Nucleic Acids Research, 1999, Vol. 27, No. 5 **1275–1282**

Escherichia coli RuvB^{L268S}: a mutant RuvB protein that exhibits wild-type activities *in vitro* but confers a UV-sensitive *ruv* phenotype *in vivo*

Christine Mézard[+], Helen George, Adelina A. Davies, Alain J. van Gool, Didier Zerbib[§], Andrzej Stasiak[1] and Stephen C. West*

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, UK and
[1]Laboratoire d'Analyse Ultrastructurale, Université de Lausanne, 1015 Lausanne, Switzerland

Received December 1, 1998; Revised and Accepted January 18, 1999

ABSTRACT

The RuvABC proteins of *Escherichia coli* process recombination intermediates during genetic recombination and DNA repair. RuvA and RuvB promote branch migration of Holliday junctions, a process that extends heteroduplex DNA. Together with RuvC, they form a RuvABC complex capable of Holliday junction resolution. Branch migration by RuvAB is mediated by RuvB, a hexameric ring protein that acts as an ATP-driven molecular pump. To gain insight into the mechanism of branch migration, random mutations were introduced into the *ruvB* gene by PCR and a collection of mutant alleles were obtained. Mutation of leucine 268 to serine resulted in a severe UV-sensitive phenotype, characteristic of a *ruv* defect. Here, we report a biochemical analysis of the mutant protein RuvB^{L268S}. Unexpectedly, the purified protein is fully active *in vitro* with regard to its ATPase, DNA binding and DNA unwinding activities. It also promotes efficient branch migration in combination with RuvA, and forms functional RuvABC–Holliday junction resolvase complexes. These results indicate that RuvB may perform some additional, and as yet undefined, function that is necessary for cell survival after UV-irradiation.

INTRODUCTION

The *ruvA*, *ruvB* and *ruvC* genes of *Escherichia coli* are required for normal levels of cellular resistance to the effects of UV- or ionising-irradiation, or to the harmful effects of DNA-damaging agents such as mitomycin C (1–3). *ruv* mutants are mildly defective in recombination (4), and this recombination defect is greatly enhanced by a mutation in *recG* (5).

Genetic studies indicate that the *ruv* genes are involved in a late step of recombination and the recombinational repair of DNA damage (6). Consistent with this, biochemical studies have shown that the RuvA, RuvB and RuvC proteins are involved in the processing of recombination intermediates (for review see 7). RuvA and RuvB interact to form a complex that drives the branch migration of Holliday junctions, a reaction that results in the extension of heteroduplex DNA. The third Ruv protein, RuvC, is an endonuclease that also specifically interacts with the junction, in this case to promote its resolution into two nicked duplex products that can be subsequently repaired by DNA ligase. RuvAB stimulate Holliday junction resolution by RuvC (8,9), indicating that resolution *in vivo* is mediated by a RuvABC complex (10–14). The formation of specific RuvABC–Holliday junction complexes was recently demonstrated *in vitro* (15).

The RuvA protein (a tetramer of four 22 kDa subunits) binds specifically to Holliday junctions, forming stable RuvA–junction complexes (16,17). The DNA within the complex exhibits four-fold symmetry, with the four arms of DNA extended towards the corners of a square (12,18,19). Two tetramers can interact with the junction such that it becomes sandwiched between the two concave protein faces (20,21). However, in the RuvABC resolvase complex it is thought that the junction is bound by only one RuvA tetramer, leaving the other face open for interaction with RuvC (8,9,12,22).

RuvA targets RuvB protein, a hexameric ring protein, to the site of the Holliday junction (23–27). RuvB (37 kDa) shows sequence homology and structural similarity to a family of ring helicases that includes *E.coli* DnaB protein (28), the bacteriophage T7 gp4 (29) and T4 gp41 helicases (30), and the viral SV40 T antigen (31). *In vitro*, RuvB exhibits DNA helicase activity in the presence of RuvA (32,33). When visualised by electron microscopy, RuvAB were shown to assemble on the Holliday junction as a tripartite complex with RuvA flanked by two hexameric rings of RuvB (18). The rings lie across the junction from each other and are oppositely oriented. The RuvB rings are thought to constitute an ATP-dependent molecular pump that promotes branch migration (26). A mutant RuvB protein (RuvB^{D113N}) has been described which forms hexameric rings in solution but is defective in DNA binding and ATP-hydrolysis (34).

To better understand the molecular mechanism used by the RuvB hexameric ring to mediate branch migration, a series of

*To whom correspondence should be addressed. Tel: +44 171 269 3868; Fax: +44 171 269 3811; Email: s.west@icrf.icnet.uk

Present addresses: [+]Laboratoire de Génétique Moléculaire de la Recombinaison, UMR 144, Institut Curie, Section de Recherche, 26 rue d'Ulm, 75248 Paris 05, France and [§]Institute de Pharmacologie et Biologie Structurale du CNRS, 205 Route de Narbonne, 31077 Toulouse, France

ruvB mutants have been obtained and selected RuvB mutant proteins purified to homogeneity. Here, we report the generation of the mutants and the biochemical characterisation of one such protein, RuvB^{L268S}.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strains HRS1004 (35) and H124 (1,36) are derived from AB1157. HRS1004 carries a deletion of the *ruvAB* operon and HI24 contains a *ruvB4* mutation.

Sequence comparisons

Protein sequences of *E.coli* RuvB (37,38) homologues in *Haemophilus influenzae* (39), *Mycobacterium leprae* (40), *Mycobacterium tuberculosis* (40), *Mycoplasma genitalium* (41), *Pseudomonas aeruginosa* (42), *Synechocystis* sp. (GenBank accession no. U38892), *Thermotoga maritima* (43) and *Thermus thermophilus* (43) were aligned using PileUp software (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI).

Random mutagenesis

Mutations were introduced into the *ruvB* gene by error-prone PCR (44). Reaction mixtures (100 µl) contained 25 mM Taps–HCl (pH 9.3), 50 mM KCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM dGTP, 1 mM dTTP, 1 mM dCTP, 0.1 mM dATP, 0.2 µM of each primer and 10 ng of template DNA. The template was plasmid pGTI19 containing the wild-type *ruvB* gene (36). Two regions of *ruvB* were amplified separately: the 5′-region between 1 and 432 bp (primers: 5′-GCACTGCA-GATGAGGTAAAGGATGATTGAAG-3′ and 5′-CGCCGGA-CCTTCACCA-3′) and the 3′-region between 346 and 1055 bp (primers: 5′-CACCGTCTATCGCCAGT-3′ and 5′-GGCATATT-GCCAGTGC-3′). *Taq* polymerase reactions were performed at 94°C for 1 min, 50°C for 1 min and 70°C for 4 min, for 30 cycles, followed by 7 min at 70°C. Amplified DNA was gel purified and digested with *Kpn*I and *Bbs*I (for the 5′-region) or *Bbs*I and *Cla*I (for the 3′-region). DNA fragments were subcloned into pGTI19 to replace the corresponding regions of *ruvB*. Ligation mixtures were then transformed into HI24 *ruvB4* strain and clones were tested for their ability/inability to restore UV resistance.

UV sensitivity

The UV sensitivities of AB1157 *ruv*$^+$ and HI24 *ruvB4* containing plasmid pGTI19 (*ruvB*$^+$), pME2 (*ruvB*^{L268S}) or pUC19 were measured as described (34).

Proteins and reagents

RecA (13), RuvA and RuvB (45) and wheat germ topoisomerase I (46) were purified as described. RuvB^{L268S} was purified from *E.coli* strain HRS1004 using a modified version of the protocol developed for the wild-type RuvB protein. In the first chromatographic step, the phosphocellulose column was omitted and the supernatant of the cell lysate was loaded directly onto a DEAE-Biogel column. RuvB^{L268S} eluted at a lower salt concentration (170–220 mM KCl) than wild-type RuvB (240–270 mM KCl). The protein was then applied to a hydroxylapatite column in buffer containing 200 mM KCl, 10 mM potassium phosphate and eluted with a 200 mM KCl, 10–150 mM potassium phosphate gradient. Fractions eluting between 35 and 50 mM potassium phosphate were pooled. RuvB^{L268S} was then purified to homogeneity by Mono Q FPLC as described for wild-type RuvB.

Protein concentrations are expressed in monomers and were determined using the Bradford assay. However, the concentrations of RecA and RuvB (or RuvB^{L268S}) were determined spectrophotometrically using $\varepsilon_{280} = 2.7 \times 10^4$ (RecA) and 1.64×10^4 M^{-1} cm^{-1} (RuvB). BSA (Gibco BRL), phosphocreatine (Sigma), phosphocreatine kinase (Sigma), proteinase K (Boehringer-Mannheim), glutaraldehyde (Sigma), T4 polynucleotide kinase (Pharmacia), *Taq* polymerase (Boehringer-Mannheim) and terminal transferase (Amersham) were purchased as indicated. Restriction enzymes were from New England Biolabs.

DNA substrates

Form I Bluescript KS, pDEA-7Z and pAKE-7Z plasmid DNAs, ØX174 single-stranded and form I duplex DNAs, gapped duplex pDEA-7Z DNA (gDNA) and linearised 3′-^{32}P-labelled pAKE-7Z DNA were prepared as described (34).

For helicase assays, a 30 nt oligonucleotide, complementary to the region 570–599 of ØX174 ssDNA, was 5′-^{32}P-end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham). It was annealed with ØX174 ssDNA as described (32) and purified by gel filtration through a 3 ml Bio-Gel A-0.5m column equilibrated with 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 100 mM NaCl.

Concentrations of ^{32}P-labelled DNA substrates were determined by calculating the specific activity using DE81 filters (47), or by measuring the absorbance at 260 nm. Unless stated otherwise, DNA concentrations are expressed in moles of nucleotide residues.

DNA binding, ATPase and branch migration assays

Reactions were carried out as described (34).

DNA unwinding assay

DNA helicase reactions (20 µl) contained 1 µM annealed DNA substrate in 50 mM Tris–HCl (pH 8.0), 15 mM $MgCl_2$, 10 mM NaCl, 2 mM ATP, 2 mM DTT and 100 µg/ml BSA. The DNA was premixed with RuvA, and the reactions initiated by addition of RuvB or RuvB^{L268S}. After 8 min incubation at 37°C, reactions were stopped and the products deproteinised and analysed by PAGE (32). ^{32}P-labelled DNA was visualised by autoradiography.

The unwinding of covalently closed DNA was carried out as described previously with a few modifications (48). Reactions contained 20 mM Tris-acetate (pH 7.5), 10 mM $Mg(OAc)_2$, 0.5 mM EDTA, 0.5 mM ATP, 0.5 mM ATPγS, 2 mM DTT, 100 µg/ml BSA and 60 µM form I ØX174 DNA. After addition of RuvA (8 µM), unwinding was initiated by addition of RuvB or RuvB^{L268S}. After 15 min at 37°C, 20 U of wheat germ topoisomerase I, an amount sufficient to relax protein-free DNA within 30 s, was added (48) and incubation at 37°C was continued for 5 min. The reactions were then stopped and deproteinised by treatment with SDS (0.8%) and proteinase K (0.8 mg/ml) for 15 min at 37°C. DNA products were purified by extraction with phenol/chloroform followed by ethanol precipitation. Agarose gel electrophoresis was carried out as described (48). Gels were

dried on DE81 paper and then washed with distilled water. The DNA was denatured by soaking the gel in 0.5 M NaOH, 150 mM NaCl for 20 min and then neutralised with 0.5 M Tris–HCl (pH 8.0), 150 mM NaCl. The DNA was hybridised with a 5′-^{32}P-end-labelled 66mer complementary to the ØX174 DNA, and the gel was dried and exposed to Kodak XAR films.

Holliday junction resolution

Synthetic Holliday junctions were prepared by annealing four oligonucleotides (66 nt in length): strand 1, 5′-TATCGAATCCG-TCTAGTCAACGCTGCCGAATTCTACCAGTGAGGATGG-ACTCCTCACCTGCAGGTT-3′; strand 2, 5′-AACCTGCAGG-TGAGGAGTCCATGGTCTTCCGTCAAGCTCGATGCCGG-TTGTATGCCCACGTTGACC-3′; strand 3, 5′-GGTCAACGT-GGGCATACAACCGGCATCGAGCTTGACGGAAGACCAT-CCCTGTCTAGAGGATCCGAC-3′; and strand 4, 5′-GTCGG-ATCCTCTAGACAGGGATCCTCACTGGTAGAATTCGGCA-GCGTTGACTAGACGGATTCGATA-3′. DNA annealing and junction purification were carried out essentially as described previously (49). Prior to annealing, strand 1 was 5′-^{32}P-end-labelled using polynucleotide kinase. Concentrations of synthetic junctions are indicated in moles of junctions.

Junctions (1 nM) were incubated with RuvC, in the presence or absence of RuvA and RuvB, in cleavage buffer [50 mM Tris-acetate (pH 8.0), 15 mM $Mg(OAc)_2$, 1 mM ATPγS, 1 mM DTT and 100 µg/ml BSA; total volume 20 µl] for 30 min at 37°C. The products were deproteinised and analysed by 8% neutral PAGE (8).

Electron microscopy

Complexes of RuvB and RuvB^{L268S} on pAKE-7Z linear duplex DNA were visualised as described (34).

Immunoprecipitation

Co-immunoprecipitation experiments with RuvA, RuvB (or RuvB^{L268S}) and RuvC were carried out using synthetic Holliday junction X0 as described (15). After incubation, protein–DNA complexes were immunoprecipitated using anti-RuvC monoclonal antibodies coupled to protein-G Sepharose beads (Pharmacia). Complexes were analysed by SDS–PAGE followed by western blotting with rabbit anti-RuvA, anti-RuvB and anti-RuvC polyclonal antibodies using ECL detection (Amersham).

RESULTS AND DISCUSSION

Random mutagenesis of the cloned *ruvB* gene and identification of mutants that confer UV^S phenotype

Error-prone PCR (44) was used to introduce random mutations into the cloned *ruvB* gene. Two different segments of *ruvB* were mutated: the 5′-region between 1 and 432 bp and the 3′-region between 346 and 1055 bp. The amplified fragments were cloned into the high copy number plasmid pGTI19 (*ruvB*$^+$), replacing the equivalent segments of the wild-type gene. The resulting plasmids were transformed into a UV-sensitive *ruvB* strain (HI24) and clones were tested for their UV sensitivity. In total, 40 out of 1070 clones were identified as being unable to complement the UV^S phenotype. Plasmids were recovered from these clones and tested for their ability to over-express soluble RuvB protein in strain HRS1004, which carries a *ruvAB* deletion. Although many plasmids expressed insoluble RuvB, 12 gave good over-expression of soluble protein and for each, the entire *ruvB* open reading frame was sequenced. Of these, eight plasmids were found to contain point mutations resulting in single amino acid changes in the RuvB protein sequence (Fig. 1). The mutants were designated F44L, V90A, K93L, T161A, D232G, F261L, F261S and L268S (Fig. 1).

Sequence analysis of RuvB indicates the presence of a number of motifs that are characteristically found in DNA/RNA helicases (50). Helicase domains I and II (two motifs involved in nucleotide binding and hydrolysis; 51,52) and helicase motifs III and VI are highly conserved whereas motifs IV and V are not (Fig. 1). Surprisingly, only one of the eight mutants obtained (T161A) contained a mutation within a helicase motif (motif III). When the mutant RuvB proteins were purified, we observed that one, RuvB^{L268S}, was exceptional in that it acts as a wild-type protein *in vitro*, although strains carrying it exhibit a severe UV^S phenotype.

Dominant negative phenotype of RuvB^{L268S}

Plasmid pME2, carrying the *ruvB*^{L268S} gene under the control of the p*lac* promoter, was transformed into *E.coli* strains HI24 *ruvB4* and AB1157 *ruv*$^+$ to assess how expression of the mutant protein affected their UV sensitivity. Due to the absence of *lacI*^{q}, RuvB^{L268S} was expressed constitutively in both strains. As expected, in view of the selection screen used after mutagenesis, expression of RuvB^{L268S} in HI24 failed to complement the *ruvB4* mutation, even at low UV doses (Fig. 2A). Expression of RuvB^{L268S} in AB1157 resulted in a dominant negative phenotype, such that the wild-type strain carrying pME2 became sensitive to UV irradiation (Fig. 2A). In contrast, over-expression of wild-type RuvB (pGTI19) in AB1157 did not affect UV sensitivity.

Purification of RuvB^{L268S} protein

To characterise the biochemical defects associated with the mutation in RuvB^{L268S}, the mutant protein was over-expressed in the *ruvAB* deletion strain *E.coli* HRS1004. The procedure used to purify RuvB^{L268S} was similar to the protocol developed for wild-type RuvB. However, during its purification, we observed that RuvB^{L268S} eluted from DEAE-Biogel at a significantly lower salt concentration (170–220 mM KCl) than that of wild-type protein (240–270 mM KCl), suggesting that the L268S mutation may have produced a change in the conformational state of the protein. After purification, RuvB^{L268S} protein was found to be >95% homogeneous when analysed by SDS–PAGE (Fig. 2B).

DNA binding and ATPase activities of RuvB^{L268S}

To determine whether RuvB^{L268S} bound DNA, wild-type and mutant proteins were incubated with supercoiled circular duplex DNA in the presence of 15 mM Mg^{2+} and ATPγS. The resulting protein–DNA complexes were fixed with glutaraldehyde and analysed by agarose gel electrophoresis. The band-shift patterns obtained with RuvB or RuvB^{L268S} were similar, such that the degree of DNA retardation was directly proportional to the protein concentration until saturation was reached (Fig. 3A). Similar results were obtained without glutaraldehyde fixation (data not shown). Electron microscopic analysis of glutaraldehyde-fixed complexes, formed between RuvB^{L268S} and linear duplex DNA, showed that the mutant protein formed double rings on

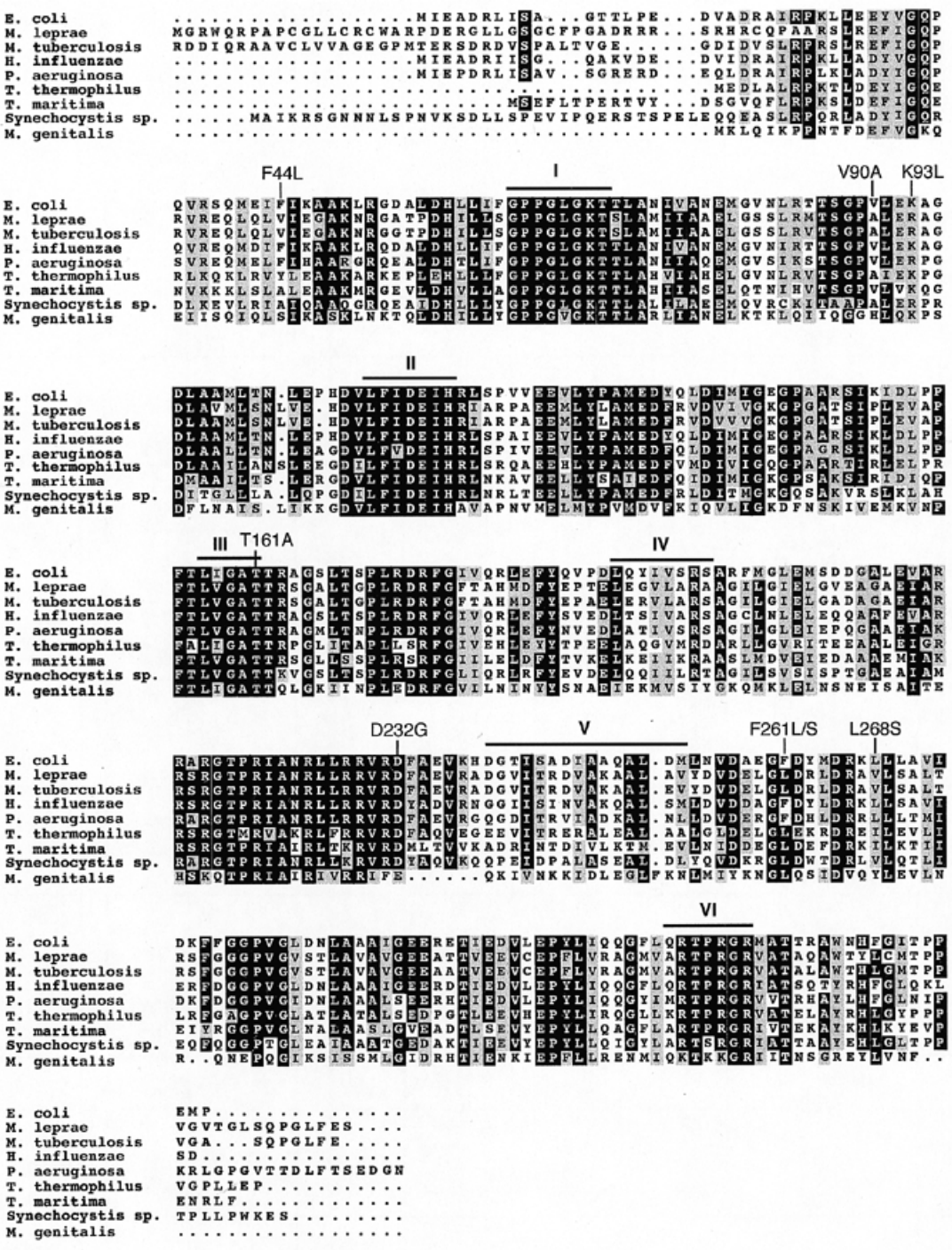

Figure 1. Alignment of the *E.coli* RuvB sequence with eight homologues. Identical and similar amino acids are boxed in black and grey, respectively. DNA helicase motifs I–VI are indicated above the alignment. The positions and the nature of the substitutions obtained by random mutagenesis are indicated. Their numbering relates to the amino acid sequence of *E.coli* RuvB.

DNA (Fig. 3C) that were indistinguishable from the double hexameric rings made by wild-type protein (Fig. 3B).

In previous studies, it was shown that RuvB exhibits a weak affinity for DNA at low Mg^{2+} concentrations (≤10 mM Mg^{2+}) and that under these conditions its interaction with DNA requires RuvA. Analysis of the RuvA-directed binding of RuvB or $RuvB^{L268S}$, in the presence of 5 mM Mg^{2+}, indicated that wild-type and mutant proteins bound with similar affinities (data not shown).

The DNA-dependent ATPase activities of RuvB and $RuvB^{L268S}$ were also found to be comparable. In both cases, the amount of

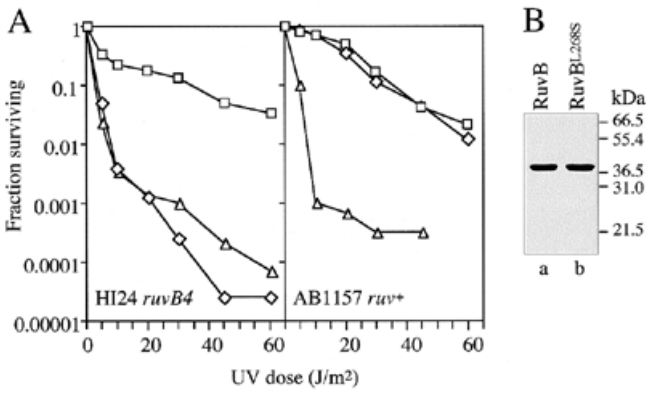

Figure 2. *In vivo* complementation assays and SDS–PAGE analysis of purified RuvB^{L268S}. (**A**) UV survival curves of HI24 and AB1157 following transformation with plasmids pME2 (*ruvB^{L268S}*, Δ), pGTI19 (*ruvB^{+}*, □) or pUC19 (◇). (**B**) SDS–PAGE of RuvB and RuvB^{L268S} proteins. Proteins (3 µg) were analysed on a 10% polyacrylamide gel and visualised by staining with Coomassie Blue. The positions of the molecular mass markers are indicated.

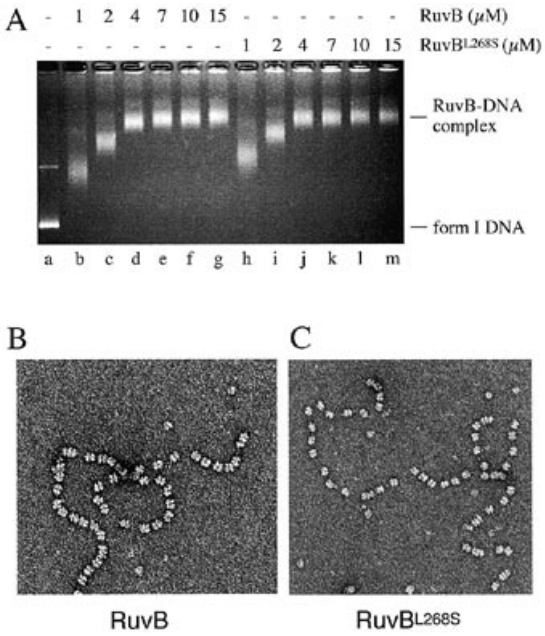

Figure 3. Duplex DNA binding by RuvB and RuvB^{L268S}. (**A**) DNA binding assays were carried out as described in Materials and Methods using the indicated concentrations of RuvB or RuvB^{L268S}. (**B** and **C**) Electron microscopic visualisation of RuvB and RuvB^{L268S}, respectively, in the presence of linear duplex DNA.

ATP hydrolysed in a given time was proportional to protein concentration (Fig. 4A). In the absence of RuvA, rates of hydrolysis in the order of 4 mol ATP/min/mol RuvB were observed (Fig. 4B). The rates of hydrolysis catalysed by RuvB or RuvB^{L268S} were stimulated to the same extent by the presence of RuvA (Fig. 4B). These results indicate that the DNA binding and DNA-dependent ATPase activities of RuvB and RuvB^{L268S} are indistinguishable and that both proteins interact with RuvA.

DNA unwinding properties of RuvB^{L268S}

Two assays were used to determine whether RuvB^{L268S} possessed the ability to promote DNA unwinding. First, we analysed its

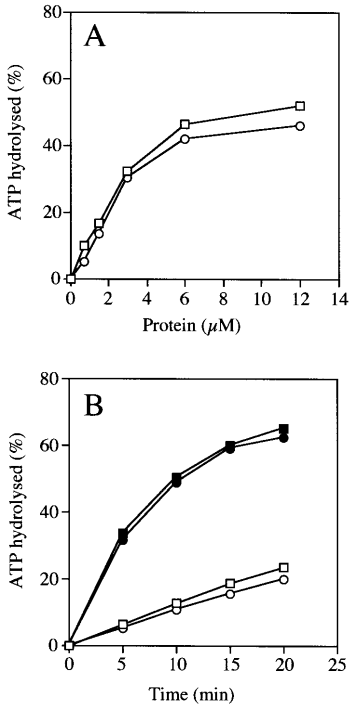

Figure 4. ATPase activity of RuvB^{L268S} compared to wild-type RuvB. (**A**) ATPase activity analysed as a function of protein concentration. Reactions contained the indicated concentrations of RuvB (□) or RuvB^{L268S} (○) and were carried out as described in Materials and Methods. Incubation was for 20 min at 37°C. (**B**) Effect of RuvA on RuvB- or RuvB^{L268S}-mediated ATP hydrolysis. Large scale reactions (100 µl) contained either RuvB (3 µM) with (■) or without (□) RuvA, or RuvB^{L268S} with (●) or without (○) RuvA. RuvA (1 µM) was added to the preincubation mixtures immediately before initiation. Aliquots were taken at the indicated times and the percentage of ATP hydrolysed was determined. Background levels of ADP (~3%) have been subtracted.

activity in a DNA helicase assay, which measured the displacement of a short (30 nt) ^{32}P-labelled oligonucleotide annealed to circular single-stranded DNA (32). In the presence of RuvA, we observed that RuvB^{L268S} displaced the 30mer with the same efficiency as wild-type RuvB (Fig. 5A, compare lanes c–f with h–k). Secondly, we used a sensitive topological assay to measure protein-induced DNA underwinding (48). In this assay, the underwinding of covalently closed duplex DNA by RuvAB results in the introduction of positive superhelical turns that are removed by eukaryotic topoisomerase I. Upon deproteinisation, the underwound DNA is observed after agarose gel electrophoresis as moderately negatively supercoiled DNA (Fig. 5B, lane h). In contrast, DNA that has not been underwound is seen at the position of relaxed DNA (lane b). With both wild-type RuvB and RuvB^{L268S}, we found that the degree of unwinding was directly proportional to the protein concentration (compare lanes d–h with i–m). We conclude that RuvB^{L268S} fails to exhibit any defect in its ability to promote DNA unwinding.

Effect of RuvB^{L268S} on RuvB- and RuvAB-mediated branch migration

Using ^{32}P-labelled recombination intermediates (α-structures) prepared by RecA-mediated strand exchange reactions (13) between gapped circular pDEA-7Z plasmid DNA and ^{32}P-labelled *Pst*I linearised duplex pAKE-7Z DNA which contains a heterologous

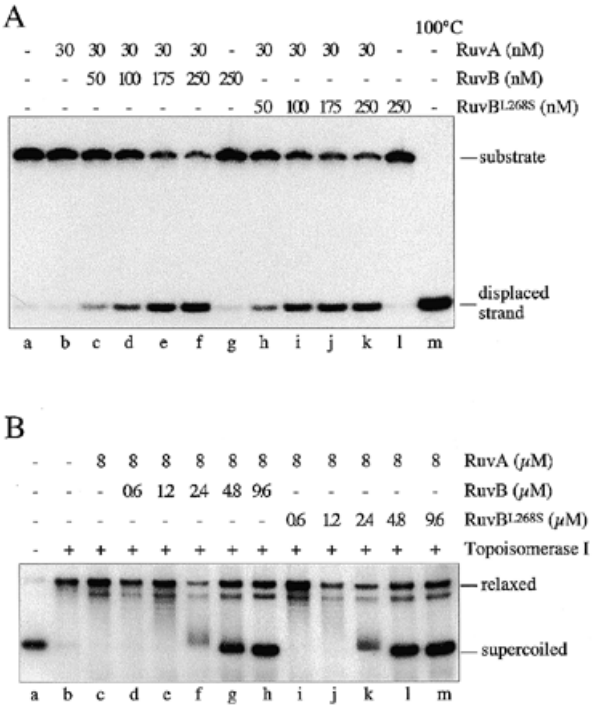

Figure 5. DNA unwinding activity of RuvB^{L268S} compared to wild-type RuvB. (**A**) Displacement of a 30 nt fragment annealed to circular single-stranded DNA. Reactions were carried out as described in Materials and Methods, using the indicated amounts of RuvA and RuvB/RuvB^{L268S}. Products were analysed by 8% PAGE. Lane m, control in which the DNA was heat-denatured at 100°C for 3 min prior to loading. (**B**) Unwinding of circular duplex DNA. Assays were carried out as described in Materials and Methods using the indicated concentrations of RuvA and RuvB proteins. Topoisomerase was added where indicated. DNA products were deproteinised and analysed on a 1% agarose gel.

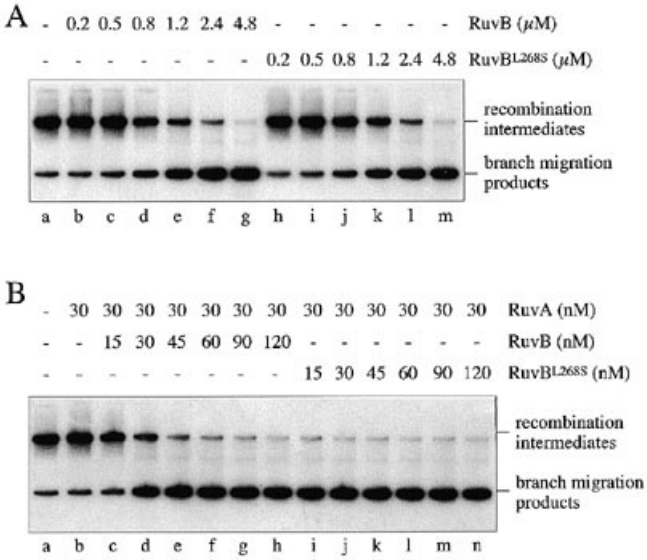

Figure 6. Branch migration activities of RuvB and RuvB^{L268S}. (**A**) ^{32}P-labelled recombination intermediates were incubated with the indicated concentrations of RuvB or RuvB^{L268S}. Reactions were stopped and DNA products were visualised by agarose gel electrophoresis followed by autoradiography. (**B**) Assays were carried out as in (A), except that RuvA (30 nM) was added immediately before RuvB or RuvB^{L268S}.

block, we observed that wild-type RuvB catalysed branch migration to produce ^{32}P-labelled linear duplex products (Fig. 6A, lanes b–g). As shown previously (24,53), the wild-type protein promoted branch migration in the absence of RuvA with an efficiency directly proportional to its concentration. Under the same conditions, similar results were obtained with RuvB^{L268S} (lanes h–m).

Branch migration experiments were also carried out in the presence of RuvA. Because RuvA promotes the specific targeting of RuvB to the Holliday junction, these experiments were performed at low RuvB concentrations. RuvB^{L268S} was found to promote efficient branch migration, such that >90% of the recombination intermediates were processed into branch migration products (Fig. 6B). In a series of experiments, the efficiency of branch migration by RuvB^{L268S} and wild-type RuvB were compared over a range of protein concentrations. The two proteins were found to promote branch migration with similar efficiencies (data not shown).

RuvABC complex formation

Since RuvB^{L268S} failed to show any obvious defect in its ability to promote branch migration, we next tested to see whether it was capable of forming functionally-active RuvABC complexes. First, we used immunoprecipitation assays to analyse whether RuvB^{L268S} was able to form RuvABC–Holliday junction complexes *in vitro* (Fig. 7A), and secondly, we tested whether RuvB^{L268S} was capable of stimulating Holliday junction resolution catalysed by the RuvC protein (Fig. 7B).

Immunoprecipitation experiments were carried out by mixing RuvA, RuvB^{L268S} (or RuvB as a control) and RuvC with Holliday junction DNA, and the resulting complexes were immunoprecipitated using monoclonal antibodies (MAbs) raised against RuvC (15). As shown in Figure 7A, the anti-RuvC MAbs pulled down a complex that contained all three Ruv proteins. Similar results were obtained using either RuvB or mutant RuvB^{L268S}.

The efficiency of Holliday junction resolution by RuvC (Fig. 7B, lane b) can be stimulated by the presence of RuvB (lane c), or more efficiently by RuvAB (lane d). Stimulation is thought to involve formation of a functional RuvABC–Holliday junction resolvase complex (8,9). When reactions were carried out using RuvB^{L268S}, we found that the mutant protein stimulated RuvC-mediated Holliday junction resolution (lanes f and g). The sites of RuvC-mediated resolution were essentially the same in the presence of RuvB or RuvB^{L268S} (data not shown). RuvB^{L268S} was also found to stimulate the resolution of supercoiled figure-8 DNA molecules containing Holliday junctions (data not shown). We conclude that RuvB^{L268S} forms RuvABC–junction complexes and that these complexes exhibit normal resolution activities *in vitro*.

Conclusions

In this paper we have described the isolation of a mutant *ruvB* allele that encodes a protein, RuvB^{L268S}, which exhibits wild-type RuvB activities *in vitro*. The *ruvB^{L268S}* mutation results in a substitution of an aliphatic amino acid (leucine 268) for a polar amino acid (serine) positioned between the poorly-conserved

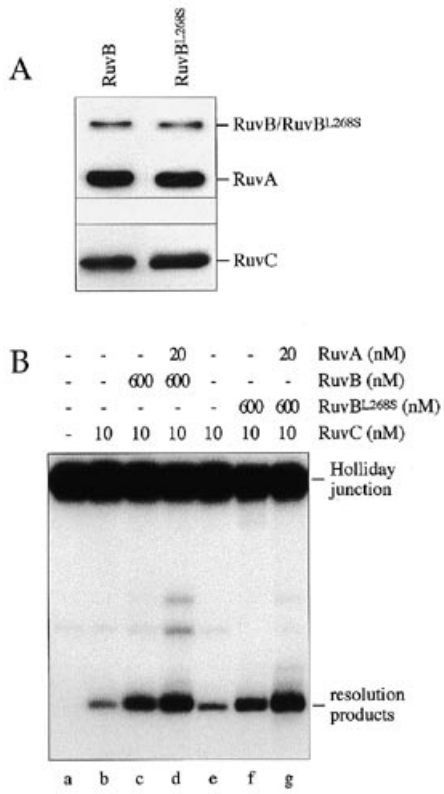

Figure 7. Formation of functional RuvABC complexes by RuvB^{L268S}. (**A**) Co-immunoprecipitation of RuvABC–Holliday junction complexes using wild-type RuvB or RuvB^{L268S}, as indicated. Complexes were prepared and pulled down using anti-RuvC MAbs as described in Materials and Methods. Aliquots were analysed by SDS–PAGE and the gels were immunoblotted with a mixture of anti-RuvA and anti-RuvB polyclonal antibodies (upper panel) or anti-RuvC antibodies (lower panel). Proteins were detected by ECL. (**B**) Stimulation of RuvC-mediated Holliday junction resolution by RuvAB or RuvAB^{L268S}. Resolution reactions contained ^{32}P-labelled synthetic Holliday junctions and were carried out as described in Materials and Methods using RuvA, RuvB and RuvC as indicated. Resolution products (^{32}P-labelled nicked duplex DNA) were analysed by 8% neutral PAGE and visualised by autoradiography.

helicase domain V and the well-conserved helicase domain VI. In eight out of the nine RuvB sequences shown in the alignment in Figure 1, an aliphatic amino acid is found at position 268. The surrounding amino acids are relatively well-conserved, with 66% similarity in the 57 amino acids between the helicase domains V and VI. Since no crystallographic data are available with RuvB, the structure and function of this region is presently unknown.

Although RuvB^{L268S} exhibits wild-type biochemical activities *in vitro* with regard to DNA binding, ATPase activity, ring formation, DNA unwinding, branch migration and ability to form functional RuvABC–Holliday junction complexes, the L268S substitution has a dramatic effect *in vivo*. Indeed, the *ruvB^{L268S}* allele was unable to complement the *ruvB4* mutation, and over-expression of RuvB^{L268S} exerted a dominant negative effect in wild-type cells. Since associations between mutant (RuvB^{D113N}) and wild-type RuvB proteins have been observed previously (34), it is possible that RuvB^{L268S} interacts with wild-type RuvB to form a hetero-hexamer that is inactive for some critical RuvB function.

Although RuvB^{L268S} appeared normal when assayed *in vitro*, during its purification RuvB^{L268S} eluted from the DEAE-Biogel column at lower salt concentrations than the wild-type protein, suggesting that the mutation caused a conformational change that might affect protein–protein or protein–DNA interactions. Since we have not observed any defects in self-association of the RuvB hexamer, interactions with RuvA or RuvC, nor assembly of the RuvABC–Holliday junction complex, one possibility to be considered is that RuvB associates with some other protein in *E.coli* and that leu268 is critical for this interaction.

Alternatively, subtle changes in the way that RuvB^{L268S} interacts with RuvA and/or RuvC, may lead to the observed cellular defect. Because our extensive *in vitro* analysis of RuvB^{L268S} failed to identify the biochemical defect in this protein, the data expose the limitations of such *in vitro* assays which, by their necessary simplicity, can only partially reproduce the complexity of reactions that take place *in vivo*. For example, recombination intermediates made *in vitro* by RecA are naked, relaxed DNA molecules that possess free ends, whereas the natural substrates for RuvABC are likely to be supercoiled and have many associated proteins. Indeed, target DNAs may be actively undergoing DNA replication, transcription and repair, and it is possible that RuvB^{L268S} is unable to act within the context of a DNA processing machine.

ACKNOWLEDGEMENTS

We thank David Adams, Angela Eggleston, Irina Tsaneva and other members of the laboratory for their interest and providing protein reagents. This work was supported by the Imperial Cancer Research Fund, the Human Frontiers Science Program, and the Swiss–British Joint Research Program and the Swiss National Foundation. C.M. and A.J.v.G. were supported in part by fellowships from the European Molecular Biology Organisation, and D.Z. by the Centre National de la Recherche Scientifique.

REFERENCES

1 Otsuji,N., Iyehara,H. and Hideshima,Y. (1974) *J. Bacteriol*., **117**, 337–344.
2 Stacey,K.A. and Lloyd,R.G. (1976) *Mol. Gen. Genet*., **143**, 223–232.
3 Lloyd,R.G., Benson,F.E. and Shurvinton,C.E. (1984) *Mol. Gen. Genet*., **194**, 303–309.
4 Lloyd,R.G., Buckman,C. and Benson,F.E. (1987) *J. Gen. Microbiol*., **133**, 2531–2538.
5 Lloyd,R.G. (1991) *J. Bacteriol*., **173**, 5414–5418.
6 Benson,F.E., Collier,S. and Lloyd,R.G. (1991) *Mol. Gen. Genet*., **225**, 266–272.
7 West,S.C. (1997) *Annu. Rev. Genet*., **31**, 213–244.
8 van Gool,A.J., Shah,R., Mézard,C. and West,S.C. (1998) *EMBO J*., **17**, 1838–1845.
9 Zerbib,D., Mézard,C., George,H. and West,S.C. (1998) *J. Mol. Biol*., **281**, 621–630.
10 Shah,R., Bennett,R.J. and West,S.C. (1994) *Cell*, **79**, 853–864.
11 Mandal,T.N., Mahdi,A.A., Sharples,G.J. and Lloyd,R.G. (1993) *J. Bacteriol*., **175**, 4325–4334.
12 Rafferty,J.B., Sedelnikova,S.E., Hargreaves,D., Artymiuk,P.J., Baker,P.J., Sharples,G.J., Mahdi,A.A., Lloyd,R.G. and Rice,D.W. (1996) *Science*, **274**, 415–421.
13 Eggleston,A.K., Mitchell,A.H. and West,S.C. (1997) *Cell*, **89**, 607–617.
14 Whitby,M.C., Bolt,E.L., Chan,S.N. and Lloyd,R.G. (1996) *J. Mol. Biol*., **264**, 878–890.
15 Davies,A.A. and West,S.C. (1998) *Curr. Biol*., **8**, 725–727.
16 Parsons,C.A., Tsaneva,I., Lloyd,R.G. and West,S.C. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 5452–5456.
17 Iwasaki,H., Takahagi,M., Nakata,A. and Shinagawa,H. (1992) *Genes Dev*., **6**, 2214–2220.
18 Parsons,C.A., Stasiak,A., Bennett,R.J. and West,S.C. (1995) *Nature*, **374**, 375–378.

19 Hargreaves,D., Rice,D.W., Sedelnikova,S.E., Artymiuk,P.J., Lloyd,R.G. and Rafferty,J.B. (1998) *Nature Struct. Biol.*, **5**, 441–446.
20 Yu,X., West,S.C. and Egelman,E.H. (1997) *J. Mol. Biol.*, **266**, 217–222.
21 Roe,S.M., Barlow,T., Brown,T., Oram,M., Keeley,A., Tsaneva,I.R. and Pearl,L.H. (1998) *Mol. Cell*, **2**, 361–372.
22 West,S.C. (1998) *Cell*, **94**, 699–701.
23 Parsons,C.A. and West,S.C. (1993) *J. Mol. Biol.*, **232**, 397–405.
24 Mitchell,A.H. and West,S.C. (1996) *J. Biol. Chem.*, **271**, 19497–19502.
25 Stasiak,A., Tsaneva,I.R., West,S.C., Benson,C.J.B., Yu,X. and Egelman,E.H. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 7618–7622.
26 West,S.C. (1996) *Cell*, **86**, 177–180.
27 Egelman,E.H. (1996) *Structure*, **4**, 759–762.
28 Bujalowski,W., Klonowska,M.M. and Jewewska,M.S. (1994) *J. Biol. Chem.*, **269**, 31350–31358.
29 Patel,S.S. and Hingorani,M.M. (1993) *J. Biol. Chem.*, **268**, 10668–10675.
30 Dong,F., Gogol,E.P. and von Hippel,P.H. (1995) *J. Biol. Chem.*, **270**, 7462–7473.
31 Mastrangelo,I.A., Hough,P.V.C., Wall,J.S., Dobson,M., Dean,F.B. and Hurwitz,J. (1989) *Nature*, **338**, 658–662.
32 Tsaneva,I.R., Müller,B. and West,S.C. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1315–1319.
33 Tsaneva,I.R. and West,S.C. (1994) *J. Biol. Chem.*, **269**, 26552–26558.
34 Mézard,C., Davies,A.A., Stasiak,A. and West,S.C. (1997) *J. Mol. Biol.*, **271**, 704–717.
35 Iwasaki,H., Shiba,T., Nakata,A. and Shinagawa,H. (1989) *Mol. Gen. Genet.*, **219**, 328–331.
36 Sharples,G.J., Benson,F.E., Illing,G.T. and Lloyd,R.G. (1990) *Mol. Gen. Genet.*, **221**, 219–226.
37 Benson,F.E., Illing,G.T., Sharples,G.J. and Lloyd,R.G. (1988) *Nucleic Acids Res.*, **16**, 1541–1550.
38 Shinagawa,H., Makino,K., Amemura,M., Kimura,S., Iwasaki,H. and Nakata,A. (1988) *J. Bacteriol.*, **170**, 4322–4329.
39 Fleischmann,R.D., Adams,M.D., White,O., Clayton,R.A., Kirkness,E.F., Kerlavage,A.R., Bult,C.J., Tomb,J.F., Dougherty,B.A. and Merrick,J.M. (1995) *Science*, **269**, 496–512.
40 Philipp,W.J., Poulet,S., Eiglmeier,K., Pascopella,L., Balasubramanian,V., Heym,B., Bergh,S., Bloom,B.R., Jacobs,W.R. and Cole,S.T. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 3132–3137.
41 Fraser,C.M., Gocayne,J.D., White,O., Adams,M.D., Clayton,R.A., Fleischmann,R.D., Bult,C.J., Kerlavage,A.R., Sutton,G. and Kelley,J.M. (1995) *Science*, **270**, 397–403.
42 Hishida,T., Iwasaki,H., Ishioka,K. and Shinagawa,H. (1996) *Gene*, **182**, 63–70.
43 Tong,J. and Wetmur,J.G. (1996) *J. Bacteriol.*, **178**, 2695–2700.
44 Leung,P.C., Teplow,D.B. and Harshey,R.M. (1989) *Nature*, **338**, 656–658.
45 Tsaneva,I.R., Illing,G.T., Lloyd,R.G. and West,S.C. (1992) *Mol. Gen. Genet.*, **235**, 1–10.
46 Dynan,W.S., Jendrisak,J.J. and Hager,D.A. (1981) *J. Biol. Chem.*, **256**, 5860–5865.
47 Sambrook,E.F., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
48 Adams,D.E. and West,S.C. (1995) *J. Mol. Biol.*, **247**, 404–417.
49 Parsons,C.A., Kemper,B. and West,S.C. (1990) *J. Biol. Chem.*, **265**, 9285–9289.
50 Gorbalenya,A.E. and Koonin,E.V. (1993) *Curr. Opin. Struct. Biol.*, **3**, 419–429.
51 Gorbalenya,A.E., Koonin,E.V., Donchenko,A.P. and Blinov,V.M. (1988) *Nature*, **333**, 22.
52 Walker,J.E., Saraste,M., Runswick,M.J. and Gay,N.J. (1982) *EMBO J.*, **1**, 945–951.
53 Müller,B., Tsaneva,I.R. and West,S.C. (1993) *J. Biol. Chem.*, **268**, 17179–17184.