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Inactivation of *recG* stimulates the RecF pathway during lesion-induced recombination in *E. coli*

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Abstract

Lesions that transiently block DNA synthesis generate replication intermediates with recombinogenic potential. In order to investigate the mechanisms involved in lesion-induced recombination, we developed an homologous recombination assay involving the transfer of genetic information from a plasmid donor molecule to the *Escherichia coli* chromosome. The replication blocking lesion used in the present assay is formed by covalent binding of the carcinogen *N*-2-acetylaminofluorene to the C8 position of guanine residues (G-AAF adducts). The frequency of recombination events was monitored as a function of the number of lesions present on the donor plasmid. These DNA adducts are found to trigger high levels of homologous recombination events in a dose-dependent manner. Formation of recombinants is entirely RecA-dependent, the RecF and RecBCD sub-pathways accounting for about 2/3 and 1/3, respectively. Inactivation of *recG* stimulates recombinant formation about five-fold. In a *recG* background, the RecF pathway is stimulated about four-fold, while the contribution of the RecBCD pathway remains constant. In addition, in the *recG* strain, a recombination pathway that accounts for about 30% of the recombinants and requires genes that belong to both RecF and RecBCD pathways is revealed.

Keywords: *E. coli*; Recombination; DNA lesions; RecG helicase; RecF pathway; RecBCD pathway

1. Introduction

DNA in bacteria is repeatedly subjected to chemical and physical assaults and repair of the resulting lesions is important if DNA is to be used for transcription or replication. Numerous repair systems have evolved to maintain the integrity of the genome. One of the main systems in *Escherichia coli*, the UvrABC-mediated Nucleotide Excision Repair (NER) system, deals with bulky DNA lesions, such as G-AAF adducts or UV-induced pyrimidine dimers [1]. Despite the presence of the NER system, some lesions are occasionally left unrepaired and their presence in DNA may trigger transient replication fork blockage. At least two strategies can be used by cells to overcome this blockage: the first one, Trans Lesion Synthesis (TLS), involves specialized DNA polymerases that are able to copy past the damaged template with a high risk of fixing mutations; while the second strategy, called Damage Avoidance (DA), uses homologous recombination to restore the blocked replication fork in an error-free manner [2].

Homologous recombination involves exchanges between parent DNA molecules that have identical or nearly identical sequences. This process is achieved by means of pairing complementary single strands from each molecule, thus forming a heteroduplex joint; in *E. coli* this process is mediated by RecA protein. Homologous recombination in wild-type *E. coli* strains is sub-divided in two pathways, both of which depend upon the *recA* gene and are named for the critical genes that distinguish between them, namely *recBCD* and *recF* (for reviews see [3,4]). Blockage of the replisome by DNA lesions may result in the formation of Daughter Strand Gaps (DSG's) that will be further processed via the RecF recombination pathway. Regions of single stranded DNA occur as a consequence of the functional uncoupling of leading and lagging strand synthesis when the replication machinery encounters a blocking lesion in either the

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leading or the lagging strand [5]. In *E. coli*, DSG repair involves RecA promoted homologous pairing and strand exchange with the undamaged sister molecule. Mutations in either *recA*, *recF*, *recO*, *recR*, *recJ*, *ruvA*, *ruvB*, *ruvC*, or *ssb* genes result in a deficiency of the RecF recombination pathway [3,4,6].

The RecBCD pathway is known to process Double Strand Breaks (DSB's) that generally occur when a replication fork encounters a single strand interruption in one of the template strands. In an excision repair-proficient background, DNA replication on template undergoing excision repair of DNA lesions has been shown to cause DSB's [4]. Alternatively, recent data suggest that replication blocks may be converted into DSB's as a consequence of the processing of the Holliday junction that is formed by annealing of the newly synthesized strands during a reaction called fork reversal [7]. The double stranded ends thus formed are processed by the RecBCD enzyme to initiate homologous recombination. Briefly, the enzymatic complex binds specifically to DNA double stranded ends, then simultaneously unwinds and degrades the DNA. Upon encountering a specific sequence named chi site, the polarity of degradation is switched from $3' \rightarrow 5'$ to $5' \rightarrow 3'$, leading to the formation of a $3'$ single stranded DNA-end that allows formation of a RecA nucleoprotein filament. This filament can then invade a homologous molecule, generating a D-loop with an invading $3'$OH extremity that can be used as a primer for DNA synthesis.

The existence of a recombination pathway involving gene products of both the RecBCD and the RecF pathways has been postulated, defining the RecBC–F pathway in which an interplay of RecF and RecJ with proteins associated with the RecBCD pathway [8–13] is suggested.

In vitro, RecG protein has been shown to be a structure-specific DNA helicase [14–16] with propensity to convert blocked synthetic replication forks into Holliday junction structures [17–19]. In vivo experiments suggest that RecG may target intermediates formed upon replication blockage that would be detrimental to the cell if processed by the *priA* gene product. RecG would thus be involved in the conversion of these potentially toxic intermediates into substrates suitable for PriA-dependent loading of the replication apparatus ([20] and for a review, see [21]).

Lesion-induced recombination between the bacterial chromosome and extrachromosomal genetic elements has been used as an indicator of the recombinogenic effects of various mutagens (for reviews see [22,23]). In the present assay, we used a lesion resulting from the covalent binding of the model chemical carcinogen *N*-2-acetylaminofluorene to the C8 position of guanine residues (G-AAF adducts). These lesions have been shown to block DNA synthesis in vitro [24] and in vivo [5], promote slippage at frameshift mutation hot spots [25] and induce homologous recombination in vivo [26,27].

In this paper, we show that G-AAF lesions strongly induce homologous recombination in a dose-dependent manner. Formation of recombinants is entirely RecA+ dependent. RecF and RecBCD pathways contribute to homologous recombination about 70 and 30%, respectively. When RecG is inactivated, RecF-dependent recombination is further stimulated, RecBCD-dependent recombination remains unchanged, and a pathway involving gene products of both the RecBCD and the RecF pathways is revealed.

2. Materials and methods

2.1. Plasmids

Plasmid pCULlacZ::kan (Fig. 1) was constructed starting from pCUL-, a derivative of pUC8 in which both the origin of replication and the *lacZ*′ gene have been inverted [28]. A pBR329 derivative carrying the complete *lacZ* gene was digested with *Eco*RI, filled-in, and digested by *Fsp*I, yielding a large (3 kb) fragment containing the complete *lacZ* gene except for a small segment on the *Fsp*I side. The large *Fsp*I–*Eco*RI restriction fragment, containing *lacZ*, was purified on agarose gel, and was ligated into pCUL- which had been digested by *Sap*I, filled in and digested by *Fsp*I. The result is a 5.6 kb plasmid containing the colE1 origin of replication, the ampicillin resistance marker, the pUC8 polylinker sites and a functional *lacZ* gene. A kanamycin marker cassette, isolated by *Hin*cII digestion of plasmid pUC4K (Pharmacia), was ligated into the *Eco*RV site present within the *lacZ* gene of plasmid pCULlacZ to give rise to a 6.7 kb plasmid referred to as pCULlacZ::kan (Fig. 1).

N-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF) was synthesized as described previously [29]. The reaction of plasmid DNA with *N*-AcO-AAF, and the determination of the extent of modification, has been previously described [25,30].

2.2. AAF-induced recombination as a tool for gene targeting

All the strains of the IP series, (see Table 1) were constructed using AAF-induced recombination as a tool for gene replacement. Construction of IP1 (AB1157 *recG*::*Cm)* is described as an illustration of this new methodology (Fig. 2). The *recG* gene was PCR amplified from AB1157 chromosomal DNA using primers recGU and recGL (see below). The amplified fragment was cloned into the *Hin*cII site of pUC8 giving rise to p-recG. A chloramphenicol cassette was inserted in a unique BsaAI site of p-recG to generate p-recG::Cm2, which was modified with *N*-Aco-AAF to various extents and used to transform AB1157 strain. Colonies selected for chloramphenicol resistance (CmR) were individually toothpicked on ampicillin (Ap) plates. The loss of the incoming plasmid when modified with AAF during the recombination reaction (see results above) allows us to use the detection of Ap sensitive colonies as a first screen towards the identification of the recombinants colonies. Four Ap sensitive colonies were obtained among 71 CmR colonies following transformation with the most heavily modified plasmid (70 AAF/plasmid). The chromosomal DNA of one of these colonies (named IP1) was PCR probed for the presence of the *recG*::*Cm2* allele using recGU2 primer located 400 bp from recGU on the chromosomal DNA but not present on pUC-recG::Cm2 plasmid (see Fig. 2, part I). As expected (Fig. 2, part II), PCR using recGU2/recGL primers gave rise to a DNA fragment of 3544 bp from IP1 chromosomal DNA template confirming that the *recG* interrupted allele is present on the chromosome of IP1 (lane D); no product was obtained when the same primers were used on the plasmid DNA template (lane F). IP1 was then tested for UV resistance (lane 1 on Fig. 2, part III) along with AB1157 (lane 2) and N4452 (lane 3), a derivative of AB1157 carrying Δ*recG265*::*Cm* allele (see Table 1). As both *recG*::*Cm* alleles exhibit the same UV sensitivity, which is higher than the sensitivity of their wild-type counterpart, we considered that the strain IP1 was defective for RecG activity. Strains IP5 and IP7, which both carry the recG::Cm2 allele, were constructed in the same way.

We used a similar strategy to construct strain IP10, defective in the *recF* gene, by first using primers recFU1 and recFL (see below) to clone the *recF* gene and construct the plasmid p-recF, and then subsequently to interrupt the *recF* gene with a tetracycline cassette inserted within the blunt-ended EagI site to create p-recF::Tc. The PCR analysis of IP10 at the chromosome level using recFU2, was essentially performed as described for IP1. To validate the *recF* deficiency of IP10, AAF-induced recombination was measured in IP10 and shown to be comparable to that measured in JC9239 (AB1157 *recF143*, cf. Table 1).

For the construction of strains IP1, IP5, IP7, the *recG* gene was PCR amplified from using primers recGU-5′-CAGCATGTTGCCGGAAGC-3′; and recGL-5′-TGAATCGCATCCGGCAGG-3′. The chromosomal DNA of IP1 was