

# RecA-mediated SOS induction requires an extended filament conformation but no ATP hydrolysis

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## Summary

The *Escherichia coli* SOS response to DNA damage is modulated by the RecA protein, a recombinase that forms an extended filament on single-stranded DNA and hydrolyzes ATP. The RecA K72R (*recA2201*) mutation eliminates the ATPase activity of RecA protein. The mutation also limits the capacity of RecA to form long filaments in the presence of ATP. Strains with this mutation do not undergo SOS induction *in vivo*. We have combined the K72R variant of RecA with another mutation, RecA E38K (*recA730*). *In vitro*, the double mutant RecA E38K/K72R (*recA730,2201*) mimics the K72R mutant protein in that it has no ATPase activity. The double mutant protein will form long extended filaments on ssDNA and facilitate LexA cleavage almost as well as wild-type, and do so in the presence of ATP. Unlike *recA* K72R, the *recA* E38K/K72R double mutant promotes SOS induction *in vivo* after UV treatment. Thus, SOS induction does not require ATP hydrolysis by the RecA protein, but does require formation of extended RecA filaments. The RecA E38K/K72R protein represents an improved reagent for studies of the function of ATP hydrolysis by RecA *in vivo* and *in vitro*.

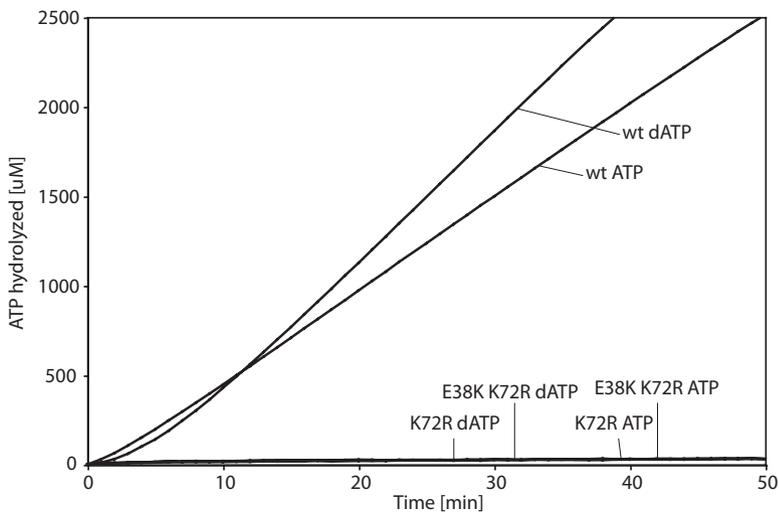
## Introduction

The RecA protein plays a central role in recombination, SOS induction and DNA repair in *Escherichia coli* and many other *bacteria*. Critical in these functions is the ability of RecA (bound to ATP) to polymerize onto single-stranded DNA (ssDNA), creating a helical nucleoprotein filament. This filament is a dynamic structure where RecA is added to the 3'-proximal end and dissociates through ATP hydrolysis from the 5'-proximal end. The ability to hydrolyze ATP in a DNA-dependent manner is crucial to RecA function in the cell. However, the way in which ATP hydrolysis contributes to RecA function(s) is not fully understood. This study focuses on the role of ATP hydrolysis in facilitating LexA auto-cleavage that is necessary for induction of the SOS response.

*In vitro*, RecA must first bind ATP or a non-hydrolyzable analogue to adopt a high affinity conformation for ssDNA. This ATP-activated form of RecA constitutes an extended nucleoprotein filament documented by electron microscopic observation and analysis (Yu and Egelman, 1992; Egelman and Stasiak, 1993) and is almost certainly equivalent to the RecA\* form mentioned in the earlier literature (Roberts *et al.*, 1978; Clark and Sandler, 1994). Other nucleotides are available *in vivo*, and *in vitro* experiments show that RecA can bind several of them and retain at least some key functions (McEntee *et al.*, 1981; Weinstock *et al.*, 1981a,b,c; Menge and Bryant, 1988; 1992a,b). The nucleotide dATP substitutes best for ATP *in vitro*, and several RecA functions are enhanced when dATP replaces ATP in reaction mixtures (Menetski and Kowalczykowski, 1989; Shan *et al.*, 1997; Ellouze *et al.*, 1999; Robu *et al.*, 2001).

Many *recA* mutants have advanced our understanding of the roles of RecA protein in the cell. A new double mutant combination, RecA E38K/K72R (*recA730,2201*), has been constructed and is the focus of the current report. The first mutation is RecA E38K (*recA730*). *In vitro*, a RecA protein with this single mutation is able to bind ssDNA coated with SSB better than is wild-type RecA (Lavery and Kowalczykowski, 1992). *In vivo*, *recA* E38K mutant cells are Rec<sup>+</sup>, UV<sup>R</sup> and exhibit constitutive SOS (SOS<sup>C</sup>) expression (Cazaux *et al.*, 1993; Wang *et al.*, 1993; Ennis *et al.*, 1995). This same mutation acts as a suppressor of *recFOR* mutations (Wang *et al.*, 1993). The capacity of RecA

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**Fig. 1.** The ATPase activity of RecA E38K/K72R compared with the activity of RecA K72R and wild-type RecA. The RecA-catalysed ATP and dATP hydrolysis in the presence of *cssDNA* was monitored over time. The reactions were performed as described in *Experimental procedures*. The two top curves represent hydrolysis of ATP and dATP for wild-type RecA protein, and the lower curves represent hydrolysis of ATP and dATP for RecA K72R and RecA E38K/K72R. Time zero corresponds to the time of ATP/SSB or dATP/SSB addition.

E38K for unassisted displacement of SSB and binding to ssDNA may explain the constitutive SOS phenotype (Lavery and Kowalczykowski, 1992). The other mutant, RecA K72R (*recA2201*), has a change in a highly conserved lysine residue in the Walker A motif, and it binds but does not hydrolyze ATP. *In vitro*, this mutant protein functions poorly in the presence of ATP, but will form long filaments and promote DNA pairing in the presence of dATP (Rehrauer and Kowalczykowski, 1993; Shan *et al.*, 1996). It is not clear if the enhancements to RecA function seen in the presence of dATP are physiologically relevant, as mixtures of dATP and ATP designed to approximate the *in vivo* ratios of these nucleotides (~1/10) produce a RecA with the properties associated with ATP, not dATP binding (Shan *et al.*, 1997). The LexA repressor of the SOS response undergoes autocatalytic cleavage in the presence of the RecA K72R protein, but at a much reduced rate (Shan *et al.*, 1996). These observations lead to an expectation that RecA K72R should exhibit limited function and particularly should facilitate some SOS induction *in vivo*. However, *in vivo* the *recA* K72R (*recA2201*) mutant behaves like a null *recA* mutant with respect to sensitivity to DNA damaging agents and recombination (Konola *et al.*, 1994). The *recA* K72R mutant does not promote SOS induction *in vivo*, suggesting that ATP hydrolysis is required for SOS induction (Renzette and Sandler, 2008). This conclusion is discrepant from the *in vitro* LexA cleavage properties of RecA K72R, and is complicated by the previously unaddressed observation that this mutant protein does not form extended filaments in the presence of ATP *in vitro*. Thus, *in vivo* results obtained with *recA* K72R, in particular its inability to induce SOS expression, could be due to its inability to hydrolyze ATP, its inability to adopt an extended conformation or due to other functional shortcomings when associated with ATP (Rehrauer and Kowalczykowski, 1993).

The RecA E38K/K72R was designed to improve the functional characteristics of the RecA K72R mutant protein, and to permit a more unambiguous determination of *in vivo* deficiencies that can be traced uniquely to a lack of ATP hydrolytic activity. This necessitates a thorough *in vivo* and *in vitro* characterization of this mutant protein.

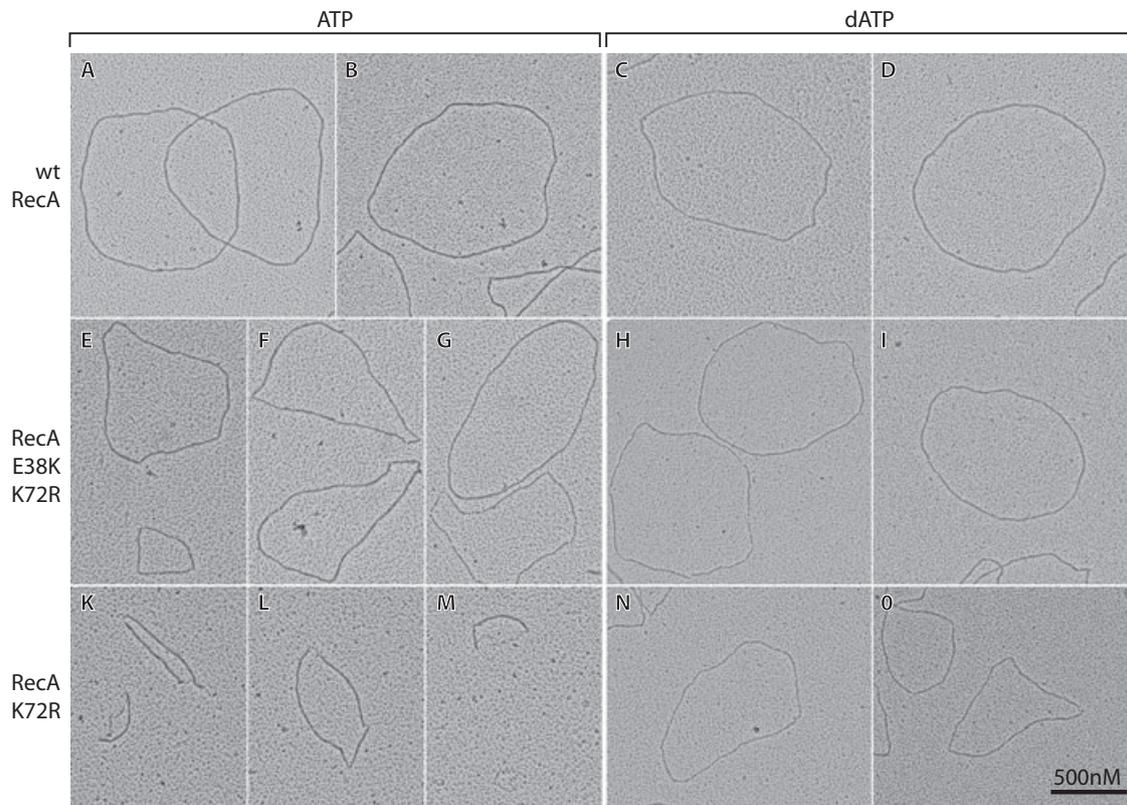
## Results

### Experimental rationale

In principle, the RecA E38K/K72R double mutant protein should retain an inability to hydrolyze ATP, while at the same time exhibiting improved DNA binding and filament formation activities in the presence of ATP. First, we thoroughly characterize the double mutant protein *in vitro* to document its properties relative to the wild-type RecA protein and RecA K72R. We then explore the properties of the RecA E38K/K72R protein *in vivo*, along with the phenotypes of the cells that express it. Throughout, the major focus is the capacity of the mutant protein to hydrolyze ATP, to form filaments on DNA in the extended conformation, to facilitate LexA repressor cleavage and to induce the SOS response.

### The RecA E38K/K72R protein is deficient in ATP and dATP hydrolysis

First, it is critical to determine if the addition of the E38K mutation had any effect on the ATP hydrolytic function of the K72R mutant protein. The ATP or dATP hydrolytic activity of RecA E38K/K72R was examined and the results are depicted in Fig. 1. The RecA E38K/K72R double mutant, like the K72R single mutant protein, exhibits a (d)ATPase activity best described as low to almost undetectable. Both the K72R single mutant and the E38K/



**Fig. 2.** RecA E38K K72R forms filaments similar to wild-type RecA on *cssDNA* with ATP or dATP. All samples were fixed by the addition of ATP $\gamma$ S prior to spreading. Due to the large variety of filaments observed, representative molecules are shown here. All pictures were taken at a magnification of 15000 $\times$  and the scale bar shown in (O) also applies to all other Panels. The contrast of individual images was adjusted uniformly using Adobe Photoshop.

K72R double mutant exhibited an apparent  $k_{\text{cat}}$  between 0.15  $\text{min}^{-1}$  and 0.30  $\text{min}^{-1}$  in the presence of ATP or dATP. This finding is comparable to previous measurements of ATPase activity of the RecA K72R single mutant, with a reported  $k_{\text{cat}}$  of 0.032  $\text{min}^{-1}$  in the presence of ATP, and a  $k_{\text{cat}}$  of 0.052  $\text{min}^{-1}$  in the presence of dATP (Rehrauer and Kowalczykowski, 1993). Small differences may reflect the different reaction conditions employed. Wild-type RecA, in the presence of either ATP or dATP, is included as a positive control and hydrolyzes ATP with a  $k_{\text{cat}}$  of 32.10 ( $\pm 0.34$ )  $\text{min}^{-1}$  and dATP with a  $k_{\text{cat}}$  of 42.24 ( $\pm 1.23$ )  $\text{min}^{-1}$ , in good agreement with reported values (Brenner *et al.*, 1987; Shan and Cox, 1997). The similarity of the ATPase properties of the two RecA mutants indicates that the E38K mutation does not improve the capacity of the RecA E38K/K72R double mutant to hydrolyze ATP or dATP when compared with the RecA K72R single mutant.

*The RecA E38K/K72R protein forms filaments similar to wild-type RecA on circular single-stranded DNA (cssDNA)*

We next determined if the double mutant RecA protein can adopt the extended conformation in the presence of

ATP. To test this, the capacity of RecA E38K/K72R and RecA K72R to form filaments on *cssDNA* in the presence of ATP and dATP was observed by electron microscopy (EM) and was compared with filaments of wild-type RecA protein. In the first set of experiments, each sample was briefly incubated with ATP $\gamma$ S before spreading, a strategy that has been used previously to stabilize RecA filaments during the spreading process (Shan and Cox, 1997; Webb *et al.*, 1997; Bork *et al.*, 2001; Lusetti *et al.*, 2003; Drees *et al.*, 2004). Representative filaments of each protein are shown in Fig. 2, and overall results are summarized in Table 1. The electron micrographs show that in the presence of ATP, the RecA E38K/K72R double mutant forms mostly fully extended filaments (Fig. 2E–G), whereas the RecA K72R mutant protein mostly forms short filaments with large regions of *cssDNA* coated with SSB, referred to as gaps (Fig. 2K–M). The RecA E38K/K72R protein formed about 61% full filaments without gaps and 39% filaments with 1 or more small gaps (total of 364 circular filaments counted). The average length ( $n = 57$ ) of RecA E38K/K72R filaments was 2827 ( $\pm 564$ ) nm, which is 78% of the average ( $n = 19$ ) wild-type RecA filament length of 3634 ( $\pm 62$ ) nm. No full filaments were detected for RecA K72R and the average ( $n = 85$ ) filament length was

**Table 1.** Summary of filament length and filament extension (Pitch) measurements.

	Filament length 3 min ATP $\gamma$ S		Pitch 3 min ATP $\gamma$ S		Pitch no ATP $\gamma$ S	
	ATP	dATP	ATP	dATP	ATP	dATP
WT	3634 $\pm$ 62	N.D.	10.70 $\pm$ 1.03	10.85 $\pm$ 1.23	10.48 $\pm$ 1.12	10.18 $\pm$ 1.27
E38K K72R	2827 $\pm$ 564	N.D.	10.52 $\pm$ 1.03	10.41 $\pm$ 0.96	10.79 $\pm$ 1.57	10.36 $\pm$ 1.27
K72R	780 $\pm$ 592	N.D.	10.53 $\pm$ 0.92	10.36 $\pm$ 1.24	N.A. <sup>a</sup>	10.55 $\pm$ 1.13

a. N.A. is not available as RecA K72R did not form filaments under these conditions.

All measurements are on M13mp18 cssDNA and lengths are in nm. For filament length measurements  $n = 19$  for wt,  $n = 57$  for E38K K72R and  $n = 85$  for K72R. For pitch measurements 50 individual pitches from 6 to 10 different molecules were measured for all conditions. N.D., is not determined.

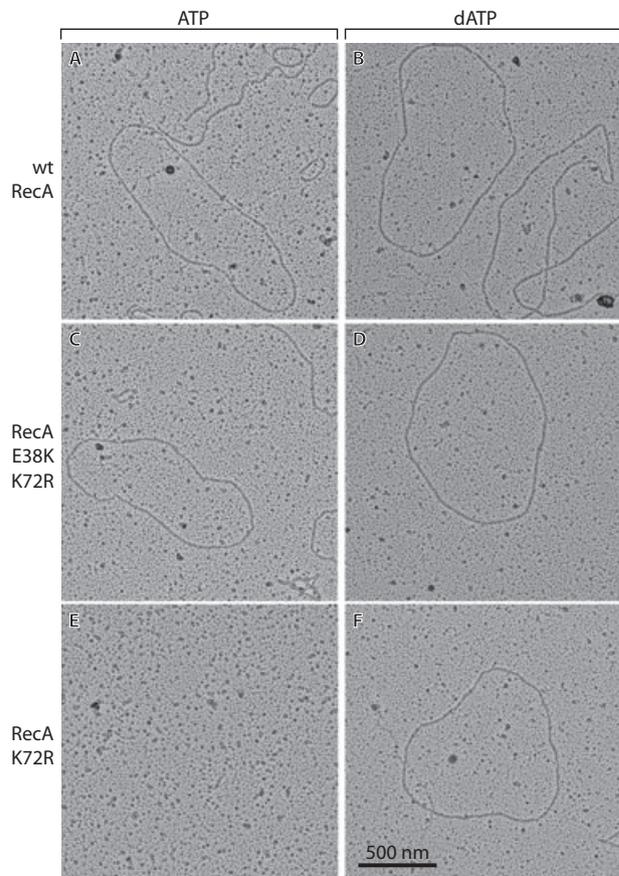
780  $\pm$  592 nm (Table 1). When dATP is present, the filament formation by both mutant proteins is enhanced. These results are consistent with the *recA* E38K mutation suppressing the inability of RecA to adopt an extended conformation and completely coat the cssDNA in the presence of ATP when the *recA* K72R mutation is also present.

Further EM experiments were carried out without adding ATP $\gamma$ S prior to spreading, to determine if the ATP $\gamma$ S addition was affecting the results obtained with RecA K72R. The results are shown in Fig. 3. The wild-type, RecA K72R and RecA E38K/K72R mutant proteins all exhibit good filament formation in the presence of dATP. In the presence of ATP, full filaments were observed for the wild-type and RecA E38K/K72R mutant proteins, although the wild-type RecA filaments showed some small discontinuities without the ATP $\gamma$ S addition. Filament extension measured by determining the pitch of the helical RecA filaments did not change significantly, for the wild-type and double mutant proteins with ATP or dATP, or with RecA K72R in the presence of dATP (Table 1). Strikingly, filament formation was almost entirely absent when RecA K72R was incubated with ssDNA and ATP, with no ATP $\gamma$ S addition (Fig. 3E). This suggests that the short RecA K72R filaments seen in Fig. 2 were dependent on the added ATP $\gamma$ S, and that this mutant protein has limited or even no capacity to form filaments, extended or otherwise, in the presence of ATP.

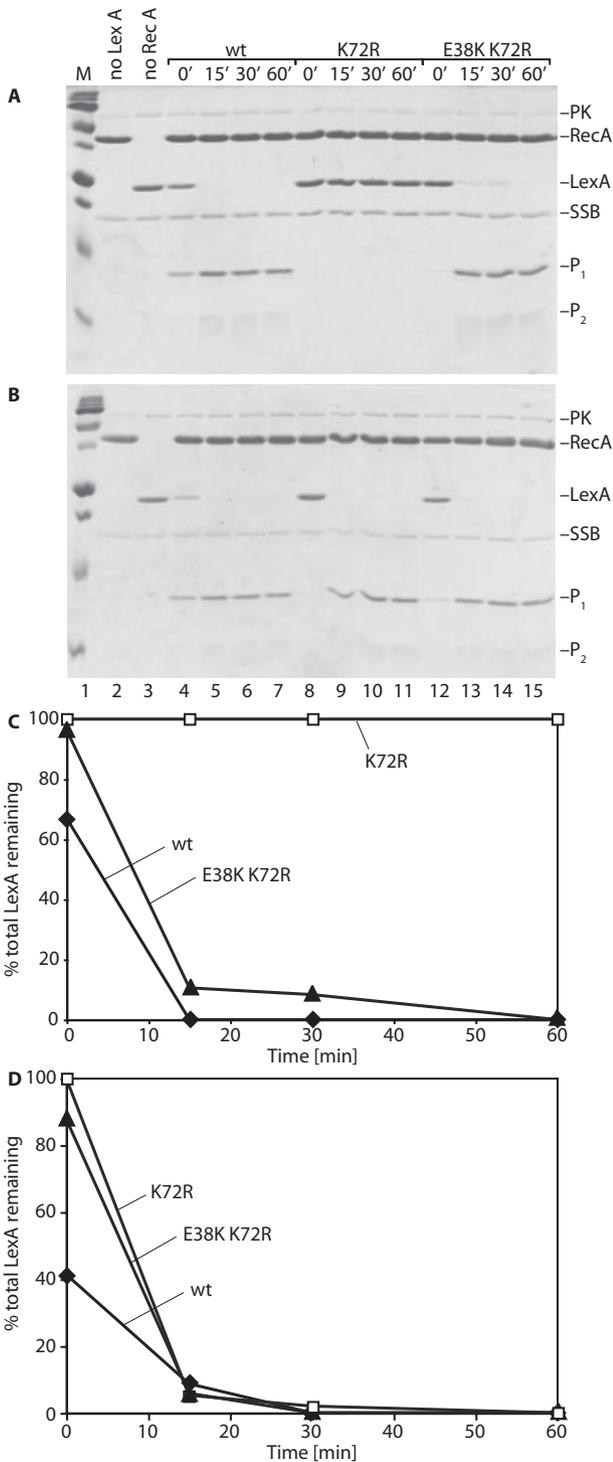
*The RecA E38K/K72R protein facilitates cleavage of the LexA repressor protein in the presence of ATP or dATP*

As combining the *recA* E38K mutation with the *recA* K72R mutation did not increase the level of ATPase activity and allowed RecA E38K/K72R to adopt the extended conformation in the presence of ATP, we asked whether the E38K alteration would also increase the rate at which LexA could undergo auto-cleavage in the presence of ATP (versus dATP). In the presence of ATP, RecA E38K/K72R mediates LexA cleavage almost to the extent of wild-type RecA (Fig. 4A and C). Proficient cleavage occurs 15 min

after LexA addition, and complete cleavage is achieved at the 60 min time point. In contrast, RecA K72R does not facilitate LexA cleavage when ATP is present under these conditions. In the presence of dATP both RecA mutants



**Fig. 3.** Formation of extended filaments is abolished for RecA K72R in the presence of ATP when not fixed by ATP $\gamma$ S. Filament fixation by ATP $\gamma$ S was omitted in all samples to prevent RecA binding to ATP $\gamma$ S from interfering with binding to ATP or dATP. Due to the large variety of filaments observed, representative molecules are shown here. All pictures were taken at a magnification of 15000 $\times$  and the scale bar shown in (F) also applies to all other Panels. The contrast of individual images was adjusted uniformly using Adobe Photoshop.

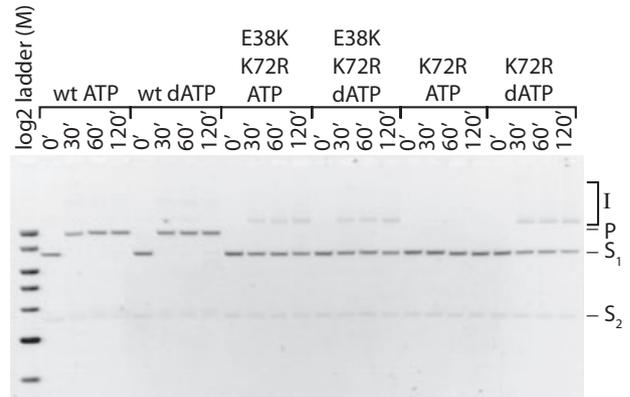


**Fig. 4.** RecA E38K/K72R mediates LexA cleavage in the presence of ATP and dATP. Reactions (50  $\mu$ l) were carried out as described under *Experimental procedures*. Reactions for Fig. 3A and B are set up the same way except ATP was used in the experiment depicted in Fig. 3A, and dATP was used in Fig. 3B. Lane 1 contains a Bio-Rad precision plus protein standard. Lane 2 shows a control reaction lacking LexA protein and lane 3 contains a control reaction without RecA protein. Figures 3C and D are quantitative analyses of the gels shown in Fig. 3A and B respectively. The graphs show the percentage of uncleaved LexA protein remaining at the different time points. PK stands for pyruvate kinase, included in the reactions as part of the ATP regeneration system. P<sub>1</sub> and P<sub>2</sub> represent the cleaved LexA fragments.

*The RecA E38K/K72R protein promotes homologous pairing and formation of joint molecule intermediates, but not nicked circular product formation in the presence of ATP or dATP*

Finally, to assess the *in vitro* recombination activity of the RecA E38K/K72R protein, its ability to carry out DNA three-strand-exchange reactions was analysed and compared with the strand-exchange activity of RecA K72R and wild-type RecA (Fig. 5). The RecA E38K/K72R double mutant converted some of the DNA substrates (S<sub>1</sub> and S<sub>2</sub>) to joint molecule intermediates in the presence of either ATP or dATP. No nicked circular product (P) formation was observed in the reactions with RecA E38K/K72R under the conditions tested here. The RecA K72R mutant formed joint molecule intermediates only in the presence of dATP, not ATP. The results obtained for RecA K72R

facilitate LexA cleavage to a similar level as the wild-type RecA protein (Fig. 4B and D). These results suggest that restoring the capacity to bind ATP and adopt an extended conformation, but not ATP hydrolysis, is central to the mechanism by which RecA E38K suppresses the inability of RecA K72R to induce the SOS response.



**Fig. 5.** The RecA E38K/K72R double mutant promotes homologous pairing in the presence of ATP and dATP. RecA E38K/K72R, RecA K72R and *wild-type* RecA *in vitro* recombination activities were evaluated as follows. Reactions (50  $\mu$ l) were carried out as described under *Experimental procedures*. Time points were taken at 0, 30, 60 and 120 min. The image was taken with a digital CCD camera utilizing GelExpert software (Nucleotech) and was inverted using Adobe Photoshop. The band labels mean the following: I, joint molecule intermediates; P, nicked circular product; S<sub>1</sub>, linear double-stranded substrate; S<sub>2</sub>, circular single-stranded substrate. The molecular weight marker used is a 2-Log DNA Ladder purchased from NEB.

are consistent with previous findings (Rehrauer and Kowalczykowski, 1993; Shan *et al.*, 1996). Included as a control, the nicked circular product of DNA strand exchange was evident at 30 min for wild-type RecA. The 30 min point was the earliest time point taken in this experiment. The ability of RecA E38K/K72R to form joint molecules in the presence of ATP as well as dATP distinguishes this double mutant from RecA K72R. These results suggest that upon ATP binding, the RecA E38K mutation enables a conformational state that is not adopted in the RecA K72R single mutant in presence of ATP. This conformational state allows homologous pairing to take place.

#### *recA E38K suppresses the defect in SOS induction after UV treatment of recA K72R*

We also explored the phenotypes of the *recA* E38K/K72R double mutant *in vivo*. The *recA* E38K mutant exhibits an SOS<sup>C</sup> expression phenotype in un-irradiated cells and requires no other cofactors (Witkin and Kogoma, 1984; Sommer *et al.*, 1993). To test if *recA* E38K can suppress *recA* K72R, the two mutations were combined on a plasmid and then transferred to the chromosome. The strain also contains a *sulAp-gfp* reporter system (McCool *et al.*, 2004). Therefore, the relative fluorescence intensity (RFI) is a measure of SOS expression. One can then measure the RFI in individual cells in a population and analyse the distribution of cells with a particular intensity in the population as well as the average RFI of the population (Fig. 6).

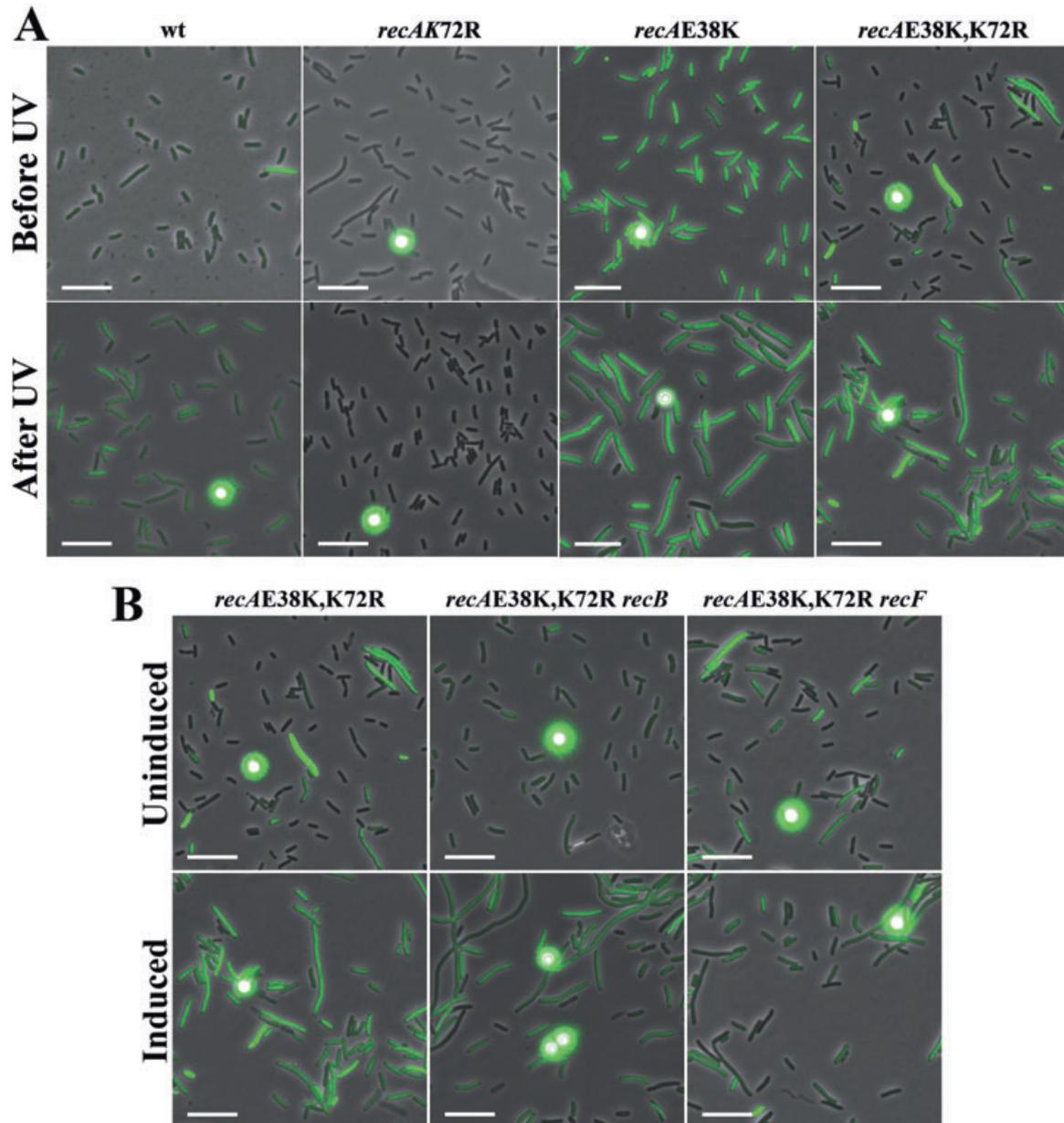
Table 2 shows that about 28% of the cells in the population expressing the *recA* double mutant have a significant level of SOS<sup>C</sup> expression (defined as sixfold above background) and the average across the population is about sixfold above the levels seen for wild-type *E. coli*. This level of SOS<sup>C</sup> expression is decreased from *recA* E38K levels whereas no SOS<sup>C</sup> is seen for the *recA* K72R strain. The mechanism of how the double mutant attains SOS<sup>C</sup> will be tested below. Given this modest background of SOS<sup>C</sup> expression, it is possible to test if the SOS response is inducible in the remainder of the population. To do this, cells were grown in minimal media and treated with 5 J m<sup>-2</sup> of UV light. Cells were then incubated for 90 min and the amount of fluorescence was measured. Table 2 shows that about 90% of the double mutant cells are now induced for SOS expression with an average RFI of 20. In comparison, about 70% of the wild-type cells are induced to an average RFI of 8. Typical cell populations are shown in Fig. 6A. This shows that SOS induction can occur in a timely manner in the *recA* E38K/K72R double mutant cells. Given the background levels, the final proportion of induced and uninduced cells in the population and the increase in average RFI is similar to wild-type.

To further test if the mechanism of SOS induction of the *recA* E38K K72R double mutant is similar to that of wild-type, the dependence of SOS induction on *recF* was tested. It is known that SOS induction after UV treatment is attenuated in a *recF* mutant (McPartland *et al.*, 1980; Thomas and Lloyd, 1983; Wackernagel, 1987; Hegde *et al.*, 1995). Therefore, *recA* E38K/K72R was combined with *recF4115*. This *recF4115* strain, in which the RecF protein is inactivated, showed approximately the same levels of SOS<sup>C</sup> expression as the *recF*<sup>+</sup> strain (Table 2 and Fig. 6B). After UV treatment, no SOS induction was detected. This suggests that the *recA* E38K/K72R mutant is using a mechanism similar to that used by wild-type *recA* to induce the SOS response after UV treatment.

Upon characterizing other phenotypes of the *recA* E38K/K72R mutant, Table 3 shows that conjugational recombination and resistance to UV irradiation are slightly enhanced relative to the null allele but are still well below the wild-type levels (Table 3). This suggests that *recA* E38K is able to suppress the SOS deficiencies of the *recA* K72R mutant. However, it is limited in its abilities to suppress the recombinational and repair deficiencies of the strain, presumably because these functions require ATP hydrolysis.

#### *The recA803 mutation does not suppress the recA K72R mutant phenotypes*

As mentioned above, *E. coli* strains expressing RecA E38K are SOS<sup>C</sup>, and the RecA E38K protein binds ssDNA coated with SSB better than wild-type RecA. To try to correlate which activity may be responsible for suppressing the inability of *recA* K72R to induce the SOS response, another mutation, *recA803*, was combined with *recA* K72R. *RecA803* (V37M) is a mutation in the codon adjacent to the *recA* E38K mutation. Both RecA V37M and RecA E38K compete with SSB for binding to ssDNA (Lavery and Kowalczykowski, 1992; Madiraju *et al.*, 1992). However, *recA* V37M does not display an SOS<sup>C</sup> phenotype like *recA* E38K (Madiraju *et al.*, 1988; 1992). The effect of the RecA V37M mutation on extended filament formation is not known. It is presumed that the V37M and E38K mutations of RecA differ in their capacity to bind ssDNA and form extended filaments sufficient to generate constitutive SOS induction. It is thus interesting to determine if the V37M mutation can suppress *recA* K72R in the same manner as *recA* E38K does. However, the *recA* V37M/K72R double mutant has phenotypes essentially indistinguishable from *recA* K72R (Tables 2 and 3). This experiment suggests that the E38K mutation confers a particularly robust capacity to bind ATP and/or to adopt an extended conformation sufficient to suppress the deficiencies in SOS induction of the *recA* K72R mutant.



**Fig. 6.** SOS induction is expressed in *recA* K72R,E38K cells in a *recF*-dependent manner. A *sulA::gfp* reporter construct is used such that fluorescence intensity is correlated to SOS expression. All figures are merged images between phase contrast and fluorescence microscopy. A. SOS induction in cells expressing different RecA protein variants. Typical cell populations are shown before and after treatment with 5 J m<sup>-2</sup> of UV light. B. SOS expression of a *recAE38K,K72R* strain with mutations in *recB* or *recF* before and after treatment with UV irradiation. The circular dots are calibration beads.

#### *The mechanisms of SOS<sup>c</sup> expression in recA E38K and recA E38K/K72R are different*

The decrease in SOS<sup>c</sup> expression in the *recA* E38K/K72R double mutant as compared with the *recA* E38K single mutant suggests that the ATPase activity is important for SOS<sup>c</sup> expression. In work to be published elsewhere, it is shown that *recA* E38K SOS<sup>c</sup> expression is *recBCD*-independent (E. Long, N. Renzette, C. Richard and S.J.

Sandler, unpublished). To further test if the mechanism of SOS<sup>c</sup> expression in *recA* E38K and a *recA* E38K/K72R strain is different, the *recB* dependence was tested. Table 2 and Fig. 6B show that a *recA* E38K/K72R *recB268::tet* strain has fourfold less (essentially background levels) SOS<sup>c</sup> expression than *recA* E38K/K72R *recB*<sup>+</sup>. The *recB* mutation has little effect on the ability of the strain to induce SOS after UV treatment as expected (Keller *et al.*, 2001). Thus, a *recB* mutation has a substan-

**Table 2.** The level of SOS expression before and after UV treatment.

Strain	Genotype		SOS expression			
			Before UV treatment		After UV treatment	
	<i>recA</i>	Other	Relative intensity	% Population high SOS	Relative intensity	% Population high SOS
SS996	+		1.00	0.8 ± 0.4	8.1 ± 0.3	71.0 ± 4.0
SS1426	<i>del(recA)</i>		0.3 ± 0.4	0.0 ± 0.2	0.3 ± 0.2	0.00 ± 0.1
SS4629	730 (E38K)		45.0 ± 1.3	99.0 ± 1.0	N.D.	N.D.
SS5602	2201 (K72R)		0.3 ± 0.2	0.0 ± 0.2	0.7 ± 0.2	0.00 ± 0.2
SS5690	730,2201		5.7 ± 0.9	28 ± 0.8	20 ± 1.9	91.0 ± 3.0
SS6418	803,2201		0.6 ± 0.8	0.0 ± 0.3	0.9 ± 0.7	0.1 ± 0.8
SS6441	730,2201	<i>recB268</i>	1.3 ± 0.3	2.1 ± 0.3	9.6 ± 0.9	55.0 ± 2.0
SS5691	730,2201	<i>recF4115</i>	4.7 ± 0.4	25.0 ± 0.7	4.6 ± 0.9	24.0 ± 1.0

The average of 1000–3000 total cells analysed from three different experiments. N.D., is not determined.

tially greater effect on strains expressing the double mutant RecA E38K/K72R than it has on strains expressing the RecA E38K protein.

## Discussion

There are four major conclusions arising from this work. First, ATP hydrolysis is not required for RecA to mediate SOS induction in *E. coli*. Second, efficient formation of RecA filaments in the extended conformation is necessary for SOS induction, even though ATP hydrolysis is not. Third, the filament formation deficiency of RecA K72R is suppressed by the E38K mutation. The suppression does not entail the uniformly constitutive SOS induction seen in cells expressing the E38K mutant protein. The fraction of cells expressing SOS increases substantially after UV irradiation in cells expressing the RecA E38K/K72R protein, and the expression depends on the function of RecF protein. The mechanism of SOS induction is thus largely preserved in this double mutant RecA protein. Even though SOS induction between the double mutant and wild-type strains are very similar, it is not possible to rule out that SOS induction in the mutant may occur by a different mechanism. Finally, we present the RecA E38K/K72R double mutant protein as an improved reagent for the exploration of questions focused on the *in vivo* and *in vitro* role of RecA-mediated ATP hydrolysis. Unlike the K72R single mutant, the double mutant forms long extended filaments in the presence of ATP almost as well as does the wild-type RecA protein. Given the preponderance of ATP over dATP in the intracellular environment, this allows an improvement of consistency between experiments carried out *in vivo* and *in vitro*.

ATP hydrolysis is used to enhance or promote a wide range of cellular processes. However, the role of an ATPase activity in the molecular function of a protein is not always obvious. As the discovery of the Walker Motif (Walker *et al.*, 1982) and the fact that a simple conservative change of a lysine to an arginine in a conserved GKT

portion of the motif will usually eliminate the ATPase activity, hundreds of genes/proteins with putative ATPase activities have been altered in this way. For RecA protein, the K72R mutation has provided many insights (Rehrauer and Kowalczykowski, 1993; Shan *et al.*, 1996), yet the precise role of the RecA ATPase activity in recombination and SOS induction has been elusive. This work shows the intragenic suppression of an ATPase mutant for a particular function of RecA, SOS induction, without restoration of the ATPase activity. It should be noted that while SOS induction is restored by the addition of E38K to the K72R mutation, the substantial deficiencies of *recA* K72R in the complicated recombination functions of RecA are altered very little by the same combination of mutations. The small amount of recombination ability and UV resistance seen the *recA* E38K/K72R double mutant, relative to the *recA* null mutant, may be due to the ability of the mutant protein to stabilize replication forks (Courcelle and Hanawalt, 2003), promote a limited strand exchange in certain situations, or induce compensatory activities during SOS. In some ways, the *recA* E38K/K72R mutant can be considered a separation of function mutant, allowing investigators to more readily distinguish functions that do not need ATP hydrolysis from ones that do.

**Table 3.** Summary of sensitivity to UV light and recombination phenotypes of RecA mutants.

Strain	<i>recA</i>	Survival to UV <sup>a</sup>	Recombination <sup>b</sup>
SS996	+	73 ± 2.33	8.44 ± 0.93
SS1426	<i>del(recA)</i>	< 10 <sup>-2</sup>	< 10 <sup>-4</sup>
SS4629	730 (E38K)	75 ± 4.29	9.03 ± 1.02
SS5602	2201 (K72R)	< 10 <sup>-2</sup>	< 10 <sup>-4</sup>
SS5690	730,2201	0.15 ± 0.18	0.012 ± 0.0073
SS6418	803(V37M),2201	< 10 <sup>-2</sup>	< 10 <sup>-4</sup>

a. UV Resistance is measured by percentage survival of strains after 10 J of UV irradiation.

b. Conjugation frequency reported as percentage transconjugants/100 donors.

The mechanism of suppression almost certainly involves a structural contribution of the E38K mutation that provides the capacity to make long RecA filaments in the extended conformation in the presence of ATP. RecA K72R is not an inactive protein and can bind dATP and perform a variety of functions *in vitro* (Rehrauer and Kowalczykowski, 1993; Shan *et al.*, 1996). But the capacity to form extended RecA filaments in the presence of ATP appears to be critical *in vivo*, where ATP is present at approximately 10-fold higher concentrations than dATP. The *recA* E38K/K72R double mutant demonstrates that the ATPase activity of RecA is not necessary for SOS induction as long as the active extended form of the RecA filament is accessible. An alternative possibility, that the ATPase activity is necessary for converting RecA<sup>+</sup> to the extended conformation and that the *recA* E38K mutation allows the bypass of this requirement, is rendered unlikely by the capacity of ATP<sub>γ</sub>S to induce extended filament formation in wild-type RecA *in vitro* without significant hydrolysis (Weinstock *et al.*, 1981d), a capacity seen in the current study.

RecA E38K/K72R is a hybrid that does not exhibit all of the properties of the RecA E38K single mutant protein. In a sense, the E38K mutation is enhancing the function of the K72R protein, and the K72R is weakening aspects of the function of E38K. The two mutations are not additive, but compensatory. The SOS response is not constitutively induced in the double mutant, as it is in strains expressing *recA* E38K. However, the ability of the E38K variant to suppress the inability of *recA* K72R to induce the SOS response is likely related to its capacity to form extended filaments, as exhibited by a constitutive SOS phenotype. Many other *recA* mutants have been shown to have an SOS<sup>c</sup> phenotype (McCall *et al.*, 1987; Lavery and Kowalczykowski, 1992; Tateishi *et al.*, 1992; Ennis *et al.*, 1995; McGrew and Knight, 2003; Mirshad and Kowalczykowski, 2003; O'Reilly and Kreuzer, 2004). It is plausible that other *recA* variants that confer an SOS<sup>c</sup> phenotype may be able to suppress the SOS-deficient phenotype of *recA* K72R, but it is also clear that not all of them do. In this study, a combination of *recA803* (V37M) with the K72R mutation did not lead to substantial SOS induction. We hypothesize that there is some threshold of ssDNA binding and SSB displacement capacity in a RecA variant that is needed to observe constitutive SOS expression. There may be yet a higher threshold for ssDNA binding and SSB displacement that is required to suppress the considerable deficiency in those activities displayed by RecA K72R protein. The combination of *recA803,4161* (V37M, ΔC17) exhibits SOS<sup>c</sup> expression in most cells whereas either single mutant showed very little if any SOS<sup>c</sup> expression (E. Long, N. Renzette, C. Richard and S.J. Sandler, unpublished). This double mutant supplied some ability to suppress the SOS defect

after UV exposure of *recA* K72R, but did not suppress as well as *recA* E38K (N.R. and S.J.S., unpubl. result).

One of the roles of RecA ATPase activity is to allow RecA to dissociate from the DNA. If so, then how does the RecA E38K/K72R protein come off the DNA to allow replication and cell growth? One can imagine that a protein tightly bound to replication forks might be detrimental to replication restart, or could pose a block for a subsequent replication fork. This might explain why another *recA* ATPase-deficient mutant, *recA* E96D, is lethal (Campbell and Davis, 1999). Under certain circumstances, the UvrD helicase is critical for removing RecA from replication forks where RecA binding is detrimental (Flores *et al.*, 2005; Veaute *et al.*, 2005). It is possible that UvrD is essential for this purpose in the *recA* E38K/K72R mutant strain. Testing this has shown that *uvrD* and *recA* E38K/K72R are synthetically lethal (N.R. and S.J.S., unpubl. results).

The *recA* E38K mutation acts as a suppressor of *recF* null mutations (Wackernagel, 1987; Lavery and Kowalczykowski, 1992; Wang *et al.*, 1993). It was thus somewhat unexpected that *recA* E38K/K72R requires *recF* function for SOS induction after UV treatment. This again suggests that the double mutant does not retain all the DNA binding and filament formation capacity of the E38K mutant. A requirement for *recF* function is a hallmark of SOS induction in wild-type cells. The similar requirement in strains expressing *recA* E38K/K72R provides another indication that this ATPase mutant is utilizing the normal SOS induction pathway.

Whereas this paper focuses mostly on the ability of RecA E38K to suppress aspects of the RecA K72R phenotypes, it also tests whether the RecA-ATPase function is required for any of the RecA E38K phenotypes. We found that ATP hydrolysis is very important for RecA to carry out recombination after conjugation and to repair DNA after UV treatment. Both of these functions are reduced in RecA E38K K72R by almost 500-fold compared with wild-type, but are not quite reduced to the level of a null mutant (Table 3).

Is there any role for the RecA ATPase activity in SOS induction in a wild-type cell? It is clear that RecA E38K K72R protein can respond to UV treatment in a manner very similar to the wild-type protein and induce the SOS response without ATPase activity. Thus, while it is not required for SOS induction, it may be possible that ATP hydrolysis has an indirect role in negatively regulating the SOS response. This idea stems from the observations that in a population of wild-type cells under normal growth conditions, approximately 15% of the cells have a RecA-DNA filament (Renzette *et al.*, 2005), yet less than 1% of the cells are induced for SOS (McCool *et al.*, 2004; Pennington and Rosenberg, 2007). Thus, not all RecA-DNA filaments are inducing the SOS response. It is

**Table 4.** Strains used in this work.

Strain	<i>recA</i>	Other relevant genotype	Origin or reference
GY8382	<i>938::cat</i>		Marsh and Walker (1987)
JC13509 <sup>a</sup>	+		Lab stock
JC18825	+	<i>recF4115 tnaA300::tn10</i>	Sandler (1994)
JJC315	+	<i>recB268::tn10</i>	Michel <i>et al.</i> (1997)
SS996	+	$\Omega$ <i>gfp</i> <sup>b</sup>	McCool <i>et al.</i> (2004)
SS1426	+	<i>recF4115 <math>\Omega</math>gfp</i>	JC18825→SS996
SS1433	+	<i>recB268::tn10 <math>\Omega</math>gfp</i>	JJC315→SS996
SS1436	<i>938::cat</i>	$\Omega$ <i>gfp</i>	GY8383→SS996
SS4629	<i>730</i>	$\Omega$ <i>gfp</i>	E. Long, N. Renzette, C. Richard and S.J. Sandler, unpublished
SS4660	+	<i>recB268::tn10 <math>\Omega</math>gfp</i>	pKD46→SS1433 <sup>c</sup>
SS5602	<i>2201</i>	<i>ygaD1::kan <math>\Omega</math>gfp</i>	Renzette and Sandler (2008)
SS5687	<i>730,2201</i>	<i>ygaD1::kan</i>	This work
SS5690	<i>730,2201</i>	<i>ygaD1::kan <math>\Omega</math>gfp</i>	SS5687→SS996
SS5691	<i>730,2201</i>	<i>ygaD1::kan recF4115 tnaA300::tn10 <math>\Omega</math>gfp</i>	SS5687→SS1426
SS6408	<i>803,2201</i>	<i>ygaD1::kan</i>	This work
SS6418	<i>803,2201</i>	<i>ygaD1::kan <math>\Omega</math>gfp</i>	SS6408→SS996
SS6441	<i>730,2201</i>	<i>ygaD1::kan recB268::tn10 <math>\Omega</math>gfp</i>	SS5687→SS4660 <sup>d</sup>

**a.** JC13509 has the following partial genotype: *sulB103<sup>-</sup> lacMS286  $\Phi$ 80dIIIacBK1 argE3 his-4 thi-1 xyl-5 mtl-1 rplS31 tsx*. The *lacMS286  $\Phi$ 80dIIIacBK1* codes for two partial non-overlapping deletions of the *lac* operon (see Konrad, 1977 and Zieg and Kushner, 1977).

**b.** *attL::sulAp $\Omega$ gfp-mut2* is abbreviated  $\Omega$ *gfp*.

**c.** pKD46 expresses the Lambda Red proteins (Datsenko and Wanner, 2000).

**d.** P1 transduction with SS4660 as a recipient was carried out as normal with the exception that the strain was grown in Luria broth with ampicillin (50  $\mu$ g ml<sup>-1</sup>) and induced with 0.2% arabinose 1 h before harvesting the culture.

possible that if the default function of RecA bound to DNA in cells is recombinational repair, then active engagement in recombinational repair may inhibit SOS induction. To induce SOS, one might then require the temporary suspension of functions requiring the ATPase so that LexA cleavage could occur. This idea is supported by experiments that show a competition between LexA and a homologous duplex to interact with a RecA-DNA filament (Rehrauer *et al.*, 1996). Some of the many proteins now being shown to regulate RecA function (Cox, 2007) may play a role in determining which pathway is undertaken.

The enhancement of RecA function *in vitro* with dATP might suggest that dATP plays some role in RecA function *in vivo*. This would require a locally high concentration of dATP near the sites of recombinational DNA repair. The capacity of RecA K72R/E38K (which can utilize ATP) to function in SOS induction *in vivo*, coupled with the lack of *in vivo* function of RecA K72R (which requires dATP), argues that no such local concentration of dATP exists in the cell. The role of dATP in RecA function, if any, remains a mystery.

In conclusion, this work shows that RecA E38K/K72R can serve as useful reagent to study RecA filament dynamics as the protein has many properties similar to wild-type RecA. For example, RecA E38K/K72R could be used in studies of RecA filaments bound to ATP, as was previously performed using RecA E96D (VanLoock *et al.*, 2003). However, RecA E96D retains significant residual ATPase activity (VanLoock *et al.*, 2003). The near complete inactivation of ATPase activity in the RecA E38K/

K72R protein offers a new tool to study this important process in RecA biology. Furthermore, *recA* E96D is lethal to host cells (Campbell and Davis, 1999) while *recA* E38K/K72R is not (when coupled with a *sulB103* mutation).

## Experimental procedures

### Bacterial strains

All bacterial strains used in this work are derivatives of *E. coli* K-12 and are described in Table 4. The protocol for P1 transduction has been described elsewhere (Willets and Mount, 1969). Protocols for assaying UV sensitivity and conjugal recombination have been described elsewhere (Clark and Margulies, 1965; Sandler *et al.*, 1996).

### Plasmid construction

*recA* E38K/K72R was cloned into the NdeI and HindIII sites of pET21a (Novagen), creating pEAW483. pNR114 was created by replacing the NcoI and BlnI fragment of pSJS1373 with the homologous fragment of pEAW483.

### Strain construction

The *recA* E38K/K72R mutation was placed on the chromosome similarly to other *recA* mutations (Renzette and Sandler, 2008). Briefly, pNR114 was digested with BamHI and the 4.5 kb linear fragment was purified. This fragment encodes *ygaD1::kan recA E38K/K72R recX* and was used to transform cells expressing the Red recombinase (SS1153)

(Datsenko and Wanner, 2000). Transformants were selected on Luria broth and kanamycin and screened for UV sensitivity. All strains in this study were derived from JC13509, which contains a *suIB103* mutation. *SuIB103* is an allele of *ftsZ* that causes FtsZ to be insensitive to the action of the Sula cell division inhibitor (Bi and Lutkenhaus, 1990).

### Buffers

P buffer contained 20 mM potassium phosphate (pH 6.8), 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol. R buffer contained 20 mM Tris-HCl, 80% cation (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol.

### Proteins and biochemicals

The *E. coli* wild-type RecA and single-stranded DNA binding (SSB) proteins were purified as described (Lohman *et al.*, 1986; Shan *et al.*, 1996). The concentration of the purified SSB protein was determined from the absorbance at 280 nm using the extinction coefficient of  $2.83 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  (Lohman and Overman, 1985).

*Escherichia coli* LexA repressor protein was purified using a combination of published protocols (Schnarr *et al.*, 1985; Little *et al.*, 1994) from an *E. coli* strain STL327/pT7pol26 transformed with the pEAW329 plasmid that has the *lexA* gene under the control of an isopropyl-1-thio- $\beta$ -D-galactopyranoside-inducible promoter. Cell growth and harvesting was performed as described previously (Lusetti *et al.*, 2003). About 22.9 g of cells was thawed in 100 ml Buffer A [50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 10% (w/v) sucrose, 1 mM DTT, 200 mM NaCl]. Cell lysis by addition of 5 mg ml<sup>-1</sup> lysozyme in Buffer A and subsequent sonication were performed. The cell debris was pelleted by centrifugation at 16 000 r.p.m. for 60 min. Contaminants were precipitated by adding 0.07 ml of 5% polyethyleneimine per ml of solution and pelleted by centrifugation at 12 000 r.p.m. for 10 min. Solid ammonium sulphate at a final concentration of 0.4 g ml<sup>-1</sup> was added to the supernatant and the precipitated LexA protein was washed twice with Buffer A plus 0.49 g ml<sup>-1</sup> ammonium sulphate. The final pellet was re-suspended in 10 ml of Buffer B (20 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 10% (w/v) glycerol, 80 mM NaCl) plus 500 mM NaCl. Column chromatography was carried out as described in Little *et al.* (1994) up through the hydroxyapatite purification step. The concentration of LexA protein was determined using an extinction coefficient of  $7300 \text{ M}^{-1} \text{ cm}^{-1}$ .

The RecA K72R protein was purified as described (Shan *et al.*, 1996) with the following changes. Briefly, 16 g of cell paste was thawed on ice overnight. Cell lysis, polyethyleneimine precipitation and ammonium sulphate extraction and precipitation were performed as described (Shan *et al.*, 1996). The resulting pellet was re-suspended in 70 ml of R buffer plus 1 M ammonium sulphate and loaded onto a phenyl-sepharose low sub 6 FF column. The column was washed with three column volumes of the same buffer and a 10-column volume gradient was run in R buffer, with a gradient of ammonium sulphate from 1.0 M to 0 M. SDS-PAGE analysis revealed that the RecA mutant protein was in the flow-through peak fractions, which were dialysed versus R

buffer. The RecA K72R protein then was loaded onto a Source 15 Q column, washed with three column volumes of R buffer and eluted by a 10-column volume gradient from R buffer to R buffer plus 1 M KCl. The peak fractions were analysed by SDS-PAGE, revealing that the RecA K72R protein eluted at 500 mM KCl. The RecA mutant containing fractions were pooled, dialysed against P buffer and loaded onto a ceramic hydroxyapatite column, washed with three column volumes of P buffer and eluted by a 10-column volume gradient from 20 mM phosphate to 500 mM phosphate. The peak fractions were analysed by SDS-PAGE, pooled and concentrated by ammonium sulphate precipitation (0.35 mg ml<sup>-1</sup>). The pellet was re-suspended in R buffer, dialysed against R buffer, frozen in liquid N<sub>2</sub> and stored at -80°C. The RecA K72R protein was free of detectable nuclease activity. The concentration of the RecA K72R protein was determined using the wild-type RecA protein extinction coefficient ( $2.23 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ).

RecA E38K/K72R protein was purified using modifications to previously described protocols (Cox *et al.*, 1981; Lusetti *et al.*, 2003). All the steps were carried out at 4°C. Thirteen and a half grams of cell paste was thawed on ice overnight in 45 ml of 25% (w/v) sucrose and 250 mM Tris-HCl (80% cation, pH 7.5). Cells were lysed by incubation with a solution containing 250 mM Tris-HCl (80% cation, pH 7.5) and 2.5 mg ml<sup>-1</sup> lysozyme (final concentration) followed by the addition of 0.4 ml per ml solution of 25 mM EDTA, six cycles of sonication for 1 min (0.5 s on 0.5 s off) at output 60% (Fischer Scientific Sonic Dismembrator Model 500), and 60 min of centrifugation at 16 000 r.p.m. in a Beckman JLA-16 rotor. The RecA was precipitated by drop wise addition of 0.111 ml of 5% (w/v) polyethyleneimine (pH 7.5) per ml of solution. The pellet was re-suspended in 130 ml of R buffer and the RecA protein was extracted two times with 110 ml of R buffer plus 300 mM ammonium sulphate. The RecA was precipitated by slow addition of ammonium sulphate to a final concentration of 0.28 g ml<sup>-1</sup>. The resulting pellet was washed three times with 100 ml of R buffer plus 0.28 g ml<sup>-1</sup> ammonium sulphate. The pellet was then re-suspended in 45 ml R buffer plus 1 M ammonium sulphate and loaded onto a phenyl-sepharose column, washed with two column volumes of R buffer plus 1 M ammonium sulphate and then eluted with three column volumes of R buffer. The flow-through peak fractions contained the RecA as determined by SDS-PAGE analysis. These fractions were pooled and dialysed versus P buffer. The protein was then loaded onto a ceramic hydroxyapatite column, washed with two column volumes of P buffer and eluted with a linear gradient from 20 mM to 1 M phosphate buffer over 10-column volumes. RecA eluted at 120 mM phosphate, as determined by SDS-PAGE, and was pooled and precipitated by adding 0.35 g ml<sup>-1</sup> of solid ammonium sulphate. The resulting pellet was re-suspended in 20 ml of R buffer plus 1 M arginine hydrochloride, loaded onto a Sephacryl S-100 Hi Prep 10/60 column (purchased from GE Healthcare) and washed with two column volumes of R buffer plus 1 M arginine hydrochloride. The peak fractions were analysed by SDS-PAGE. Those fractions containing RecA were pooled, dialysed into R buffer, flash-frozen in liquid N<sub>2</sub> and stored at -80°C. The concentration of the RecA E38K/K72R protein (37 896 Da) was determined from the absorbance at 280 nm using the extinction coefficient for

wild-type RecA ( $2.23 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ ). The protein was free of detectable nuclease activity.

#### DNA substrates

cssDNA from bacteriophage M13mp18 (7229 nucleotides) was prepared as described (Neuendorf and Cox, 1986). The concentration of cssDNA was determined by absorbance at 260 nm, using  $36 \mu\text{g ml}^{-1} A_{260}^{-1}$  as the conversion factor. All DNA concentrations are given in  $\mu\text{M}$  nucleotides. Linear double-stranded DNA (ldsDNA) used in the DNA strand-exchange experiments was generated by complete digestion of M13mp18 circular double-stranded DNA (cdsDNA) using PstI according to the manufacturer's instructions.

#### DNA three-strand-exchange reactions

Three-strand-exchange reactions were carried out in standard strand-exchange buffer containing 25 mM Tris-OAc (80% cation, pH 7.4), 1 mM DTT, 5% (w/v) glycerol, 3 mM potassium glutamate and 10 mM  $\text{Mg}(\text{OAc})_2$ . An ATP regeneration system consisting of 10 units  $\text{ml}^{-1}$  pyruvate kinase and 2 mM phosphoenolpyruvate was included. All incubations were carried out at 37°C, and all listed reagent concentrations are final. The wild-type RecA or RecA mutant protein (4  $\mu\text{M}$ ) was pre-incubated with 10  $\mu\text{M}$  M13mp18 cssDNA for 10 min. SSB protein (1  $\mu\text{M}$ ) and ATP (3 mM) were then added, followed by another 10 min of incubation. The reactions were initiated by the addition of 10  $\mu\text{M}$  M13mp18 ldsDNA. The reactions were incubated for 120 min and time points were taken as indicated in the figure legends. To stop the reaction, a 10  $\mu\text{l}$  aliquot was removed and added to 5  $\mu\text{l}$  of a solution containing 15% Ficoll, 0.25% xylene cyanol, 72 mM EDTA and 4% SDS. Samples were subjected to electrophoresis in 0.8% agarose gels with TAE buffer, stained with ethidium bromide, and exposed to ultraviolet light. Gel images were captured with a digital CCD camera utilizing GelExpert software (Nucleotech) and inverted using Adobe Photoshop.

#### ATPase assay

A coupled spectrophotometric enzyme assay (Morrical *et al.*, 1986; Lindsley and Cox, 1990) was used to measure the DNA-dependent ATPase activities of the wild-type RecA, the RecA E38K/K72R and the RecA K72R proteins. The regeneration of ATP from PEP and ADP was coupled to the oxidation of NADH and was observed as a decrease in absorbance at 380 nm (the maximal absorbance for NADH is at 340 nm, but 380 nm wavelength was used so that the signal remained within the linear range of the spectrophotometer for the duration of the experiment). The assays were carried out in a Varian Cary 300 dual beam spectrophotometer equipped with a temperature controller and a 12-position cell changer. The cell path length and band pass were 0.5 cm and 2 nm respectively. The NADH extinction coefficient at 380 nm of  $1.21 \text{ mM}^{-1} \text{ cm}^{-1}$  was used to convert the amount of NADH oxidized to the amount of ATP hydrolyzed. Reaction mixtures (400  $\mu\text{l}$ ) contained 5  $\mu\text{M}$  circular M13mp18 ssDNA, 3  $\mu\text{M}$  of wild-type or mutant RecA protein, and a coupling system of 1.5 mM NADH and 10 units  $\text{ml}^{-1}$  lactate dehydrogenase in

standard strand-exchange reaction buffer. The reactions were started after a 10 min incubation of the reaction mixtures by the addition of a mixture of ATP and SSB protein at 3 mM and 0.4  $\mu\text{M}$  final concentrations respectively.

#### LexA repressor cleavage assay

Reactions were carried out at 37°C and contained 25 mM Tris(OAc) (80% cation, pH 7.5), 3 mM  $\text{Mg}(\text{OAc})_2$ , 5% (w/v) glycerol, 3 mM potassium glutamate, 1 mM DTT, an ATP regeneration system (2 mM phosphoenolpyruvate, 10 units  $\text{ml}^{-1}$  pyruvate kinase), 9  $\mu\text{M}$  circular M13mp18 ssDNA and 3  $\mu\text{M}$  wild-type or mutant RecA protein. After 6 min incubation, 3 mM ATP or dATP and 1.5  $\mu\text{M}$  SSB protein were added, followed by another 6 min incubation. To initiate the reactions, 3  $\mu\text{M}$  LexA repressor protein was added. Time points were taken as indicated in the figure legends by adding 10  $\mu\text{l}$  of the reaction solutions to 5  $\mu\text{l}$  Laemmli buffer. The samples were analysed by SDS-PAGE on 17% polyacrylamide gels. Totalab TL100 v2006c software was used to quantify the bands on the gels.

#### Electron microscopy

A modified Alcian method was used to visualize RecA filaments. Activated grids were prepared as previously described (Lusetti *et al.*, 2003).

Samples for EM analysis were prepared as follows. All incubations were carried out at 37°C. Wild-type RecA protein, RecA E38K/K72R or RecA K72R protein (3  $\mu\text{M}$ ) was pre-incubated with 6  $\mu\text{M}$  M13mp18 circular ssDNA, 25 mM Tris-OAc (80% cation; pH 7.5) buffer, 5% (w/v) glycerol, 3 mM potassium glutamate and 10 mM  $\text{Mg}(\text{OAc})_2$  for 10 min. An ATP regeneration system of 10 units  $\text{ml}^{-1}$  creatine phosphokinase and 12 mM phosphocreatine was also included in the incubation. ATP and SSB protein were added to 3 mM and 0.6  $\mu\text{M}$ , respectively, and the reaction was incubated for another 10 min. Where indicated, ATP $\gamma$ S was then added to 3 mM to stabilize the filaments, followed by another 3 min incubation.

A 3  $\mu\text{l}$  sample of the reaction mixture described above was diluted eightfold with 200 mM ammonium acetate, 10 mM HEPES (pH 7.5) and 10% glycerol, and adsorbed to an activated carbon grid (Alcian grid) for 3 min. The grid was then touched to a drop of the same buffer, followed by floating on a second drop of the buffer for 1 min. The sample was then stained by touching to a drop of 5% uranyl acetate followed by floating on a fresh drop of 5% uranyl acetate solution for 30 s. Finally, the grid was washed by touching to a drop of double distilled water followed by successive immersion in two 10 ml beakers of double distilled water and one beaker of 100% ethyl alcohol. After the sample was dried, it was rotary-shadowed with platinum. This protocol is designed for visualization of complete reaction mixtures, and no attempt was made to remove unreacted material. Although this approach should yield results that provide insight into reaction components, it does lead to samples with a high background of unreacted proteins.

To determine the proportion of the molecules that was either fully or partially coated by RecA E38K K72R protein, or bound only by the SSB protein, in the presence of ATP, 423

molecules from three separate regions of the grid were counted at an identical magnification. A RecA filament was considered gapped if the ssDNA molecule it was bound to had a detectable region of SSB-coated DNA of any size.

Imaging and photography were carried out with a TECNAL G2 12 Twin Electron Microscope (FEI) equipped with a GATAN 890 CCD camera. Digital images of the nucleoprotein filaments were taken at 15 000 $\times$  magnification. Filament lengths were measured using MetaMorph analysis software. Nineteen filaments from wild-type RecA protein, 51 filaments from RecA K72R E38K and 85 filaments from RecA K72R were measured. Each filament was measured three times, and the average length was calculated. To measure the helical pitch of RecA filaments MetaMorph analysis software was used. The distance between 50 individual striations for each condition was measured and averaged. A Cytochrome C method was used to visualize M13mp18 circular ssDNA to check the purity of the DNA. Samples were prepared as described previously (Schnos and Inman, 2000) except that the spreading solutions were made by combining 32  $\mu$ l of 1 M Na<sub>2</sub>CO<sub>3</sub>, 40  $\mu$ l of 0.126 M Na<sub>2</sub>EDTA, 400  $\mu$ l of 37% HCHO, 292  $\mu$ l of 100% formamide and 10  $\mu$ l of 5 M KOH. The final DNA concentration was 0.004  $\mu$ g  $\mu$ l<sup>-1</sup>.

#### Preparation of cells for microscopy

To measure SOS expression in either log phase or UV-treated cells using quantitative fluorescence microscopy, the protocol is as described previously (McCool *et al.*, 2004). For all UV treatment experiments, cultures were irradiated with 5 J m<sup>-2</sup> of UV light at 254 nm using a germicidal lamp. Processing of images and quantification of RFI was as described previously (McCool *et al.*, 2004).

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