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Endonuclease-independent DNA mismatch repair processes on the lagging strand

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ABSTRACT

DNA mismatch repair (MMR) pathways coordinate the excision and re-synthesis of newly-replicated DNA if a mismatched base-pair has been identified by protein MutS or MutS homologues (MSHs) after replication. DNA excision during MMR is initiated at single-strand breaks (SSBs) *in vitro*, and several redundant processes have been observed in reconstituted systems which either require a pre-formed SSB in the DNA or require a mismatch-activated nicking endonuclease to introduce a SSB in order to initiate MMR. However, the conditions under which each of these processes may actually occur in living cells have remained obscured by the limitations of current MMR assays. Here we use a novel assay involving chemically-modified oligonucleotide probes to insert targeted DNA 'mismatches' directly into the genome of living bacteria to interrogate their replication-coupled repair processes quantitatively in a strand-, orientation-, and mismatched nucleotide-specific manner. This 'semi-protected oligonucleotide recombination' (SPORE) assay reveals direct evidence in *Escherichia coli* of an efficient endonuclease-independent MMR process on the lagging strand—a mechanism that has long-since been considered for lagging-strand repair but never directly shown until now. We find endonuclease-independent MMR is coordinated asymmetrically with respect to the replicating DNA—directed primarily from 3′- of the mismatch—and that repair coordinated from 3′- of the mismatch is in fact the primary mechanism of lagging-strand MMR. While further work is required to explore and identify the molecular requirements for this alternative endonuclease-independent MMR pathway, these findings made possible using the SPORE assay are the first direct report of this long-suspected mechanism *in vivo*.

1. Introduction

In a mechanism that is highly conserved from bacteria to humans, the DNA mismatch repair (MMR) pathway coordinates the excision and correct re-synthesis of tracts of DNA that contain mismatched nucleotides or insertion/deletion (indel) loops that may have been erroneously generated during replication [1,2]. At the initiation of the conserved mechanism, protein MutS or MutS homologues (MSH) bind to the replication errors, after which a latent nicking endonuclease (MutLα in humans and MutH in *Escherichia coli*, *e.g.*) is authorized to introduce single-strand breaks (SSBs) on the newly-replicated strand [3–7]. These breaks can serve as entry points for DNA helicases and exonucleases to begin excision of the DNA from the SSB through the error in a process termed "long-patch repair" (LPR), since long contiguous stretches of DNA (hundreds of nucleotides) can be digested and resynthesized between the two sites.

While disruption of MutS/MSH or nicking endonuclease activity results in strong 'mutator' phenotypes *in vivo* [4,8–10], nicking endonuclease activity is not necessary to initiate MMR *in vitro* if the mismatched DNA already contains a SSB [3,5,11,12]. Because breaks occur naturally between Okazaki fragments on the lagging strand as it is synthesized discontinuously, it has been proposed that MMR complexes may coordinate MMR on the lagging strand at those breaks in the absence of MMR endonucleases [13–15]. In particular, there are several lines of evidence of an endonuclease-independent mechanism MMR from studies in eukaryotes (reviewed in [13]) to suggest that the 5′- end of the Okazaki fragment may be used to coordinate MMR during the processing of its 5′- RNA primer and Okazaki maturation, but this mechanism has never been directly demonstrated to occur in living cells.

To investigate the possibility of an endonuclease-independent repair on the lagging strand *in vivo*, we require a newly-developed assay, which we call a 'semi-protected oligonucleotide recombination' (SPORE) assay [16], that uniquely allows us to introduce targeted

Abbreviations: MMR, mismatch repair; LPR, long-patch repair; VSP, very-short patch repair; MSH, MutS homologue; SSB, single-strand break; SPORE, semi-protected oligonucleotide recombination

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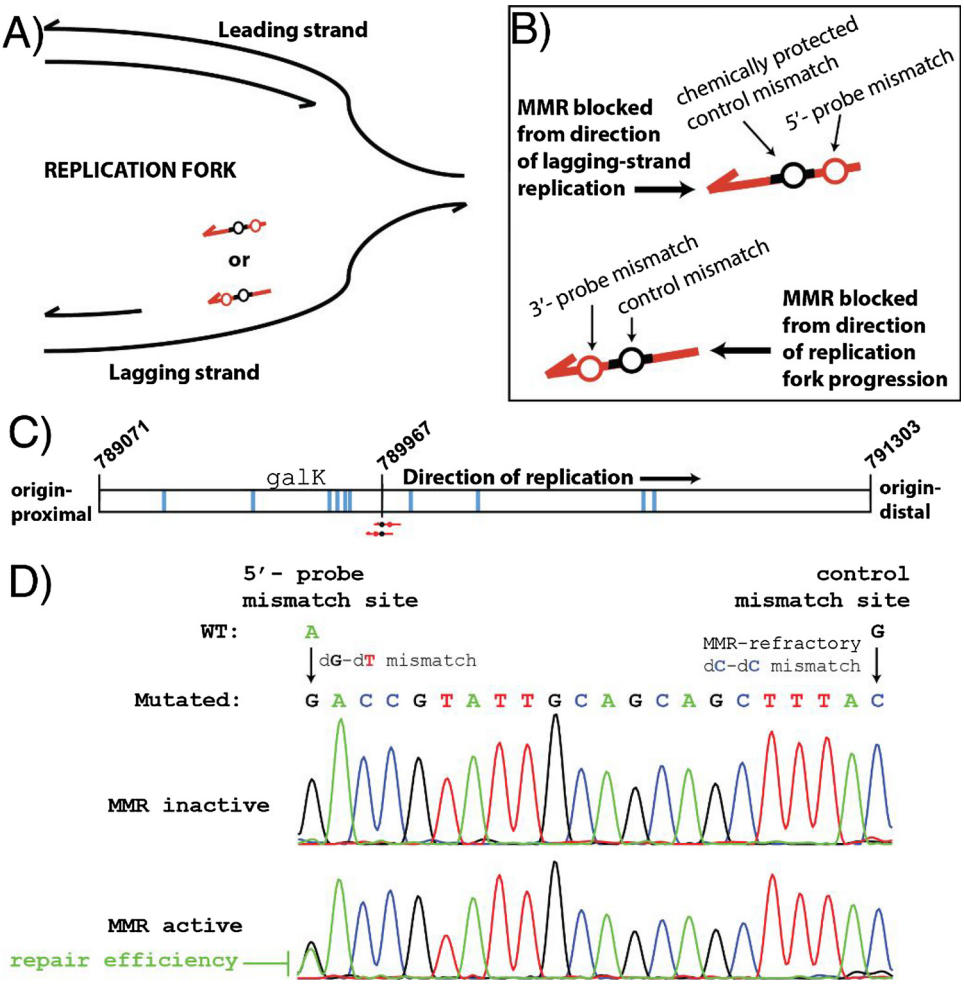

Fig. 1. A "semi-protected oligonucleotide recombination" (SPORE) assay to quantify mismatch repair (MMR) efficiency *in vivo* (see text for details). (A) Chemically modified oligonucleotides that are mostly complementary to the lagging strand template are transfected into *E. coli* and become incorporated into the lagging strand at the replication fork in a process that is proofread by the MMR pathway (Ref. [16] and references therein). (B) The SPORE oligonucleotide (oligo) contains a 'probe' mismatch that introduces a silent mutation in the *galK* gene if left unrepaired, as well as a 'control' mismatch that is weakly recognized and not repaired by *E. coli* MMR (a dC-dC mismatch [17]) but designed to introduce a selectable mutation, by correcting an amber mutation in the galactose kinase gene *galK* [18]. The control mismatch is flanked by multiple phosphorothioate bonds [19] (black) to protect against degradation during MMR and to block long-patch repair that initiates from the opposite side as the probe mismatch. (C) Genomic map of the local d(GATC) sites (blue lines) near the sites complementary to the SPORE oligos. (D) In the SPORE assay, successful transformants are screened for a MMR-refractory control mutation by enrichment in a galactose media. The directional repair efficiency is determined from the sequencing signal at the site of the 'probe' mismatch in a manner that robustly accounts for imperfect transformation efficiencies, non-specific SPORE oligo degradation, and chromatogram noise that may occur (see Supplementary Methods).

'replication errors' directly into genomic DNA at the replication fork and to quantify their repair in a strand-, orientation-, and mismatched nucleotide-specific manner (Fig. 1). By performing this SPORE assay in *E. coli*—a model organism for MMR—and inserting a 'mismatch' onto the replicating lagging strand, here we are able to directly identify an efficient MMR-endonuclease-independent mismatch repair in *mutH*-knockout strains. Endonuclease-independent repair of lagging-strand mismatches is coordinated primarily from 3′- of the mismatch and weakly contributes to repair initiated from the 5′- end of the Okazaki fragment. Repair coordinated from the 5′- end of the error is strongly MMR endonuclease-dependent; however evidence from the SPORE assay suggests that LPR coordinated from the 3′- polarity—in the direction of lagging-strand replication (Fig. 1B) and which does not require MutH—represents the primary mechanism of MMR on the lagging strand. While further work is required to specifically identify the molecular requirements and epigenetic repair signals used to coordinate endonuclease-independent MMR, these findings provide the first direct report in living cells of what appears to be the primary mechanism for lagging-strand MMR.

2. Material and methods

2.1. Materials

E. coli strains SIMD50 (W3110 $galK_{tyr145UAG}$ *ΔlacU169* [λ cI857 *Δ(cro-bioA) (int-cIII < > bet)*]), SIMD90 (SIMD50 *mutS < > cat*), and HME61 (W3110 $galK_{tyr145UAG}$ *ΔlacU169* [λ cI857 *Δ(cro-bioA) (mutH < > amp)*] were obtained as a generous gift of the laboratory of Don Court (National Cancer Institute, Frederick, MD). M9 minimal salts (5x) were obtained from Sigma-Aldrich Co. M63 media (3% KH_2PO_4 w/w, 7% K_2HPO_4 w/w, 2% $(NH_4)SO_4$ w/w, 2% D-galactose w/w, 1 mM $MgSO_4$, 0.5 mg/L $FeSO_4$, 1 mg/L D-biotin) was prepared as previously described [20]. For galactose-selective M63 media, 2% D-galactose w/w was added, and for galactose-selective agar Plates 15 g of agar was added to 1 L of this solution. For 2-deoxygalactose (DOG) agar plates [21], we used 1 L M63 without galactose, 15 g agar, 2 mL glycerol (0.2%; Fischer), and 2 g DOG. Taq 2X MasterMix was obtained by New England Biolabs (Ipswich, MA) and used for all PCR reactions. Gene Pulser(R)/MicroPulser(tm) Electroporation Cuvettes, 0.1 cm gap were obtained from Bio-Rad Laboratories. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) without 5′-phosphorylation (as these ends are readily generated *in vivo* by nucleolytic trimming [22]) and with standard desalting, and they were used without further purification.

2.2. Generation of E. coli strain variants

Generation of strain SIMD50 MutSD835R was described previously [16]. Strains SIMD50 *galK* g.348G > A and SIMD50 *galK* g.158G > C 348G > A, with the first and first two origin-distal d(GATC) sites (Fig. 1C) removed by synonymous mutations, respectively, were generated by Red-mediated oligo-mediated recombination according to the standard protocol [23,24] as previously described [16]. First SIMD50 was transformed *via* electroporation with a phosphorothioated version of oligo *144* from [25] to correct the amber mutation in the *galK* gene (see Table S1, Supplementary Materials), and plated on galactose-selective agar plates immediately after 30 min outgrowth followed by a wash with M9 media. Colonies were picked and sequenced to confirm,