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The dCMP transferase activity of yeast Rev1 is biologically relevant during the bypass of endogenously generated AP sites

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ABSTRACT

The bypass of AP sites in yeast requires the Rev1 protein in addition to the Pol ζ translesion synthesis DNA polymerase. Although Rev1 was originally characterized biochemically as a dCMP transferase during AP-site bypass, the relevance of this activity *in vivo* is unclear. The current study uses highly sensitive frameshift- and nonsense-reversion assays to monitor the bypass of AP sites created when uracil is excised from chromosomal DNA. In the frameshift-reversion assay, an unselected base substitution frequently accompanies the selected mutation, allowing the relative incorporation of each of the four dNMPs opposite endogenously created AP sites to be inferred. Results with this assay suggest that dCMP is the most frequent dNMP inserted opposite uracil-derived AP sites and demonstrate that dCMP insertion absolutely requires the catalytic activity of Rev1. In the complementary nonsense-reversion assay, dCMP insertion likewise depended on the dCMP transferase activity of Rev1. Because dAMP insertion opposite uracil-derived AP sites does not revert the nonsense allele and hence could not be detected, it also was possible to detect low levels of dGMP or dTMP insertion upon loss of Rev1 catalytic activity. These results demonstrate that the catalytic activity of Rev1 is biologically relevant and is required specifically for dCMP insertion during the bypass of endogenous AP sites.

1. Introduction

Apurinic/apyrimidinic (AP) sites are the most common endogenous DNA lesion [1] and are formed by hydrolysis of the glycosidic bond between the base and deoxyribose sugar of a nucleoside. Base loss can occur spontaneously or through enzymatic removal of damaged bases by specialized DNA N-glycosylases of the base excision repair (BER) pathway [2]. Because an unrepaired AP site is a potent block to replicative DNA polymerases, bypass is critical for maintaining genetic integrity and completing genome duplication. Translesion synthesis (TLS) is one of two general tolerance/bypass pathways that circumvent the replication blocks caused by AP sites, as well as a variety of other DNA lesions (for recent reviews, see [3,4]). TLS can be divided into two steps: an insertion step in which a nucleotide is incorporated opposite the lesion and an extension step in which the unpaired primer-template terminus is extended. While the overall efficiency of TLS is determined by the extension step, whether or not the bypass event is mutagenic depends on the nucleotide inserted opposite a non-instructive AP site. Under conditions where AP-site repair is compromised, mutagenesis increases in a TLS-dependent manner [5].

The yeast *Saccharomyces cerevisiae* contains three, highly conserved TLS polymerases that potentially can participate in AP-site bypass: Pol η, Pol ζ and Rev1. Pol η is a Y-family DNA polymerase whose loss results in a variant form of the human cancer-predisposition syndrome Xeroderma Pigmentosum, which is characterized by extreme sensitivity to UV light [6,7]. In yeast, Pol η is encoded by the *RAD30* gene and its absence is associated with enhanced UV-induced sensitivity and mutagenesis [8]. Pol ζ is a B-family DNA polymerase comprised of two subunits: the Rev3 catalytic and Rev7 accessory proteins (reviewed in [9]). Pol ζ is required for most induced, as well as a substantial fraction of spontaneous, mutagenesis in yeast and is essential in mammalian cells [10,11]. While it is capable of independently bypassing lesions *in vitro*, the primary role of Pol ζ *in vivo* is thought to reflect its unique ability to extend an unpaired primer-template terminus [12,13]. Finally, Rev1 is a Y-family DNA polymerase that is required for Pol ζ-dependent mutagenesis. It was initially described biochemically as a deoxycytidyl (dCMP) transferase, specifically inserting cytosine opposite template lesions [14]. In addition to the catalytic activity, an N-terminal BRCT domain is important for DNA binding [15] and a C-terminal scaffolding domain interacts with Rev3 and Rev7 [16,17].

In contrast to the general biological significance of the BRCT and C-terminal domains of Rev1, the relevance of the dCMP

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transferase activity *in vivo* appears to be lesion-specific. This activity, for example, is not required for survival or Rev1-dependent mutagenesis following UV irradiation, but is important for surviving 4-nitroquinoline-1-oxide (4-NQO)-induced damage [18]. With regard to AP-site bypass, there are conflicting data concerning the relevance of the Rev1 dCMP transferase activity. Early experiments examined genomic mutations induced by the base-alkylating agent methyl methane sulfonate (MMS), which generates AP sites primarily at purines. Most MMS-induced mutations were GC > TA transversions, a mutation pattern inconsistent with dCMP insertion opposite AP sites and shown not to require Rev1 catalytic activity [13]. While these data were used to argue for a dAMP insertion bias during Rev1-dependent bypass of guanine-derived AP sites, it should be noted that dCMP insertion would not have been mutagenic and hence could not have been detected in these experiments (see [19]). A study of mutagenesis associated with expression of T- or C-specific glycosylases reported Rev1-dependent mutation patterns consistent with dCMP insertion opposite AP sites [5], as did a study examining the mutagenic consequence of uracil-derived AP sites [20]. Neither of these studies, however, could have detected non-mutagenic dAMP insertion opposite thymine-derived AP sites and neither examined the relevance of the protein's catalytic activity. As an alternative to studying the bypass of physiologically produced AP sites, oligonucleotides or gapped plasmids containing a single, defined AP site have been used in transformation-based studies. These analyses have reported preferential insertion of dCMP opposite an engineered AP site [19,21–24], and have implicated the catalytic activity of Rev1 during bypass [24].

We previously described very sensitive frameshift- and nonsense-reversion assays that monitor the bypass of AP sites produced when uracil is excised from highly transcribed DNA [25,26]. Because uracil specifically replaces thymine in these assays, the base substitution pattern at AT base pairs provides a read-out of nucleotides inserted opposite thymine-derived AP sites. In contrast to previous assays, where non-mutagenic AP-site bypass via dAMP insertion could not be detected, the frequent occurrence of non-selected base substitutions in the frameshift-reversion assay allows the relative insertion efficiencies of all four dNMPs to be inferred. The data reported here suggest that dCMP is the predominant nucleotide inserted opposite uracil-derived AP sites in this system and confirm that dCMP insertion, but not that of alternative dNMPs, requires the catalytic activity of Rev1. Accompanying shifts in mutation spectra suggest that back-up activities compensate for dCMP transferase loss by inserting primarily dAMP. When considered together with the nonsense-reversion assay, results indicate that the insertion preference opposite AP sites in yeast is dCMP > dAMP ≫ dGMP ∼ dTMP. Finally, we extend the importance of the BRCT DNA-binding and C-terminal scaffold domains of Rev1 to include the bypass of AP sites.

2. Materials and methods

2.1. Media and growth conditions

All growth of yeast strains was at 30 °C. Cells were grown non-selectively in YEP medium (1% yeast extract, 2% peptone; 1.5% agar for plates) supplemented with 2% dextrose (YEPD) or 2% each of glycerol and ethanol (YEPGE). It should be noted that under these growth conditions, *pTET* (whose repression requires doxycycline) is maximally activated. Selective growth was on synthetic, 2% dextrose (SD) medium supplemented with all but the one relevant amino acid or base. The presence of a hygromycin-resistance marker was selected by plating transformants on YEPD plates supplemented with 300 μg/ml hygromycin.

2.2. Strain constructions

All yeast strains were derived from YPH45 (*MATα ura3-52 ade2-101$_{oc}$ trp1Δ1*). Wild-type (WT) strains containing the *pTET-lys2ΔA746* [27] or *pTET-lys2-TAA* [26] allele near *ARS306* on chromosome III were previously described. The *rev1-BRCT* (*rev1-G192A,G193A*), *rev1-AA* (*rev1-D467A,E468A*) or *rev1-CDEL* (*rev1-Y914Stop,L915Stop*) allele was introduced into these strains by two-step replacement using plasmids pSR836, pSR838 or pSR986, respectively. To construct these plasmids, *REV1* sequences were subcloned into the *URA3*-marked integrating vector pRS306 [28]. The desired sequence changes were then introduced using the Quik-Change Site-Directed Mutagenesis kit (Stratagene; see Table S1 for mutagenic oligonucleotides used). Deletion strains were derived by one-step gene disruption using PCR-generated cassettes containing an appropriate selectable marker flanked by ∼60 bp of homology bordering the coding region of the relevant gene [29]. When appropriate, the marker gene was subsequently deleted using a Cre/*loxP*-mediated procedure [30]. A complete list of strains is provided in Table S2.

2.3. Mutation rates and spectra

For mutation rate determinations, 1-ml YEPGE cultures were inoculated with 250,000 cells from an overnight culture grown in the same medium. After 3 days growth, appropriate dilutions were plated on SD-Lys and YEPD plates to determine the number of Lys$^+$ revertants and the total number of cells, respectively, in each culture. Mutation rates were calculated using the method of the median and the corresponding 95% confidence intervals were calculated as described previously [31]. Ten to 24 cultures were used for each rate determination.

To isolate independent Lys$^+$ revertants, 1-ml YEPGE cultures were started from independent colonies. Following 2–3 days growth, an appropriate fraction of each culture was plated on SD-Lys medium. A single revertant from each culture was purified and genomic DNA was prepared using an enzymatic lysis method modified for a 96-well format. The relevant region of *LYS2* was amplified using primers LYSWINDF (5′-GCCTCATGATAGTTTTTCTAACAAATACG) and LYSWINDR (5′-CCCATCACACATACCATCAAATCCAC), and sequenced by the Duke University DNA Analysis Facility.

3. Results

3.1. AP sites in the pTET-lys2ΔA746 assay are produced by Ung1 and require Pol ζ for bypass

The *lys2ΔA746* allele contains a 1-bp deletion in a nonessential region of the *LYS2* coding sequence and reverts by any net +1 frameshift mutation that restores the correct reading frame of the gene [32]. The Pol ζ TLS polymerase generates a distinctive mutation signature in this assay: "complex" 1-bp insertions that additionally contain a nearby, unselected base substitution. Such complex events are proportionally enhanced in repair-defective backgrounds, indicating that most are produced during the Pol ζ-dependent bypass of unrepaired lesions [33]. Because changing the identity of the base pair most often mutated at complex hotspots eliminates the selected frameshift, we have proposed that complex mutations are produced by a misincorporation-slippage mechanism [32]. In this mechanism, misinsertion opposite a discrete lesion precedes and may facilitate slippage in a short homopolymer run that follows, thereby generating the selected frameshift mutation. A key feature of misincorporation-slippage is that the position of the base substitution at complex-mutation hotspots marks the