Mutagenesis in eukaryotes dependent on DNA polymerase zeta and Rev1p

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DNA polymerase zeta (Pol ζ) and Rev1p carry out translesion replication in budding yeast, *Saccharomyces cerevisiae*, and are jointly responsible for almost all base pair substitution and frameshift mutations induced by DNA damage in this organism. In addition, Pol ζ is responsible for the majority of spontaneous mutations in yeast and has been proposed as the enzyme responsible for somatic hypermutability. Pol ζ , a non-processive enzyme that lacks a 3' to 5' exonuclease proofreading activity, is composed of Rev3p, the catalytic subunit, and a second subunit encoded by *REV7*. In keeping with its role, extension by Pol ζ is relatively tolerant of abnormal DNA structure at the primer terminus and is much more capable of extension from terminal mismatches than yeast DNA polymerase alpha (Pol α). Rev1p is a bifunctional enzyme that possesses a deoxycytidyl transferase activity that incorporates deoxycytidyl opposite abasic sites in the template and a second, at present poorly defined, activity that is required for the bypass of a variety of lesions as well as abasic sites. Human homologues of the yeast *REV1* and *REV3* have been identified and, based on the phenotype of cells producing antisense RNA to one or other of these genes, their products appear also to be employed in translesion replication and spontaneous mutagenesis. We suggest that Pol ζ is best regarded as a replication enzyme, albeit one that is used only intermittently, that promotes extension at forks the progress of which is blocked for any reason, whether the presence of an unedited terminal mismatch or unrepaired DNA lesion.

Keywords: DNA polymerase zeta; mutagenesis; Rev1p; yeast; human cells

1. INTRODUCTION

In budding yeast *Saccharomyces cerevisiae*, the activities of DNA polymerase zeta (Pol ζ) and Rev1p are responsible for a high proportion of the base pair substitutions and small frameshift mutations induced by unrepaired DNA damage. Their role in translesion replication, the process that generates these mutations, is currently the major focus in studies of these enzymes (reviewed in Lawrence 1994; Lawrence & Hinkle 1996; Lawrence *et al.* 2000). Unrepaired damage in the DNA template often blocks or severely impedes chromosome replication, a consequence that is potentially lethal. Translesion replication, one of the strategies used to overcome this inhibition, employs specialized DNA polymerases to extend the nascent strand at blocked forks. In most cases in yeast, such extension is likely to be performed by Pol ζ , though DNA polymerase eta (Pol η), encoded by *RAD30* (McDonald *et al.* 1997), is probably used with some lesions, such as a bipyrimidine cyclobutane dimer (Johnson *et al.* 1999). While translesion replication using Pol ζ and Rev1p can promote viability, it nevertheless carries the penalty of

reduced fidelity, the combined result of using a less accurate enzyme on a distorted template; the process is therefore appreciably mutagenic.

Although its role in translesion replication has received the most attention, the activity of Pol ζ is also responsible for the majority of spontaneous mutations in yeast, most of which are probably not induced by unrepaired DNA damage, suggesting a more general role for the enzyme. Moreover, and of particular relevance to the topic of this discussion meeting, it has been speculated that a human homologue of the yeast gene might be concerned with hypermutation in antibody genes (Diaz *et al.* 1999). However, a number of other candidate DNA polymerases that might be responsible for this phenomenon have been discovered following this suggestion (reviewed in Woodgate 1999; Gerlach *et al.* 1999), and the identity of the enzyme or enzymes that account for somatic hypermutation will no doubt be resolved by studies of this process in cells deficient for one of the various candidate activities. In the meantime, the aim of this paper is to review the role of yeast Pol ζ and Rev1p in translesion replication and DNA-damage-induced mutagenesis, to examine the reasons for the involvement of Pol ζ in spontaneous mutagenesis, and to discuss the properties of this enzyme, particularly those that might suit it for a possible

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role in somatic hypermutation. Finally, we also review evidence suggesting the presence of similar activities in humans and discuss the biological roles that these enzymes might play in eukaryotic cells.

2. MUTAGENESIS IN YEAST

Genetic studies indicate that DNA-damage-induced mutagenesis in yeast depends on the functions of at least three genes, *REV1*, *REV3* and *REV7* (Lemontt 1971; Lawrence *et al.* 1985a), none of which is essential for viability. As discussed in §4, *REV3* encodes the catalytic subunit of DNA Pol ζ (Morrison *et al.* 1989; Nelson *et al.* 1996a), a distant relative of DNA polymerase delta (Pol δ) (Braithwaite & Ito 1993), and *REV7* encodes another subunit of this enzyme (Torpey *et al.* 1994; Nelson *et al.* 1996a). *REV1* encodes a protein that possesses a deoxycytidyl transferase activity used in replication past an abasic site, but Rev1p function is also required for mutagenesis induced by other lesions—the nature of this, its principal enzymatic activity, is still unclear. Ultraviolet (UV)-induced mutagenesis is substantially reduced in *rev* mutant strains, and such strains are also mildly hypersensitive to the lethal effect of the radiation, indicating that the normal functions of the *REV* genes contribute a small component to the ability of yeast cells to tolerate DNA damage. UV-induced reversion frequencies of a set of 13 defined test alleles in *REV+* and null *rev* mutant strains indicates that more than 95% of the base pair substitution mutations induced by this mutagen depend on *REV1* and *REV3* functions (Lawrence & Christensen 1978, 1979), while a smaller set of data suggests that the phenotype of *REV7* is like that of *REV3* (Lawrence *et al.* 1985a,c). Similar experiments with a set of eight frameshift test alleles indicate that more than 90% of this type of mutation depend on *REV3* activity, and the same is true of *REV1* for frameshift induction at some genetic sites, although at most the dependence is much less or non-existent (Lawrence & Christensen 1978, 1979; Lawrence *et al.* 1984). Mutagenesis induced by a variety of other mutagens also depends on *REV* gene products (McKee & Lawrence 1979; Lawrence *et al.* 1985c), suggesting that Pol ζ is responsible for replication past a broad range of DNA lesions. It is possible that the functions of other genes, such as *REV6* and *NGM2*, are also employed in translesion replication by Pol ζ (Lawrence *et al.* 1985b; Nisson & Lawrence 1986).

In addition to its role in DNA-damage-induced mutagenesis, Pol ζ is also responsible for the majority of spontaneous mutations in yeast. Indeed, Von Borstel and colleagues (Quah *et al.* 1980) isolated a *rev3* mutation in a genetic screen for antimutators. Based on their data, and that of Kunz and colleagues (Roche *et al.* 1994), between 50 and 75% of spontaneous mutations appear to be *REV3* dependent. *REV3* is also responsible for the increased frequency of spontaneous mutations resulting from transcription (Datta & Jinks-Robertson 1995), recombination (Holbeck & Strathern 1995), the overexpression of 3-methyl adenine glycosylase (Glassner *et al.* 1998) and for a substantial fraction of the enhanced spontaneous mutagenesis that occurs in an excision-defective strain (Roche *et al.* 1994).

3. PROPERTIES OF YEAST DNA POLYMERASE ZETA AND Rev1p

The enzymatic properties of yeast Pol ζ have been investigated, with the aim of exploring the kinds of qualities that might differentiate such a specialized enzyme concerned with translesion replication and a replicase responsible for normal chromosome replication. Since it has not yet proved possible to purify Pol ζ from normal yeast strains, its properties were examined using an enzyme produced by co-expression of a glutathione-S-transferase-Rev3 fusion protein (Gst-Rev3p) and normal Rev7p in yeast, and purifying the complex by affinity chromatography on glutathione-Sepharose (Nelson *et al.* 1996a). Rev7p associates strongly with Rev3p giving a complex that is 20–30-fold more active than Gst-Rev3p alone, but the Rev7p subunit has no other known enzymatic activity. A plasmid expressing Gst-Rev3p fully complemented the mutant phenotype of a *rev3* Δ strain, and cleavage of the Gst moiety from the fusion protein only increased its activity by about twofold, indicating that the fusion protein retains a substantial level of activity *in vivo*. However, in view of its method of production, we cannot rule out the possibility that this enzyme lacks other subunits that may be present in the natural enzyme occurring in normal yeast strains in the absence of overproducing plasmids.

Pol ζ exhibits a number of properties that might be expected to be appropriate in an enzyme concerned with translesion replication. It does not appear to possess 3' to 5' exonuclease proofreading activity and is a poorly processive enzyme, dissociating from half of the template molecules after adding only ≥ 3 nucleotides under conditions in which extension mostly resulted from a single polymerase binding event. However, in some instances up to ca. 200 nucleotides can be added. Of particular significance to its role in translesion replication, Pol ζ appears to be much more capable than the major replicases in extending nascent chains despite abnormal structure in the template and at the primer terminus. For example, Pol ζ extended the primer past a thymine–thymine *cis-syn* cyclobutane dimer with ca. 10% efficiency, under conditions in which principally single binding events appear to have occurred. In contrast, with yeast Pol α the efficiency was less than 1% in these conditions (Nelson *et al.* 1996a). Even though it is now known that Pol ζ is not primarily responsible for dimer bypass *in vivo*, such a result nevertheless indicates that Pol ζ possesses a superior capability for tolerating template distortion. Furthermore, Pol ζ extends a terminally mismatched primer more efficiently than Pol α on damage-free templates, again indicating a reduced requirement for normal duplex structure at the primer terminus (Lawrence *et al.* 2000). Apparent extension efficiencies, $V_{\max}/K_m$ (mismatch) normalized to $V_{\max}/K_m$ (match) were determined by Goodman's method (Boosalis *et al.* 1987) for all paired or mispaired combinations of nucleotides at the primer terminus and in the template. In one set of primer–template combinations, the next nucleotide beyond the primer terminus was dA, whereas in the second set it was dG, and extension was driven by increasing concentrations of dTTP or dCTP, respectively. Using Pol ζ , apparent extension efficiencies were from

twofold to more than 1000-fold higher than those for Pol α for any given mismatch. Actual efficiencies ranged from 0.3 to 54% with Pol ζ , compared with 0.002 to 3% for Pol α . Clearly Pol ζ has an unusually high capability for extending terminally mismatched primers, suggesting that it is less accurate than the major replicases, not only by virtue of a lack of proofreading but also because it lacks some of the stringent requirements for correct primer–template structure.

In addition to Pol ζ , translesion replication in yeast also requires the function of Revlp, a protein that possesses at least two enzymatic activities. One of these is a deoxycytidyl (dC) transferase activity that inserts dCMP opposite an abasic site in the template and, to a much lesser extent, opposite template guanine and adenine nucleotides (Nelson *et al.* 1996b). Revlp is therefore also a DNA polymerase, albeit of a highly unusual kind that only incorporates dCMP—it possesses no detectable ability to incorporate any of the other nucleotides. This activity is responsible for the preferential insertion of dCMP opposite abasic sites *in vivo* (Gibbs & Lawrence 1995), a result unlike that in *E. coli* where preferential insertion of dAMP is observed (Lawrence *et al.* 1990). Revlp strongly stimulates replication past an abasic site by Pol ζ *in vitro* (Nelson *et al.* 1996b) and the same is probably true *in vivo*. Even though the Revlp dCMP transferase activity has received the greatest attention, no doubt because other members of the Rev1/UmuC/DinD/Rad30 superfamily have subsequently been found to be DNA polymerases (reviewed in Woodgate 1999; Gerlach *et al.* 1999), this activity is unlikely to constitute the main Revlp function, as required for the bypass of most lesions other than abasic sites, and the protein appears to possess a second activity the enzymatic basis of which is at present poorly understood.

The evidence supporting this conclusion was obtained by measuring lesion bypass frequencies in *REV+* or *rev* mutant yeast strains transformed with duplex DNA plasmids that carried either a thymine–thymine (6-4) UV photoadduct or abasic site at a specific site within a 28 nucleotide single-stranded region (Nelson *et al.* 2000). Placing the lesion in such a region makes it immune to excision repair and simplifies the analysis of DNA sequence in replicated plasmid products. Two observations indicate the existence of a second Revlp function. First, bypass of a thymine–thymine (6-4) UV photoadduct (T–T (6-4) adduct) was found to be Revlp dependent, even though it almost never entails the incorporation of dCMP opposite the lesion, the hallmark of the dC transferase activity. The presence of the T–T (6-4) adduct in the plasmid strongly inhibits the formation of transformants, presumably because the lesion blocks chain elongation, gap filling and thus plasmid replication; even in a *REV+* strain only 17% of the plasmids were completely replicated. This replication was nevertheless largely dependent on Revlp, since the fraction of fully replicated plasmids fell to 1% in the *rev1Δ* strain. However, incorporation of dCMP opposite one or the other of the thymine nucleotides occurred in only one of 72 *REV+* transformants analysed, suggesting that the transferase activity was at best rarely employed during bypass of this lesion. A low frequency of dCMP insertion opposite a T–T (6-4) adduct was also seen in a previous study (Gibbs *et al.*

1995). Bypass of the T–T (6-4) adduct was also much reduced in the *rev1-1* (G193R) and *rev3Δ* strains.

In addition to this evidence, the existence of a second Revlp function is also supported by the observation (Nelson *et al.* 2000) that bypass of an abasic site is much reduced in a *rev1-1* mutant, even though the Rev1-1 mutant protein retains a substantial fraction of the normal level of dC transferase activity. Like the T–T (6-4) adduct, an abasic site strongly blocks fork progression and only 23% of plasmid molecules were fully replicated in the *REV+* strain. Such bypass was also found to be Revlp dependent, with only 0.4% of the plasmid molecules fully replicated in a *rev1Δ* strain, but such a result might reflect loss of the dC transferase activity, loss of a second activity or loss of both of these activities. Results from the *rev1-1* mutant show, however, that even if the dC transferase is necessary for efficient bypass of an abasic site, it is not sufficient. Analysis of the G193R Rev1-1 mutant protein *in vitro* shows that it retains *ca.* 60% of the wild-type dC transferase activity (Nelson *et al.* 2000). Significantly, however, bypass of the abasic site occurred in only 2.3% of the plasmids transformed into the *rev1-1* mutant strain, i.e. only 10% of the wild-type fraction, indicating that a second *REV1*-encoded activity is required. dCMP is incorporated opposite the abasic site in roughly the same proportion of plasmids in the *rev1-1* mutant (*ca.* 75%) as seen in the *REV+* strain (90%), showing that the Rev1-1 dC transferase is active *in vivo*. dCMP incorporation was entirely absent in the *rev1Δ* mutant, however. It therefore appears that bypass of an abasic site requires both the transferase and a second Revlp function. Finally, similar experiments with plasmids carrying a T–T cyclobutane dimer showed that bypass of this lesion required neither Revlp nor Rev3p function; bypass of this lesion is presumably carried out by another DNA polymerase, most probably by Pol η (Johnson *et al.* 1999). Conversely, the results described above also show that Pol η is not capable of replication past either a T–T (6-4) adduct or abasic site *in vivo*.

The enzymatic nature of the second Revlp function is not yet understood. Although both Pol ζ and Revlp appear to be employed in most translesion replication events, no evidence for their association can be found when Revlp, Gst-Rev3p and Rev7p are simultaneously overproduced in yeast, followed by glutathione-Sepharose chromatography of cleared cell extracts. However, association between Pol ζ and Revlp may require other subunits, found in the native enzyme but not in the Rev3p–Rev7p complex used. Alternatively, Pol ζ and Revlp may be recruited to a stalled replication fork separately, resulting in only a transient association. Whether the association is permanent or transient, Revlp is likely to promote nucleotide insertion and primer extension by Pol ζ since it is more concerned with base substitution than with misalignment and frameshift mutagenesis.

4. PROPERTIES OF HUMAN Rev3p AND Rev1p

Inspection of the databases suggests that homologues of the yeast *REV* genes are found in almost all eukaryotes, including mammals and specifically humans. Homologues of yeast *REV1* and *REV3* have been jointly found in humans, the mouse, *Arabidopsis thaliana*, *Drosophila*

melanogaster (J. Eeken, P. van Sloun and R. Romeijn, personal communication), and *Schizosaccharomyces pombe*. In addition, *Leishmania major* has also been found to possess a *REV3* gene, though not as yet a *REV1* locus. *Caenorhabditis elegans* possesses a *REV1* gene but not, apparently, a *REV3* gene and since almost the entire genome of this organism has been sequenced, it appears to lack an enzyme of the Pol ζ type. Translesion replication in the worm may therefore be accomplished by Revlp in combination with a different DNA polymerase such as Pol δ , the enzyme to which Pol ζ is most closely related. At the present, putative Rev7p homologues have been discovered only in *Drosophila* and humans (Murakumo *et al.* 2000), perhaps because of poor conservation of sequence.

Because of their possible clinical significance, we have used sequence information from yeast Revlp and Rev3p to isolate cDNA clones that encode the human counterparts (Gibbs *et al.* 1998, 2000). The human *REV3* gene encodes an expected protein of 353 kD, about twice the mass of yeast *REV3* (173 kD), with regions of significant identity principally at the amino terminus and at the carboxy terminus. The carboxy-proximal polymerase domain is more closely related to that in yeast Rev3p than to its counterparts in the human replicases and is clearly of the Pol ζ type. As with yeast *REV3*, several features of the human gene predict that cellular levels of hRev3p are low. About 40% of human *REV3* mRNA contains a 128 nucleotide insertion, probably the result of alternate splicing, at position +139/140 of the open reading frame, which introduces a stop codon. *hREV3* also possesses several upstream out-of-frame ATG codons, which are likely to reduce the translational efficiency of the *hREV3* open reading frame. The *hREV1* gene encodes a 138 kD protein with significant identity to the 112 kD yeast protein over the amino two-thirds of the protein (Gibbs *et al.* 2000). Comparisons between the Revlp from a variety of species identifies nine sequence motifs, the first of which lies in a region with weak homology to the BRCT domain of the *BRCA1* gene, which may be concerned with protein–protein interactions. This region contains G76, the residue homologous to G193 in yeast and the site of the G193R mutation found in the Revl-1 mutant protein, which substantially lacks the second function of yeast Revlp but retains much of the dC transferase activity. The highly conserved DXD and DE sequences found in motifs III and VI, respectively, appear to be concerned with polymerase function (Johnson *et al.* 1999; Gerlach *et al.* 1999). As with the *REV3* locus, the human *REV1* gene contains an upstream out-of-frame ATG in a good context, again suggesting that cellular levels of Revlp may be low. Essentially identical *REV3* and *REV1* clones have also been isolated by other groups (Morelli *et al.* 1998; Lin *et al.* 1999a,b).

In addition to possessing significant homology to the yeast proteins, the human Rev proteins also appear to perform similar functions to their fungal counterparts. To investigate these functions, we transformed cells derived from MSU-1.2—an infinite life span, non-tumorigenic, near diploid, human fibroblast cell line—with plasmids expressing high levels of either a *REV1* or *REV3* antisense RNA fragment and assayed the cells for UV-induced mutagenicity and cytotoxicity (Gibbs *et al.* 1998, 2000). The frequency of 6-thioguanine resistant mutants

induced by UV was much reduced in cells expressing either of the antisense RNAs, compared with those seen in control cells in which antisense RNA was absent, suggesting that the functions of hRevlp and hRev3p, like those of their yeast counterparts, are concerned with translesion replication. The similarity with yeast also extends to spontaneous mutagenesis; expression of *REV3* antisense RNA also reduces spontaneous mutation frequencies in an *msh6* mutant strain (Z. Li and V. M. Maher, unpublished data). Reinforcing the similarity between the yeast and human proteins, Zigang Wang and co-workers have additionally shown that hRevlp possesses a dC transferase activity (Lin *et al.* 1999b). However, whether this activity is used *in vivo* is unclear; although dCMP is predominantly inserted opposite an abasic site *in vivo* in yeast (Gibbs & Lawrence 1995), incorporation of dAMP is the predominant event in monkey cells (Takeshita & Eisenberg 1994). A further difference from yeast concerns cytotoxicity; while yeast *rev* mutants exhibit modest hypersensitivity to killing by UV, human cells expressing *REV* antisense RNA showed no increase in sensitivity to the cytotoxic effects of the UV radiation, perhaps because the unrepaired damage was channelled into another repair or tolerance pathway.

5. BIOLOGICAL FUNCTIONS OF DNA POLYMERASE ZETA

In light of the information reviewed above, it seems likely that almost all eukaryotes, and perhaps all mammals, possess a translesion replication pathway that employs Pol ζ and Revlp. At the same time, it appears that Pol ζ is not only concerned with translesion replication and the tolerance of unrepaired DNA damage originating from exogenous mutagens but is also involved in processes that generate spontaneous mutations; yeast *rev3* mutants are strong antimutator strains (Quah *et al.* 1980; Roche *et al.* 1994; Datta & Jinks-Robertson 1995; Holbeck & Strathern 1995) and spontaneous mutation frequencies are reduced in *msh6* mutant human cells producing high levels of *REV3* antisense RNA (Z. Li and V. M. Maher, unpublished data). Although replication past spontaneously arising lesions may generate some of these mutations, it is unlikely that sufficient damage occurs, or fails to be repaired, to explain more than a small fraction of them.

These observations raise two questions: first, what is the biological function of Pol ζ and second, why do the multicellular eukaryotes make use of translesion replication? With respect to the first question, concerning the biological function of Pol ζ , this enzyme is perhaps best thought of as a polymerase engaged principally in normal replication, albeit only intermittently. In this context, its function is to restart replication at forks that are stalled for any reason, whether by virtue of a terminal mismatch, hairpin structure, or refractory DNA sequence, as well as by virtue of unrepaired DNA damage. Of these, the terminal mismatch and template damage are likely to have the greatest propensity to generate mutations, but other causes of stalling may also result in replication errors because Pol ζ lacks proofreading. If this is correct, the majority of spontaneous mutations produced by Pol ζ may be generated when this enzyme is called upon to

restart replication at forks where extension is blocked by the presence of an unedited terminal mismatch. As discussed above, Pol ζ can perform such extension from mismatches efficiently. A similar suggestion was made by Roel Schaaper and co-workers (Fijalkowska *et al.* 1997) to explain the enhanced spontaneous mutagenesis resulting from derepression of the SOS regulon in *E. coli*. Such a viewpoint is much more in keeping with the changing perspective on DNA repair and damage tolerance systems that has been emerging over the last few years. Although in an earlier period repair and tolerance processes were investigated in an isolated context, perhaps because experiments often involved unnaturally high doses of mutagen, it is now realized that all DNA transactions form a seamless web that encompasses various modes of replication and recombination as well as repair, and that intersects with the central activities of the cell, such as transcription.

A second question raised by the information reviewed above concerns the reasons for the presence of a translesion replication pathway in multicellular eukaryotes. In the single-celled eukaryotes, such a process is clearly useful; the advantage of completing replication is likely to outweigh the disadvantage of possibly generating mutations. In mammalian cells, which have the additional option of apoptosis, the balance in favour of translesion replication is at first sight less clear. However, use of translesion replication in mammals may in part be because the adverse consequences of lesion bypass are not necessarily high. The error rate of translesion replication is usually quite low and even if mutations are produced, their occurrence in non-informational DNA, or the formation of synonymous changes in coding sequences, and the tolerance of many proteins towards residue substitutions, are all factors that diminish the adverse effects of translesion replication. At the same time, the occurrence of unscheduled apoptosis may also itself carry risks, particularly in early development, and it is perhaps significant that a *REV3* knockout in the mouse is lethal (P. P. H. van Sloun, R. J. Romeijn, N. de Wind and J. C. J. Eeken, personal communication), though the reasons for this are not yet known. At first sight, the observation that cultured human fibroblasts apparently containing very little Rev3p or Revlp are fully viable and grow at a normal rate (Gibbs *et al.* 1998, 2000) appears to be at variance with the supposed risks, but such cells are in fact relatively resistant to the induction of apoptosis. Interrelationships of the regulation of Pol ζ , apoptosis, tumour suppression and cell cycle control is perhaps suggested by the observation that the candidate tumour suppressor p33^{ING1} strongly associates with Rev3p in a two-hybrid assay (R. Murante, unpublished data). p33^{ING1} has been reported to negatively regulate cell proliferation and apoptosis to facilitate transcription from the p21/WAF1 promoter and to physically associate with p53, indicating that it is part of the p53 signalling pathway (Garkavtsev *et al.* 1998).

This work was supported by US Public Health Service grants GM21858 to C.W.L. and CA56796 and ES09822 to V.M.M., from the National Institutes of Health.

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