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*Eco*KI with an amino acid substitution in any one of seven DEAD-box motifs has impaired ATPase and endonuclease activities

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ABSTRACT

For type I restriction systems, recently determined nucleotide sequences predict conserved amino acids in the subunit that is essential for restriction but not modification (HsdR). The conserved sequences emphasize motifs characteristic of the DEAD-box family of proteins which comprises putative helicases, and they identify a new candidate for motif IV. We provide evidence based on an analysis of *Eco*KI which supports both the relevance of DEAD-box motifs to the mechanism of restriction and the new definition of motif IV. Amino acid substitutions within the newly identified motif IV and those in six other previously identified DEAD-box motifs, but not in the original motif IV, confer restriction-deficient phenotypes. We have examined the relevance of the DEAD-box motifs to the restriction pathway by determining the steps permitted *in vitro* by the defective enzymes resulting from amino acid substitutions in each of the seven motifs. *Eco*KI purified from the seven restriction-deficient mutants binds to an unmethylated target sequence and, in the presence of AdoMet, responds to ATP by undergoing the conformational change essential for the pathway of events leading to DNA cleavage. The seven enzymes have little or no ATPase activity and no endonuclease activity, but they retain the ability to nick unmodified DNA, though at reduced rates. Nicking of a DNA strand could therefore be an essential early step in the restriction pathway, facilitating the ATP-dependent translocation of DNA, particularly if this involves DNA helicase activity.

INTRODUCTION

Enzymes that bind ATP include a motif defined by two component sequences, 'A' and 'B' (1). Many of these enzymes comprise a 'super family' within which members share a number of sequences including GxGKS/T, an abridged version of the A component of the ATP-binding motif, the sequence DEAD, DExH (e.g. DEAH and DECH) or DExx (e.g. DEYQ) as the B component of the ATP-binding motif, and five additional conserved sequences (2). Some of these proteins are known to be helicases (3,4). Mutational analyses of DEAD-box proteins have been reported, the most extensive being for an RNA helicase, the mammalian translation initiation factor eIF-4A (5). The results suggest that the seven highly conserved regions in the DEAD-box proteins are critical for helicase activity. Each of these conserved sequences will be referred to as a motif, identified as I, Ia and II–VI (6).

Some members of the DEAD-box proteins have been shown to be DNA, rather than RNA, helicases. These include Rad3 of yeast (7), and RecG (8) and RecQ (9) of *Escherichia coli*, all three of which are involved in recombination and repair processes. RecG includes the seven motifs characteristic of the DExH family (10) and catalyses branch migration of Holliday junctions in a reaction that requires ATP hydrolysis. A mutation affecting motif III, in which the sequence TAT was changed to TVT, was shown to reduce ATP hydrolysis and to block branch migration (8). For RecG, it is concluded that branch migration of Holliday junctions is related to an ATP-dependent helicase activity.

Type I restriction and modification (R–M) enzymes, like RecG, are members of the DExH subgroup of DEAD-box proteins, and although evidence for a helicase activity is lacking, like RecG, they are believed to translocate DNA (11–16). Type I R–M enzymes are complex proteins comprising three different subunits, HsdR, HsdM and HsdS. Each system recognizes its specific target sequence, the methylation status of which determines whether the R–M complex responds by modifying (methylating) or restricting (cutting) the DNA. Restriction, the response to unmethylated target sequences, is ATP dependent and involves the cutting of non-specific sequences remote from the target sequences. The restriction enzyme remains bound to its target sequence (17) and is believed to translocate DNA past the bound enzyme in an ATP-dependent process (12). Experiments using a linear DNA substrate led to a model (13) in which it was proposed that cutting is stimulated when two translocating complexes collide. This model is consistent with *in vivo* evidence that cutting occurs between target sequences (18).

The predicted amino acid sequence of *Eco*KI, the type I R–M enzyme found in *E.coli* K-12, includes the A and B components of the ATP-binding motif; motifs I and II of DEAD-box proteins. These sequences are in the HsdR subunit of the complex, consistent with the ATP dependence of the restriction reaction *in vitro* (19) and the demonstration that the HsdR subunit of type I R–M enzymes is essential for restriction but not modification (20,21). The B component of the ATP-binding motif in *Eco*KI is

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DExH; this, and the putative presence of the other sequences characteristic of the DExH subgroup of DEAD-box proteins in the HsdR subunits of *Eco*KI and *Eco*R124I, was recognized by Gorbalenya and Koonin (6). Sequence comparisons of the very dissimilar HsdR subunits of different type I R–M enzymes identified the DEAD-box motifs as their most noticeable common features, and this observation prompted the speculation that these motifs are relevant to the DNA translocation that is believed to precede restriction (14).

Conservative mutations in motifs I, II and III of *Eco*KI have been shown to impair DNA restriction *in vivo* (22). In this paper we extend the analysis to mutations in motifs Ia, IV, V and VI, as identified by Gorbalenya and Koonin (6), and an additional sequence (Y) located between motifs III and IV (23). The mutations in region Y and motifs Ia, V and VI, but not IV, confer a restriction-deficient phenotype (r_K^-) and we now propose that region Y identifies the correct motif IV. *Eco*KI has been purified from seven r_K^- mutants, one for each of the seven DEAD-box motifs including region Y as motif IV, and from two r_K^+ mutants. The proteins recognize their DNA target sequence, but those from r_K^- strains are unable to cut DNA and they have low or negligible levels of ATPase activity. These analyses identify seven regions of the HsdR polypeptide of *Eco*KI, which share sequence similarities with DEAD-box motifs, as being relevant to the restriction activity of *Eco*KI.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages

AB1157 (24) was used as a restriction-proficient ($r_K^+ m_K^+$) *E.coli* K-12 strain. NM795 and NM802 are r_K^- derivatives of AB1157; NM795 has missense mutations in *hsdR* (22) and NM802 an internal deletion, *hsdR*Δ4 (21), that removes the DNA specifying the DEAD-box motifs, with the possible exception of motif I. A strain deleted for the *hsd* genes, NM679 (25), was the host for propagating *hsdR*$^-$*M*$^+$*S*$^+$ plasmids for DNA and the production of mutant *Eco*KI for purification.

The origins of the mutations in motifs I, II and III have been described previously (22). pSB2, a plasmid including *hsdR* but not *hsdM* and *S* (22), was the template for amplification by PCR to obtain mutations in motif Ia. This site-directed mutagenesis used the recombinant PCR technique as described by Higuchi (26). Amplified DNA fragments were substituted for the relevant wild-type sequence of the *hsdR* gene in pSB2 and the complete nucleotide sequences of inserts were determined to identify the anticipated base changes and demonstrate the absence of additional mutations. Mutations in motifs IV, V, VI and region Y were made directly in pSB2 using the Quickchange™ procedure of Stratagene. The mutations were identified from the nucleotide sequence. For each base change, duplicate isolates were selected, one from each of two separate reactions to ensure that each mutant was of independent origin. The nucleotide sequences of oligonucleotides used to make changes in the motifs are given in Table 2. Some mutations were transferred to p*hsd*$^+$ to make *hsdR*$^-$*M*$^+$*S*$^+$ plasmids, the source of mutant enzymes; the 2.2 kb fragment (*Asp*718–*Sma*I; Fig. 1) that includes the region encoding all the DEAD-box motifs was excized from pSB2 derivatives and used to replace the wild-type sequence of p*hsd*$^+$ (see Table 2 for plasmids).

The *hsdR*$^-$ derivatives of pSB2 were used for complementation tests in the *hsdR*$^-$ strain NM795, and for dominant negative

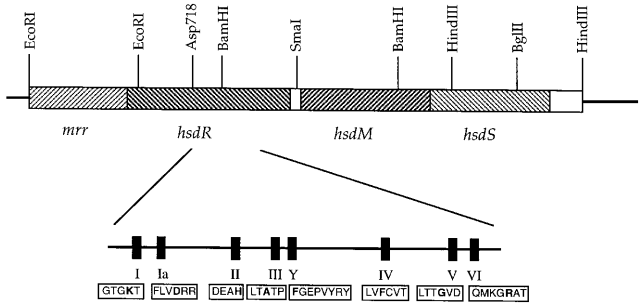

Figure 1. Restriction map of the *hsd* region cloned in p*hsd*$^+$. *mrr* is a gene encoding a methylation-dependent restriction system. The locations of the coding sequences for the DEAD-motifs are indicated below the map. The amino acid sequences of the motifs in *Eco*KI are given; bold type identifies the amino acids for which substitutions were made.

complementation tests in the *hsd*$^+$ strain AB1157. λ virulent (laboratory collection), either unmodified (λ*vir*.0) following propagation on NM679 or modified (λ*vir*.K) following propagation on AB1157, was used to measure restriction phenotypes. Phages λNM1265 (27) and λNM1347 (22) were used to clone mutant *hsdR* genes within a *Sma*I–*Eco*RI DNA fragment and facilitate their transfer from the plasmid to the chromosome of AB1157. λNM1266 retains one target for *Sma*I, the leftmost, and λNM1347 retains only the leftmost *Eco*RI site.

DNA manipulation

Preparation and manipulation of DNA and nucleotide sequencing were carried out as described previously (25). The nucleotide sequence for Ia is between unique *Kpn*I (*Asp*718) and *Bam*HI targets in the *hsdR* gene of plasmid pSB2 (Fig. 1). The oligonucleotide primers used in the PCR to amplify this 550 bp region were 5′-CGATGGCGACCGGTACCG and 5′-GGCGGATCCTGGTCGATC, in which the *Kpn*I (*Asp*718) and *Bam*HI targets, respectively, are underlined. The *hsdR* fragment was excised by *Bam*HI in combination with *Asp*718.

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

Transfer of mutations to the bacterial chromosome

The λ phages including *hsdR* encode a temperature-sensitive repressor (*cI*857) and are defective in their attachment site (*b*527). Lysogens selected as immune colonies at 32°C frequently result from homology-dependent recombination but they occasionally lose their prophage by homologous recombination. These ‘cured’ clones may be selected at 42°C. The *b*527 mutation greatly reduces the frequency of site-specific integration but does not entirely

prevent it. Lysogens resulting from site-specific recombination only rarely produce cured clones and liberate no or few progeny phage.

λ phages in which the *hsdR* mutation has conferred restriction deficiency gave both r_K^+ and r_K^- lysogens of AB1157, depending on the location of the integrative cross-over with respect to the mutation. Approximately 50% of the cured colonies selected at 42°C had replaced the chromosomal *hsdR* allele with that of the phage. As expected, the two restriction-proficient mutants (A619G and F730S) failed to give r_K^- lysogens in an *hsdR*$^+$ host; lysogens were made, therefore, in an *hsdR*$^-$ host (NM802) and on curing these lysogens yielded r_K^+ derivatives.

Reagents

DNA sequencing kits, sequenase and [γ-^{32}P]ATP were obtained from Amersham International, type II restriction enzymes from Boehringer Mannheim and New England BioLabs, DNA ligase and Vent DNA polymerase from New England BioLabs and Red Hot DNA polymerase from Advanced Biotechnologies. Polynucleotide kinase was supplied by S.Bruce and K.Murray. ATP was obtained from Sigma and AdoMet from New England BioLabs. Quik-Change™ Site-Directed Mutagenesis kits were bought from Stratagene. Synthetic oligonucleotides were supplied by OSWEL DNA, University of Southampton.

Protein purification and analysis

The enzymes were purified as described by Dryden *et al*. (28). DNA binding and the stability of the nuclease ($R_2M_2S_1$) complex were checked by gel retardation of the unmodified specific 45 bp duplex DNA containing the *Eco*KI recognition site (0.1 nM) by *Eco*KI (0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 nM). Synthetic oligonucleotide (45mers) were end-labelled and hybridized to yield the 45 bp duplex as described by Powell *et al*. (29) except [γ-^{33}P]ATP (ICN) was used instead of [γ-^{32}P]ATP. The protein was preincubated on ice with AdoMet (100 μM), or AdoMet (100 μM) and ATP (2 mM), for 10 min prior to DNA addition. The binding buffer was 20 mM Tris–HCl pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 7 mM β-mercaptoethanol and 5% glycerol. After DNA addition, the samples were incubated for 10 min at 22°C prior to running on a 5% non-denaturing gel for 2 h at 35 mA. Gels were dried for 75 min at 80°C and exposed to Kodak Biomax MR film for 24–48 h.

Exonuclease III footprinting was carried out essentially as described by Powell *et al.* (30). Protein (100 nM) was preincubated for 10 min on ice with AdoMet (100 μM) or AdoMet and ATP (2 mM), prior to DNA addition. The samples were incubated for a further 10 min at 22°C in the buffer used for gel retardation, to allow DNA–protein complex formation. After this incubation, 1.5 U exonuclease III was added to 60 μl of each reaction, and digestion was for 10 min at 37°C. The samples were processed as described by Powell *et al*. (30) and run on a 12% urea denaturing gel which was dried prior to autoradiography. Gel retardation confirmed that the *Eco*KI complex (wild-type or mutant) used in each footprinting reaction remained intact, with no dissociation of the R subunit to yield $R_1M_2S_1$ and M_2S_1.

Endonuclease activity was assayed by a modification of the method of Dryden *et al*. (28). The reactions were stopped by the addition of 0.5 vol 'stop' solution [0.1 M EDTA, 0.1 M Tris–HC1 pH 8.0, 40% (w/v) sucrose], and the products of the reaction were separated by electrophoresis through a 1.2% agarose gel in TAE buffer (31).

Two assays were used for ATPase. One followed the release of inorganic phosphate from γ-labelled ATP directly (32), the second was the pyruvate kinase/lactate dehydrogenase-linked assay of Ali *et al*. (33). Both assays were done at 37°C in buffer A from Boehringer Mannheim (33 mM Tris–acetate, 10 mM magnesium acetate, 66 mM potassium acetate and 0.5 mM dithiothreitol, pH 7.9) and were initiated by the addition of ATP. The rates are initial velocities based on a period of linear reduction of A_{340}, within 3 min of starting the reaction for active enzymes and within 15 min for impaired enzymes. All assays were done in triplicate, and corrected for background activity in the absence of AdoMet. The two assays gave very similar results, the second being preferable for the determination of rates, since ADP does not accumulate and inhibit the reaction.

RESULTS

Mutations in the DEAD-box motifs

The mutations affecting motifs I, II and III were made in plasmid pSB2, a plasmid in which the entire *hsdR* gene is located downstream of the T7 promoter in pT7-7, and have been described previously (22). The mutations in motif Ia were made by site-directed mutagenesis (SDM) using the polymerase chain reaction (PCR); a 550 bp *Asp*718–*Bam*HI fragment was amplified (Fig. 1) and substituted for the wild-type sequence in plasmid pSB2. Clones with each of three alternative mutations were identified from the nucleotide sequences of the *Asp*718–*Bam*HI fragment. No additional mutations were created during the amplification procedure. Mutations in motifs IV, V and VI and in region Y were made directly during amplification of pSB2 using the Quickchange™ procedure of Stratagene. This method uses *Pfu* DNA polymerase, a polymerase of high fidelity, and rather than sequence each mutant *hsdR* gene, a sequence of >4 kb, duplicate mutations for each amino acid change were identified and isolated from independent reactions; the phenotypes of these duplicate mutants were checked and found to be consistent.

The amino acid substitutions made are listed in Table 1. Motifs I, Ia, II, IV, V and VI are as identified by Gorbalenya and Koonin (6). Motif III (22) and region Y (23) are within the corrected sequence for *hsdR* in which a short frame-shifted segment in the original *hsdR* sequence was identified (34).

Phenotypes of mutants

Tests dependent on multicopy plasmids. Plasmid pSB2 includes *hsdR* cloned in the absence of *hsdM* and *S* (22). This *hsdR*$^+$ parent plasmid, when maintained in a strain encoding a defective HsdR subunit (e.g. NM795), compensates for the chromosomal *hsdR* mutation and the restriction-proficient phenotype is recognized by the low efficiency of plaque formation (e.o.p.) of λ*vir*.0. The phenotypes associated with the presence of the mutant derivatives of pSB2 were tested in the same way; a low e.o.p. of unmodified phages would be indicative of a functional HsdR polypeptide. On the basis of this test, one of three substitutions in motif III (22) and all three of those in motif IV failed to inactivate the HsdR polypeptide (Table 1). The phenotypes of the other mutants are consistent with defects in restriction. The test, however, is not reliable, because any cell lacking the plasmid will fail to restrict λ*vir*.0 and progeny phages will be modified by the methyltransferase

component of *Eco*KI. It is, therefore, critical that the vast majority of cells retain the plasmid, otherwise a false indication of restriction deficiency will result.

Table 1. The effects of changes in the DEAD-box motifs on the restriction phenotype

Motif	Sequence	Mutation on plasmid in *hsdR*$^-$ host[a]	in *hsdR*$^+$ host[a]	Mutation on chromosome[b]
(I)[c]				
	K477I	rk$^-$	rk$^-$	*
GTG**K**T	K477R	rk$^-$	rk$^-$	rk$^-$
	K477T	rk$^-$	rk$^-$	*
(Ia)				
	D502H	rk$^-$	rk$^-$	*
FLV**D**RR	D502N	rk$^-$	rk$^-$	*
	D502Y	rk$^-$	rk$^-$	rk$^-$
(II)[c]				
	H577D	rk$^-$	rk$^-$	rk$^-$
DEA**H**	H577N	rk$^-$	rk$^-$	*
	H577Y	rk$^-$	rk$^+$	rk$^-$
(III)[c]				
	A619D	rk$^-$	rk$^-$	*
LT**A**TP	A619V	rk$^-$	rk$^-$	rk$^-$
	A619G	rk$^+$	rk$^+$	rk$^{+/-}$
(IV)[d]				
	F730C	rk$^+$	rk$^+$	*
LV**F**CVT	F730S	rk$^+$	rk$^+$	rk$^+$
	F730T	rk$^+$	rk$^+$	*
(V)				
	G799C	rk$^-$	rk$^-$	rk$^-$
LTT**G**VD	G799R	rk$^-$	rk$^-$	*
	G799S	rk$^-$	rk$^-$	*
(VI)				
	R826H	rk$^-$	rk$^-$	rk$^-$
QMKG**R**AT	R826L	rk$^-$	rk$^-$	*
	R826P	rk$^-$	rk$^-$	*
(Y)				
FGEPVYRY	F629C	rk$^-$	rk$^+$	*
	F629Y	rk$^-$	rk$^-$	rk$^-$

[a]The *hsdR*$^-$ strain, NM795, is a derivative of the *hsdR*$^+$ strain, AB1157.
[b]The mutations were transferred to the chromosome of AB1157 via a λ*hsdR* phage.
[c]Data presented by Webb *et al*. (22).
[d]Original suspect motif IV, designated 'IV' in Figures 2–4.
rk$^-$, inferred from an e.o.p. of ~1 relative to the rk$^-$ strains NM795 and NM802.
*, not tested.

Positive evidence of a restriction-deficient phenotype is obtained if the mutant HsdR polypeptide competes with wild-type for HsdM and HsdS and, particularly when present in excess, converts an *hsd*$^+$ host into an r_K^- phenocopy. Therefore, each of the plasmids carrying a mutant *hsdR* gene was used to transform the r_K^+ strain AB1157, and the restriction phenotypes of the transformed strains were tested. In this test an r_K^+ phenotype is consistent with either a mutant HsdR polypeptide that remains functional, or an inactive polypeptide that fails to displace the wild-type HsdR subunit. An r_K^- phenotype is good evidence of a defective HsdR subunit that retains the ability to interact with the methylase, and mutations conferring this dominant negative phenotype were isolated in each of the regions tested, other than the original motif IV. In this motif none of three different substitutions for the phenylalanine residue prevented restriction (Table 1).

Test dependent on mutations in the bacterial chromosome. Quantification of the restriction phenotype of any *hsdR* mutant is only possible if the mutation is stably maintained in single copy on the bacterial chromosome, preferably in its normal location under the control of its resident promoter. Some mutations, including those for which *Eco*KI has been purified, have been transferred to the chromosome of AB1157 and restriction quantified using λ*vir*.0. The mutations in region Y and changes in all the previously designated motifs other than IV can confer an r_K^- phenotype identical to that found for a strain lacking the *hsdR* gene (Table 1). The most conservative mutation in motif III had only a small effect on the restriction phenotype (22).

The mutants specify *Eco*KI complexes of normal stoichiometry

*Eco*KI was purified from nine mutants. The mutations chosen included one within each conserved sequence other than motif III, for which enzyme was purified from one mutant (A619V) with an r_K^- phenotype and one (A619G) that retained almost full restriction capacity. Each of the nine mutations was transferred from the pSB2 derivative, which lacks *hsdM* and *hsdS*, to p*hsd*$^+$, a plasmid that includes the three *hsd* genes (35; Fig. 1). *Eco*KI was prepared from an *hsd*Δ strain (NM679) transformed with *hsdR*$^-$ derivatives of p*hsd*$^+$ (Table 2).

For each of the mutants, the elution profiles obtained during purification of the *Eco*KI complex, including that from the size-exclusion chromatography column (results not shown), were indistinguishable from those obtained for the wild-type enzyme (28). This similarity supports the correlation of the dominant negative phenotype of the mutant strains with localized defects in the HsdR subunit of the *Eco*KI complex rather than merely the absence of a stable HsdR polypeptide or the failure to maintain the *Eco*KI complex.

It has been shown recently by gel retardation that wild-type *Eco*KI has a tendency to dissociate at very low protein concentration, particularly in the presence of ATP (36). This dissociation was shown by the detection of $R_1M_2S_1$–DNA and M_2S_1–DNA complexes as well as the major species in which the active nuclease, $R_2M_2S_1$, is associated with DNA. Each of the three complexes was excised from a non-denaturing gel and the component subunits separated on an SDS–polyacrylamide gel to permit their identification in western blots by antibody to *Eco*KI (36).

In order to check the integrity of the mutant nuclease complexes over a range of protein concentrations (5–100 nM), we examined the gel retardation of a 45 bp duplex that includes an unmodified target sequence for *Eco*KI. In the presence of 100 μM AdoMet, but in the absence of ATP, the only DNA–protein complex observed for wild-type *Eco*KI was that for intact $R_2M_2S_1$, even at protein concentrations as low as 5 nM. In the presence of ATP (2 mM), faint bands representing $R_1M_2S_1$–DNA and M_2S_1–DNA were apparent at 5 and 10 nM protein, but the major complex was $R_2M_2S_1$–DNA. Gel retardation assays in the presence and absence of ATP were carried out on all the proteins made from restriction-deficient mutants (data not shown). The assays in the absence of ATP showed that the $R_2M_2S_1$–DNA complex is the major species across the 5–100 nM range of protein. In the presence of ATP, $R_2M_2S_1$ generally remained the

main species across the 5–100 nM range. However, the enzyme preparations with substitutions in either motif II or V required 10 nM protein in order that the main species was $R_2M_2S_1$, while the concentration of the protein with a substitution in motif VI had to be raised to 20 nM to achieve the *Eco*KI complex as the major species. For this reason, the enzyme assays on *Eco*KI with a substitution in motif VI were performed using 40 nM protein as well as the 10 nM concentration used in other assays.

The mutant *Eco*KI complexes bind DNA and undergo an ATP-dependent conformational change

Exonuclease III footprinting has recently been used to investigate the effects of the cofactors AdoMet and ATP on DNA binding by the wild-type *Eco*KI endonuclease (36). With DNA containing an unmodified *Eco*KI recognition sequence, protection of 42–45 bp was observed either in the absence of any cofactor or in the presence of AdoMet alone. In the presence of ATP and AdoMet, a shorter footprint of 30 bp was observed, while in the presence of ATP alone there was no protection of the DNA. These results are consistent with an ATP-dependent change in the conformation of *Eco*KI, as previously observed by electron microscopy (17), and with AdoMet being essential for the recognition of specific unmodified DNA in the presence of ATP. The hydrolysis of ATP is not necessary for this change in the footprint as both ATPγS and ADP were able to substitute for ATP in producing this smaller footprint. No protection was observed in the presence of both ATP and AdoMet when the oligonucleotide was fully modified or when it lacked a target sequence; in the presence of both cofactors protection requires an unmodified or hemimethylated *Eco*KI target sequence.

We have used the exonuclease III footprinting assay as an indication of whether the mutant proteins, like wild-type *Eco*KI, can undergo the ATP-dependent change in conformation in response to unmodified target sequences, the step that precedes ATP hydrolysis and presumably translocation and cutting. The top strand of the 45 bp duplex that includes an unmodified target for *Eco*KI was labelled and used as substrate for footprinting experiments. The cofactor AdoMet was always present and the effect of ATP was monitored.

The footprints obtained in the absence of ATP are shown in the first 12 lanes of Figure 2, those in the presence of ATP in the second 12 lanes. In the absence of ATP, four of the mutant proteins gave footprints indistinguishable from those obtained for wild-type enzyme, with a 45mer being the major protected species. These included the two mutants with a restriction-proficient phenotype, one with a change in motif III (A619G), and the second in the wrongly identified motif IV (F730S). The same footprint was observed for proteins with substitutions in motif I (K477R) and motif V (G799C). However, for proteins with substitutions in motif Ia (D502Y), motif II (H577D), motif III (A619V), region Y (F629Y) or motif VI (R826H), the major protected species in the absence of ATP was a 43mer. This slight difference in the protection pattern would appear to indicate an alteration in the edge of the binding site of these mutant proteins.

Table 2. Summary of mutations made, their origins and the relevant plasmid used to amplify *Eco*KI

Motif	Oligonucleotide (5′→3′)	Mutation	Substitution	Plasmid
I	G ACC GGT ACC GGT A**B**A ACC CG	A **T** A	K477I	–
		A **G** A	K477R	pJW17[a]
		A **C** A	K477T	–
Ia	C TTC CTT GTC **H**AC CGC GGT TC[b]	**C** A C	D502H	pJW18
		A A C	D502N	pJW19
		T A C	D502Y	pJW20[a]
II	GA CGA AGC G**D**T TCG CGG C[b]	**G** A T	H577D	pJW21[a]
		A A T	H577N	–
		T A T	H577Y	–
III	C GCT CTC ACC G**D**C ACC CCG G[b]	G **A** C	A619D	–
		G **G** C	A619G	pJW22
		G **T** C	A619V	p*hsdR*[–a,c]
IV	CCG GAT CGC AAA AAA CGC TGG	T **G** C	F730C	–
	TCT T**V**G TGC GTC ACC[b]	T **C** C	F730S	pJW23[a]
		T **A** C	F730T	–
V	C GAC CTG CTG ACG ACC **H**GC GTC	**T** G C	G799C	pJW24[a]
	GAT ATT CCG[b]	**C** G C	G799R	pJW25
		A G C	G799S	pJW26
VI	C GAA CAG ATG AAA GGC C**H**C GCC	C **A** C	R826H	pJW27[a]
	ACG CGC TTA TGC[b]	C **T** C	R826L	–
		C **C** C	R826P	–
Y	GCG CTA CAT ACT GTG CAG	T **G** C	F629C	pGDY12
	ATT T**V**C GGC GAG CCG G[b]	T **A** C	F629Y	pGDY9[a]

[a]Plasmids used to amplify enzymes.
[b]The ‘top’ strand primer; that for the ‘bottom’ strand was the complement of the sequence given. B = C, G or T; D = A, G or T; H = A, C or T; V = A, C or G.
[c]Described by O’Neill *et al.* (35).

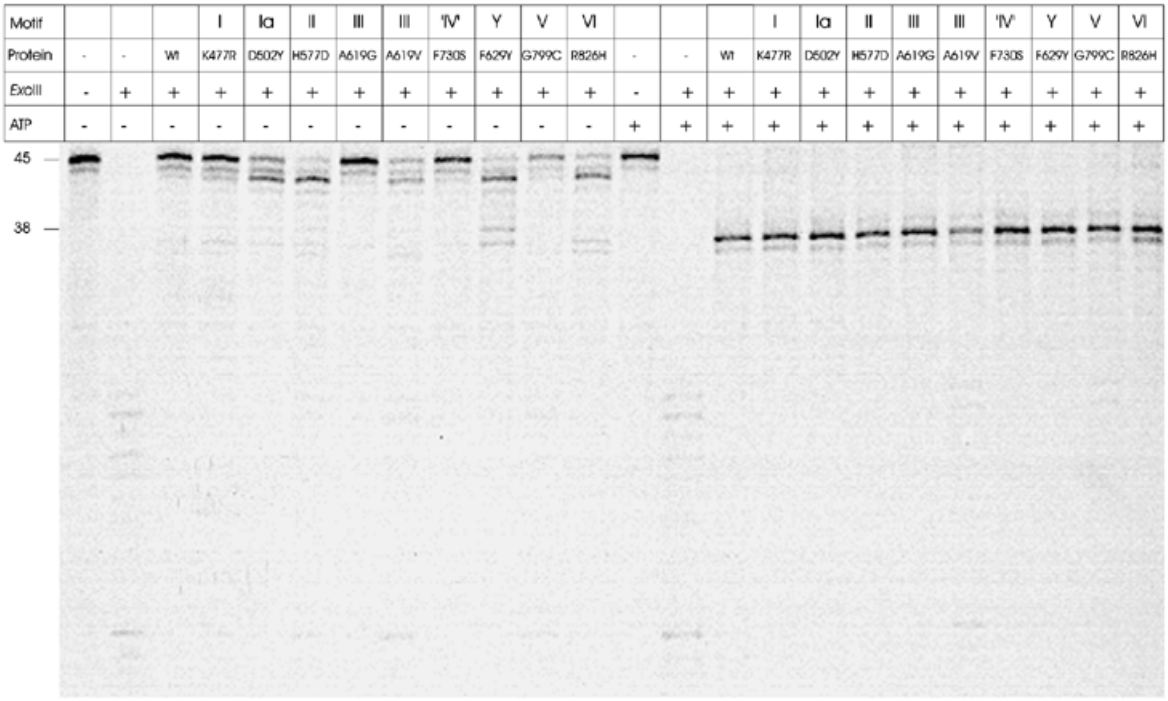

Figure 2. DNA protection defined by exonuclease III footprints of the top strand of the unmodified 45 bp substrate. 45 and 38 indicate the lengths of the major species of oligonucleotides, as identified from a sequencing ladder. See Table 1 and text for further details of the motifs.

For wild-type protein, we have identified previously the DNA fragments protected for both strands of this substrate (36). For the top strand the length of the major protected fragment was 45 bases in the absence of ATP and the presence of 100 μM AdoMet, and 38 bases in the presence of both ATP and AdoMet. For the bottom strand the protected fragments were 42–45 bases in the presence of AdoMet and absence of ATP, and 37 bases in the presence of both AdoMet and ATP. In the present experiments in which the top strand was labelled, it is striking that, in the presence of AdoMet and ATP, all of the mutants gave the same protected 38mer species as the wild-type protein. This implies that all of the mutants are able to bind ATP, although a reduction in the affinity of ATP binding would not necessarily be detected in this assay as we aimed to use an excess (2 mM), so that ATP would not be limiting. These results also confirm that the binding specificity of the mutants is not lost, as a non-specific interaction of *Eco*KI results in no protection of the DNA (36).

The major species protected from exonuclease III by the *Eco*KI methyltransferase (M_2S_1), using the 45 bp duplex with the top strand labelled as substrate, is the same length as that protected by *Eco*KI ($R_2M_2S_1$) in the presence of both cofactors (30,36). For this reason, the composition of the protein in the DNA–protein complex was validated for each of the mutant enzymes. Samples from the footprinting reactions, before and after exonuclease III treatment, were run on 5% non-denaturing polyacrylamide gels; the mobilities of the complexes confirmed that the R subunits had not dissociated from the nuclease ($R_2M_2S_1$) in the protein–DNA complexes isolated during footprinting (results not shown). The footprinting experiments, therefore, indicate that the DEAD-box mutations are exerting their effects on a step, or steps, following the conformational change of the $R_2M_2S_1$ complex associated with ATP binding.

Nuclease activity of the purified proteins

The nine proteins from mutant strains were compared with wild-type enzyme for nuclease activity. The substrate used in the assay was an unmodified plasmid 6.2 kb in length (pRH3) that has two target sequences for *Eco*KI, 2.3 kb apart. Protein (10 nM) was added to provide one molecule of *Eco*KI per target sequence (28). Endonuclease activity is indicated by conversion of the covalently-closed circular DNA to a full-length linear molecule and the subsequent degradation to DNA fragments of variable sizes (Fig. 3a). Enzymes from r_K^+ strains showed endonuclease activity, those from r_K^- strains did not; assays for one enzyme from an r_K^+ strain and one from an r_K^- mutant are shown (Fig. 3b). The assay was repeated using the same concentration of DNA but a higher concentration (40 rather than 10 nM) of the protein preparation (R826H in motif VI) for which the complex was most prone to dissociate at low concentrations. No endonuclease activity was detected even at this concentration.

DNA samples taken a few seconds after the addition of the restriction-proficient enzyme indicate that the plasmid DNA is nicked prior to linearization (Fig. 3a). This ability to relax covalently-closed circular DNA is dependent on the presence of an unmodified target sequence. A nick may be essential to permit DNA translocation by the putative helicase activity associated with the DEAD-box motifs or it could be generated in response to helicase activity. All the enzymes from the mutant strains were examined for nicking activity. All, even those from r_K^- strains, retained the ability to nick unmodified plasmid DNA, although for all the mutants, the nicking occurred more slowly than with the wild-type enzyme (data shown for one r_K^- mutant in Fig. 3b). No endonuclease activity was detected even when assays were incubated for 3 h. The finding that mutations in the DEAD-box

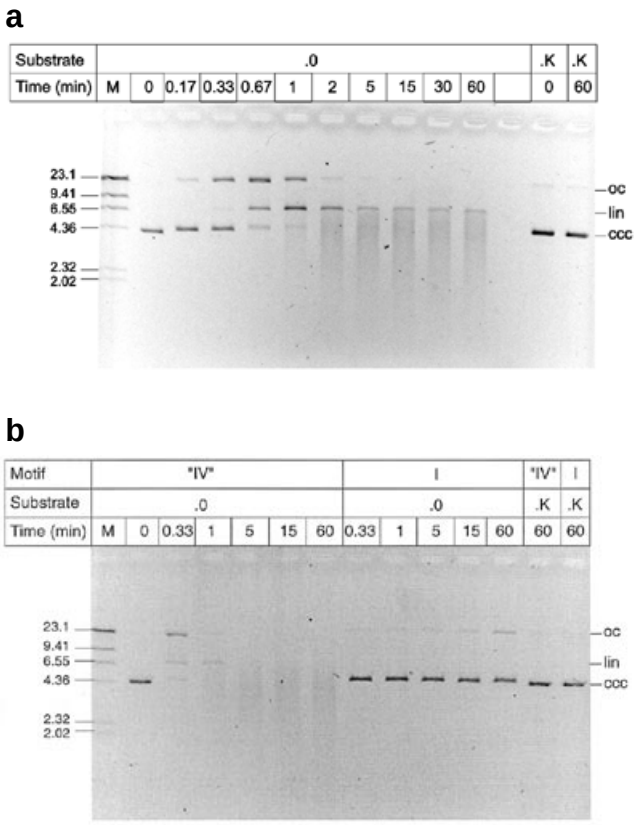

Figure 3. Nuclease assays. (**a**) Assay for wild-type enzyme. (**b**) Assays for enzymes purified from one restriction-proficient mutant (F730S), and from one restriction-deficient mutant (K477R). The substrate DNA was plasmid pRH3, either unmodified (.0) or modified (.K). Reactions were carried out as described in Materials and Methods with samples removed at the times indicated. The positions of supercoiled (ccc), linear (lin) and open-circle (oc) forms of the plasmid are shown. DNA digested with *Hin*dIII was used as marker DNA (lane M); marker sizes are given in kb.

motifs block endonuclease activity, but not the nicking of DNA, is consistent with nicking as a step in the restriction pathway that is separable from the final cutting of the DNA.

ATPase activities of proteins

The ATPase activities of the proteins purified from the nine mutants were compared with that of wild-type *Eco*KI. pRH3, the plasmid with two unmodified *Eco*KI recognition sites, was used as substrate for all the enzymes. Wild-type enzyme and protein from other mutants that retained appreciable ATPase and endonuclease activities with pRH3 as substrate were tested for activity on the 45 bp duplex containing one *Eco*KI site. Both substrates were used in an attempt to separate different ATPase activities; activities that might precede, accompany or follow DNA cleavage. The 45 bp duplex, in contrast to the plasmid, is not a substrate for either the endonuclease or nicking activity of *Eco*KI (36 and unpublished results). pRH3 was used at half the molar concentration of the 45 bp duplex to maintain a ratio of one enzyme molecule per target sequence.

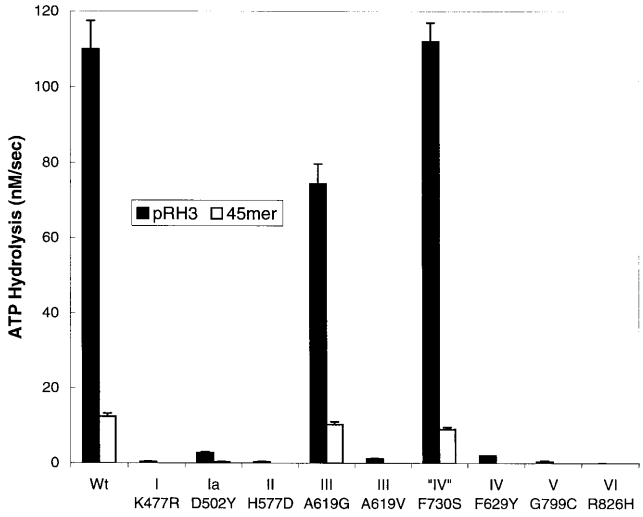

Figure 4. Rates of ATP hydrolysis for *Eco*KI. Assay carried out using pyruvate kinase/lactate dehydrogenase linked coupled assay (33). Error bars indicate standard deviation of at least three experiments.

ATPase activity was assayed directly by the release of radiolabelled inorganic phosphate from γ-labelled ATP (32), and indirectly by a pyruvate kinase/lactate dehydrogenase linked assay (33). For both assays a correction was made for a very small amount of activity observed in the absence of the cofactor AdoMet. The two assays gave consistent results, but the enzyme-linked assay provides better quantification. The rates shown (Fig. 4) are based on this assay.

Using the plasmid as substrate for ATPase activity, an enzyme from the restriction-proficient mutant with a substitution in the original, though suspect, motif IV was indistinguishable from wild-type; that with the A619G substitution in motif III was only slightly impaired (Fig. 4). The oligonucleotide substrate promoted significant hydrolysis of ATP by the three active enzymes. All the enzymes derived from restriction-deficient mutants had greatly reduced ATPase activities with the plasmid as substrate; the assays demonstrate a close correlation between the restriction phenotype and ATPase activity.

DISCUSSION

Type I R–M enzymes comprise three polypeptides, and whenever the amino acid sequences of two type I systems have been compared, the component polypeptides have been found to be either very similar (e.g. *Eco*KI and *Eco*BI) or quite dissimilar (*Eco*KI and *Eco*R124I). Enzymes of very similar sequences have been grouped together as members of one family, those of very dissimilar sequences as representatives of different families (37,38). Pair-wise comparisons of the sequences of HsdR polypeptides from different families, usually detect between 20 and 25% amino acid identity (14,23). Those motifs that identify HsdR polypeptides as members of the super family of DEAD-box proteins make the major contribution to the conserved amino acids. DEAD-box proteins include RNA and DNA helicases (2,4) though all members of this family are not necessarily helicases (39,40). There is no experimental evidence that type I R–M systems are ATP-dependent helicases, but the prominence of the DEAD-box motifs has tempted the speculation that these

motifs are relevant to the ATP-dependent translocation of DNA that has been proposed to precede DNA cutting (14). In the present paper we reconsider the identification of DEAD-box motif IV in HsdR and test the hypothesis that each DEAD-box motif in this subunit is associated with some step in the pathway of events that leads to restriction of unmodified DNA.

The definition of type I R–M systems as DEAD-box proteins was based originally on the sequences of the HsdR polypeptides of *Eco*KI and *Eco*R124I (6). Motif III in *Eco*KI and motif IV in *Eco*R124I were difficult to identify. In 1995, a short frame-shifted sequence was found in the *hsdR* gene of *E.coli* K-12 (34) and an obvious candidate for motif III was readily identified within the new segment of amino acid sequence (22). When this same segment of polypeptide sequence was compared with those of six other, known or putative, HsdR sequences, a conserved region (Y) was located between motifs III and V (23). Region Y became the preferred candidate for motif IV. This preference is consistent with evidence from the crystal structure of PcrA, a DExx helicase isolated from *Bacillus stearothermophilus* (41). In this structure a tyrosine residue in motif IV was shown to be involved in stacking the bound nucleotide. The original motif IV identified by Gorbalenya and Koonin (6) in *Eco*KI included a phenylalanine residue in the position where the consensus residue is either phenylalanine or tyrosine, but neither phenylalanine nor tyrosine was found at this position in most of the HsdR polypeptide sequences (23). In this paper we show that substitution of serine, threonine or cysteine for the tyrosine residue in the original motif IV does not confer a restriction-deficient phenotype. Region Y, as defined by the alignment of the seven HsdR polypeptides, includes a conserved tyrosine residue (23). We show that mutations in region Y, and not motif IV as originally defined, confer a restriction-deficient phenotype. In our following discussion we consider region Y to be the correct motif IV.

We find that mutations in each of the seven motifs (Table 1) can confer a restriction-deficient phenotype. In support of the relevance of these phenotypes, we show for one mutation in each of the seven motifs that the resulting proteins retain the gross configuration of the wild-type enzyme but fail to degrade unmodified plasmid DNA.

Previous knowledge of the steps in the pathway that leads to the cutting of unmodified DNA by *Eco*KI indicates that the enzyme binds to DNA in the presence of the cofactor AdoMet, but requires ATP to recognize and respond to unmethylated target sequences (17). Recognition of an unmodified target sequence elicits a conformational change, first visualized by electron microscopy (17) and recently documented by footprinting experiments (36). The hydrolysis of ATP, rather than the binding of ATP, is only essential for subsequent steps, including the formation of DNA loops that have been taken as evidence of DNA translocation (12). For *Eco*KI and *Eco*R124I or II it has been proposed that DNA cutting is elicited either when translocating complexes collide (13,15,22) or when a change in DNA topology leads to stalling of DNA translocation (13,15,16,22). There is no evidence to implicate ATP hydrolysis in the breakage of the phosphodiester bonds of the DNA chains but ATP hydrolysis has been shown to continue after DNA breakage (42,43).

All the mutant enzymes retain the ability to bind an unmodified target sequence and to protect a sequence of 43–45 bases on the top strand of a 45 bp duplex. In response to ATP each protein undergoes a conformational change and in the resulting complex, like that generated by the wild-type enzyme (36), a much shorter sequence (38 bases) of DNA is protected. The ATP-dependence of the conformational change shows that none of the mutations has prevented binding of the cofactor. It might have been anticipated that even the very conservative substitution of arginine for lysine in the ATP-binding component of the Walker motif (1) would decrease the affinity of the enzyme for ATP. This may be so, since our experiments have not quantified the affinity of the protein for ATP but, under the experimental conditions we have used (2 mM ATP), they show that ATP is able to elicit the conformational change associated with the recognition of a restriction target.

Mutations that conferred a restriction-deficient phenotype resulted in enzyme that was deficient in nuclease activity. The assays for endonuclease activity demonstrate that a covalently-closed circular DNA substrate is nicked prior to linearization. Earlier evidence for nicking has supported the simple concept that two nicks generate a break (19). This is consistent with the stimulation of nicking when two translocating complexes collide (13). Substitutions in the DEAD-box motifs block endonuclease activity but, although the rate of nicking is much reduced, none prevents nicking (compare Fig. 3a and b). A nicking activity that can be separated from the endonucleolytic cleavage could implicate nicking in an early step of the restriction pathway. A nick might be essential to relieve topological constraints and permit DNA translocation to proceed for appreciable distances. Less interestingly, the relaxed circles may merely reflect some residual nicking by bound enzyme despite the absence of the normal stimulus for generating double-strand breaks. The relevance of the nicks remains to be demonstrated. It is not yet known whether the nicks are located in specific regions, e.g. close to the target sequence as might be expected if translocation is impaired, or why the rate of nicking is much reduced by DEAD-box mutations. For *Eco*R124II there is evidence for some preferential DNA cutting close to the target sequence (44). A restriction pathway in which nicking is an essential step could enable a DNA translocation mechanism dependent on helicase activity. Alternatively, DNA nicking (44) or a topoisomerase activity (12) may be required to drive DNA translocation for appreciable distances by some other mechanism. Although topoisomerase activity was invoked for *Eco*KI (12), there is no direct evidence for this activity. Mutations that allow the accumulation of nicked DNA and others that block the nicking activity (G.P.Davies, unpublished results) should permit the identification of the role of nicks in the restriction pathway.

All the endonuclease-deficient mutants retained little or no ATPase activity. Mutations that failed to block restriction *in vivo*, including that in the original motif IV, yielded enzyme that resembled wild-type for both activities. The assays for ATP hydrolysis used two substrates, pRH3, a plasmid that would permit DNA translocation and cleavage, and a duplex oligonucleotide 45 bp in length which might permit some limited translocation but was known to be too short a substrate for DNA cleavage or nicking (36 and unpublished observations). For wild-type enzyme the short substrate elicits appreciable levels of ATPase activity. The ATP hydrolysis associated with the oligomer is dependent on an unmodified target sequence and occurs in the absence of cutting or nicking of the DNA. If we suppose that ATPase activity is a manifestation of DNA translocation, then the activity seen with the 45 bp oligonucleotide implies that translocation is possible on this short substrate. Alternative explanations are possible. For *Eco*R124II, unmodified oligonucleotides of 35 bp

support ATPase activity in the absence of DNA cleavage, with a level of activity similar to that seen with a plasmid substrate (43).

Mutations in each of the seven DEAD-box motifs have resulted in bacteria defective in restriction by the *Eco*KI system. All of the mutations impair both the ATPase and endonuclease activities of the purified enzyme. Mutants with substitutions in motifs I, II, III (A619V), IV, V and VI retain little or no ATPase activity. The substitution in motif Ia leaves appreciable (Fig. 4) ATPase activity but, nevertheless, it confers a restriction-deficient phenotype. The very conservative change in motif III, A619G, imposed a slight reduction in the efficiency of restriction *in vivo* (22), similarly ATPase activity using the plasmid substrate was slightly less than that of wild-type enzyme. Our data indicate that mutations in any of the seven motifs can affect ATPase activity, and that there is a tight correlation between ATPase activity and restriction, with a low ATPase activity being sufficient for a severe defect in restriction *in vivo*. It remains to be determined whether the reduced ATPase activities correlate with poor DNA translocation *in vivo* and whether a helicase activity is essential for restriction, possibly for DNA translocation. Translocation assays based on the entry of phage T7 DNA into a bacterium are now available (45).

Other mutational analyses of DEAD-box motifs, most commonly focusing on motifs I through IV, have demonstrated that changes in the motifs affect ATPase and related enzyme activities e.g. helicase, branch migration and endonuclease (5,8,46). Some mutations have been found to enhance rather than impair ATPase activity (5). Mutations in motif II of *Eco*P1I, like those in *Eco*KI, reduce ATPase activity, but the decrease is not necessarily associated with a block in restriction (46). As predicted from structural studies (47,48), mutations in motif I, the ATP-binding component of the Walker motif (1), can prevent the binding of ATP (49). The conservative change made in *Eco*KI did not prevent ATP binding, but resulted in much reduced ATPase activity. The same amino acid change in Rad3 of yeast abolished ATPase and helicase activities but, as with *Eco*KI, the mutant protein retained ATP-binding activity (7). Recent structures of two helicases, both DEAD-box proteins of the DExx-family (41,50), now locate the motifs in key positions with respect to each other, the cofactor and the substrate, providing a framework for functional models (51).

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