro. Sci.* 3: 296-299.

Price, C., Pripfl, T. and Bickle, T. A. 1987. *Eco*R124 and *Eco*R124/3: The first members of a new family of type I restriction and modification systems. *Eur. J. Biochem.* 167: 111-115.

Price, C., Linger, J., Bickle, T. A., Firman, K. and Glover, S. W. 1989. Basis for changes in DNA recognition by the *Eco*R124 and *Eco*R124/3 Type I restriction and modification enzymes. *J. Mol. Biol.* 205: 115-125.

Raleigh, E. A. 1992. Organization and function of the *mcr*BC genes of *Escherichia coli* K-12. *Mol. Microbiol.* 6: 1079-1086.

Ravetch, J. V., Horiuchi, K., Zinder, N. D. 1978. Nucleotide sequence of the recognition site for the restriction-modification enzyme of *Escherichia coli B. Proc. Natl. Acad. Sci. USA.*75: 2266-2270.

Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C. and Thach, R. E. 1985. ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. *J. Biol. Chem.* 260: 7651-7658.

Reiser, J. and Yuan, R. 1977. Purification and properties of the P15 specific restriction endonuclease from *Escherichia coli. J. Biol. Chem.* 252: 451-456.

Revzin, A. 1989. Gel electrophoresis assays for DNA-protein interactions. *Biotechniques.* 7: 346-355.

Reynolds, R. J. and Friedberg, E. C. 1981. Molecular mechanisms of pyrimidine dimer excision in *Saccharomyces cerevisiae*: incision of ultraviolet-irradiated deoxyribonucleic acid *in vivo. J. Bacteriol.* 146: 692-704.

Rickenberg, H. V. and Lester, G. 1955. The preferential synthesis of β-Galactosidase in *Escherichia coli. J. Gen. Microbiol.* 13: 279-284.

Roberts, R. J. and Macelis, D. 1996. REBASE-restriction enzymes and methylases. *Nucleic Acids Res.* 24: 223-235.

Roman, L. J. and Kowalczykowski, S. C. 1989. Characterisation of the helicase activity of the *Escherichia coli* RecBCD enzyme using a novel helicase assay. *Biochemistry.* 28: 2863-2873.

Rosamund, J., Endlich, B. and Linn, S. 1979. Electron microscopic studies of the mechanism of action of the restriction endonuclease of *Escherichia coli* B. *J. Mol. Biol.* 129: 619-935.

Roulland-Dussoix, D. and Boyer, H. W. 1969. The *Escherichia coli* B restriction endonuclease. *Biochem. Biophys. Acta.* 195: 219-229.

Rozen, F., Pelletier, J., Trachsel, H. and Sonenberg, N. 1989. A lysine substitution in the ATP-binding site of Eucaryotic Initiation factor 4A abrogates nucleotide-binding activity. *Mol. and Cell. Biol.* 9: 4061-4063.

Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C. and Sonenberg, N. 1990. Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell. Biol.* 10: 1134-1144.

Saha, S. and Rao, D. N. 1995. ATP hydrolysis is required for DNA cleavage by *Eco*PI restriction enzyme. *J. Mol. Biol.* 247: 559-567.

Saha, S. and Rao, D. N. 1997. Mutations in the Res subunit of the *Eco*PI restriction enzyme that affect ATP-dependent reactions. *J. Mol. Biol.* 269: 342-354.

Saiki, S. J., Gelfand, D. H. Stoffel, S., Scharf, S. J. Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA-polymerase. *Science.* 239: 487-491.

Sain, B. and Murray, N. E. 1980. The *hsd* (host specificity) genes of *E. coli* K12. *Mol. Gen. Genet.* 180: 35-46.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular cloning. A laboratory manual* (2nd Edit.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Niklen, S. and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitions. *Proc. Natl. Acad. Sci. USA.* 74: 5463-5467.

Schmid, S. R. and Linder, P. 1992. D-E-A-D protein family of putative RNA helicases. *Mol. Microbiol.* 6: 283-292.

Schroeder, C., Jurkschat, H., Meisel, A., Reich, J. G. and Kruger, D. 1986. Unusual occurrence of *Eco*PI and *Eco*P15 recognition sites and counterselection of type III methylation and restriction sequences in bacteriophage T7 DNA. *Gene.* 45: 77-86.

Schulz, G. E. 1992. Binding of nucleotides by proteins. *Curr. Opin. Struct. Biol.* 2: 61-67.

Schwer, B. and Guthrie, C. 1991. PRP16 is an RNA-dependent ATPase that interacts transiently with the spliceosome. *Nature.* 349: 494-499.

Seeley, T. W. and Grossman, L. 1989. Mutations in the *Escherichia coli* UvrB ATPase motif compromise excision repair capacity. *Proc. Natl. Acad. Sci. USA.* 86: 6577-6581.

Selby, C. P. and Sancar, A. 1993. Molecular mechanism of transcription-repair coupling. *Science.* 260: 53-58.

Sharples, G. J., Whitby, M. C., Ryder, L. and Lloyd, R. G. 1994. A mutation in helicase motif III of *E. coli* RecG protein abolishes branch migration of Holliday junctions. *Nucleic Acids Res.* 22: 308-313.

Sharrocks, A. D. and Hornby, D. P. 1991. Transcriptional analysis of the restriction and modification genes of bacteriophage PI. *Mol. Microbiol.* 5: 685-689.

Smith, H. O. and Wilcox, K. W. 1970. A restriction enzyme from *Haemophilus influenzae.* I. Purification and general properties. *J. Mol. Biol.* 51: 379-391.

Smith, J. Arber, W. and Kühnlein, U. 1972. Host specificity of DNA produced by *Escherichia coli.* XIV. The role of nucleotide methylation in *in vivo* B-specific modification. *J. Mol. Biol.* 63: 1-8.

Smith, H. O. and Nathans, D. 1973. A suggested nomenclature for bacterial host modification and restriction systems and their enzymes. *J. Mol. Biol.* 81: 419-423.

Story, R. M. and Steitz, T. A. 1992. Structure of the recA protein-ADP complex. *Nature.* 355: 374-376.

Studier, F. W. and Bandyopadhyay. 1988. Model for how type I restriction enzymes select cleavage sites in DNA. *Proc. Natl. Acad. Sci. USA.* 85: 4677-4681.

Subramanya, H. S., Bird, L. E., Brannigan, J. A. and Wigley, D. B. 1996. Crystal structure of a Dexx box DNA helicase. *Nature.* 384: 379-383.

Sung, P., Prakash, L., Weber, S. and Prakash, S. 1987a. The *Rad3* gene of *Saccharomyces cerevisiae* encodes a DNA-dependent ATPase. *Proc. Natl. Acad. Sci. USA.* 84: 6045-6049.

Sung, P., Prakash, L., Matson, S. W. and Prakash, S. 1987b. RAD3 protein of Saccharomyces Cerevisiae is a DNA helicase. *Proc. Natl. Acad. Sci.* 84: 8957-8955.

Sung, P., Higgins, D., Prakash, L. and Prakash, S. 1988. Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes the ATPase and DNA helicase activities but not the ability to bind ATP. *EMBO J.* 7: 3263-3269.

Suri, B., Nagaraja, V. and Bickle, T. A. 1984. Bacterial DNA modification. *Curr. Top. Microbiol. Immunol.* 108: 1-9.

Szczelkun, M. D., Dillingham, M. S., Janscak, P., Firman, K. and Halford, S. E. 1996. Repercussions of DNA tracking by the type IC restriction endonuclease *Eco*R124I on linear, circular and catenated substrates. *EMBO J.* 15: 6335-6347.

Szczelkun, M. D., Janscak, P., Firman, K. and Halford, S. E. 1997. Selection of non-specific DNA cleavage sites by the type IC restriction endonuclease *Eco*R124I. *J. Mol. Biol.* 271: 112-123.

Szybalski, W., Kim, S. C., Hasan, N. and Podhajska, A. J. 1991. Class-IIS restriction enzymes - a review. *Gene.* 100: 13-26.

Tabor, S. 1990. Expression using the T7 RNA polymerase/promoter system. *In* Current protocols in molecular biology. F. A. Ausubel *et al.* (eds), Greene publishing and Wiley-Interscience, NY. pp. 16.2.1.

Tao, T., Bourne, J. C. and Blumenthal, R. M. 1991. A family of regulatory genes associated with type II restriction-modification systems. *J. Bacteriol.* 173: 1367-1375.

Taylor, J. D., Badcoe, I. G., Clarke, A. R. and Halford, S. E. 1991. *Eco*RV restriction endonuclease binds all DNA sequences with equal affinity. *Biochemistry.* 30: 8743-8753.

Taylor, I., Watts, D. and Kneale, G. 1993. Substrate recognition and selectivity in the type IC DNA modification methylase M.*Eco*R124I. *Nucl. Acids Res.* 21: 4929-4935.

Thielking, V.,Selent, U., Köhler, E., Wolfes, H., Pieper, U., Geiger, R., Urbanke, C., Winkler, F. K. and Pingoud, A. 1991. Site-directed mutagenesis studies with *Eco*RV restriction endonuclease to identify regions involved in recognition and catalysis. *Biochemistry.* 30: 6416-6422.

Thorpe, P. H., Ternent, D. and Murray, N. E. 1997. The specificity of *Sty*SKI, a type I restriction enzyme, implies a structure with rotational symmetry. *Nucleic Acids Res.* 25: 1694-1700.

Titheradge, A. J. B., Ternent, D. and Murray, N. E. 1996. A third family of allelic *hsd* genes in *Salmonella enterica*: sequence comparisons with related proteins identify conserved regions implicated in restriction of DNA. *Molecular Microbiology*. 22: 437-447.

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76: 4350-4354.

Tsaneva, I. R., Müller, B. and West, S. C. 1993. RuvA and RuvB proteins of *Escherichia coli* exhibit DNA helicase activity *in vitro*. *Proc. Natl. Acad. Sci. USA*. 90: 1315-1319.

Tyndall, C., Meister, J. and Bickle, T. A. 1994. The *Escherichia coli prr* region encodes a functional type IC DNA restriction system closely integrated with an anticodon nuclease gene. *J. Mol. Biol.* 237: 266-274.

Umezu, K., Nakayama, K. and Nakayama, H. 1990. *Escherichia coli* RecQ protein is a DNA helicase. *Proc. Natl. Acad. Sci.* 87: 5363-5367.

Vovis, G. F., Horiuchi, K. and Zinder, N. D. 1974. Kinetics of methylation of DNA by a restriction endonuclease from *Escherichia coli* B. *Proc. Natl. Acad. Sci. USA*. 71: 3810-3813.

Walker, J. E., Saraste, M., Runswick, M. J. and Gay, N. J. 1982. Distantly related sequences in the α- and β- subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 1: 945-951.

Webb, J. L., King, G., Ternent, D., Titheradge, A. J. B. and Murray, N. E. 1996. Restriction by *Eco*KI is enhanced by co-operative interactions between target sequences and is dependent on DEAD box motifs. *EMBO J.* 15: 2003-2009.

Weiserova, M., Janscak, P., Benada, O., Hubacek, J., Zinkevich, V. E., Glover, S. W. and Firman, K. 1993. Cloning, production and characterisation of the R.*Eco*KI endonucleases. *Nucleic Acids Res.* 21: 373-379.

West, S. C. 1996. DNA helicases: New breeds of translocating motors and molecular pumps. *Cell.* 86: 177-180.

Whittaker, P. A., Campbell, A. J. B., Southern, E. M. and Murray, N. E. 1988. Enhanced recovery and restriction mapping of DNA fragments cloned in a new λ vector. *Nucleic Acids Res.* 16: 6725-6736.

Willcock, D. F., Dryden, D. T. F. and Murray, N. E. 1994. A mutational analysis of the two motifs common to adenine methyltransferases. *EMBO J.* 13: 3902-3908.

Winkler, F. K., Banner, D. W., Oefner, C., Tsernoglou, D., Brown, R. S., Heathman, S. P., Bryan, R. K., Martin, P. D., Petratos, K. and Wilson, K. S. 1993. The crystal structure of *Eco*RV endonuclease and of its complexes with cognate and non-cognate DNA fragments. *EMBO J.* 12: 1781-1795.

Wood, W. B. 1966. Host specificity of DNA produced by *Escherichia coli*: Bacterial mutations affecting the restriction and modification of DNA. *J. Mol. Biol.* 16: 118-133.

Yanisch-Perron, C., Viera, J. and Messing, J. 1985. Improved M13 phage cloning vectors and host strains-nucleotide sequences of the M13mp18 and pUC18 vectors. *Gene.* 33: 103-109.

Yuan, R. and Meselson, M. 1970. A specific complex between a restriction endonuclease and its DNA substrate. *Proc. Natl. Acad. Sci. USA.* 65: 357-362.

Yuan, R., Bickle, T. A., Ebbers, W. and Brack, C. 1975. Multiple steps in DNA recognition by restriction endonuclease from *E. coli* K. *Nature.* 256: 556-560.

Yuan, R., Hamilton, D. L. and Burckhardt, J. 1980. DNA translocation by the restriction enzyme from *E. coli* K. *Cell.* 20: 237-244.

Zavitz, K. H. and Marians, K. 1992. ATPase-deficient mutants of the *Escherichia coli* DNA replication protein PriA are capable of catalyzing the assembly of active primosomes. *J. Biol. Chem.* 267: 6933-6940.

Appendix

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