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Mutagenesis dependent upon the combination of activation-induced deaminase expression and a double-strand break

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Abstract

We explored DNA metabolic events potentially relevant to somatic hypermutation (SHM) of immunoglobulin genes using a yeast model system. Double-strand break (DSB) formation has been discussed as a possible component of the SHM process during immunoglobulin gene maturation. Yet, possible mechanisms linking DSB formation with mutagenesis have not been well understood. In the present study, a linkage between mutagenesis in a reporter gene and a double-strand break at a distal site was examined as a function of activation-induced deaminase (AID) expression. Induction of the DSB was found to be associated with mutagenesis in a genomic marker gene located 7kb upstream of the break site: mutagenesis was strongest with the combination of AID expression and DSB induction. The mutation spectrum of this DSB and AID-mediated mutagenesis was characteristic of replicative bypass of uracil in one strand and was dependent on expression of DNA polymerase delta (Pol δ). These results in a yeast model system illustrate that the combination of DSB induction and AID expression could be associated with mutagenesis observed in SHM. Implications of these findings for SHM of immunoglobulin genes in human B cells are discussed.

Keywords

Activation-induced deaminase; AID; base excision repair; somatic hypermutation; double-strand breaks; mutagenesis

1. Introduction

In the hypothesis proposed by Brenner and Milstein for somatic hypermutation (SHM) of immunoglobulin (Ig) genes in adaptive immunity, the process was divided into two stages: targeted DNA cleavage in specific regions of the Ig genes; and error-prone repair of the DNA break (Brenner and Milstein, 1966; Di Noia et al., 2007; Xu et al., 2005). The hypothesis is currently being investigated (Li et al., 2004; Maizels, 1995; Neuberger et al.,

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2003; Neuberger and Rada, 2007; Rajewsky, 1996; Scharff et al., 1997). While a role of single-strand DNA breaks in the variable region of Ig genes was verified in recent years, a role of double-strand DNA breaks (DSBs) has remained uncertain. Yet, SHM-proficient human B cells were shown to accumulate DSBs in the Ig locus (Bross et al., 2000; Cook et al., 2007; Papavasiliou and Schatz, 2000). It was suggested that DSBs formed secondary to activation-induced deaminase (AID) activity trigger the SHM process in the variable region of Ig genes. However, later studies demonstrated that such DSBs also occurred in AID-null mice that are SHM-deficient. Thus, a role for DSBs in AID-mediated SHM has remained unclear. Variability of antibody genes during immunoglobulin gene maturation also is increased by class switch recombination (CSR). This type of recombination is initiated by AID. In this study, we investigated a model system capable of addressing a linkage between DSBs and AID-mediated mutagenesis.

During the adaptive immune response, point mutations accumulate in the variable regions of heavy and light chain Ig genes, ultimately increasing the variability of Ig antigen-binding sites and the affinity of antigen-antibody interactions (Alt et al., 1987; French et al., 1989; Rajewsky et al., 1987). This type of mutagenesis, designated as SHM of Ig genes, is initiated by expression of AID (Muramatsu et al., 2000). This enzyme is targeted to Ig genes and converts cytosine bases to uracil, forming the uracil-DNA base lesion. The U:G mismatch initiates DNA repair processes that are involved in SHM, including base excision repair and mismatch repair (Petersen-Mahrt et al., 2002; Poltoratsky et al., 2000; Rada et al., 2004; Rada et al., 2002; Schanz et al., 2009; Xue et al., 2006).

Individuals with AID enzymatic deficiency have impaired SHM and CSR, and these individuals exhibit an immune deficiency clinical syndrome (Chelico et al., 2009; Revy et al., 2000). AID is believed to function as a homodimer of 24-kDa monomers; it catalyzes deamination of cytosine to uracil in a processive fashion and has a strict preference for single-stranded DNA as substrate (Peled et al., 2008; Pham et al., 2003). The preferred sequence motif for AID activity is WRCY (Bransteitter et al., 2003; Rogozin and Kolchanov, 1992). These DNA strand- and sequence-specificity features of AID are believed to assist it in targeting the Ig genes. Moreover, active transcription is required for both SHM and CSR (Xue et al., 2006). In a yeast system, AID-induced mutagenesis required active transcription (Gomez-Gonzalez and Aguilera, 2007).

While expression of AID is limited to a short phase in normal B cell maturation, constitutive recombinant expression of the enzyme in other cell types and organisms results in increased genomic mutagenesis (Harris et al., 2002; Martin et al., 2002; Okazaki et al., 2002; Poltoratsky et al., 2004). This indicates that AID expression can be used as a tool for understanding features of genomic mutagenesis processes involving the AID substrate, stretches of single-stranded DNA. For example, AID acts on cytosine bases in single-stranded DNA generated during the transcription process (Bransteitter et al., 2003; Pham et al., 2003). Mutations in non-B cells expressing AID were observed in the non-transcribed strand of expressed genes, where the DNA remains single-stranded as the other strand is being copied (Boursier et al., 2004; Pham et al., 2003). Extended stretches of single-stranded DNA also can be formed during repair of DSBs (Yang et al., 2008). These long single-stranded DNA stretches could be targets for AID activity, and this idea was investigated in the present experiments. Our experiments were based on the premise that activated B-cells contain a significant level of double-strand breaks and at the same time, AID expression is known to occur in these cells. We took advantage of the genetically-tractable yeast model system previously established to study AID-mediated mutagenesis (Poltoratsky et al., 2004). Using this system we examined the role of an induced DSB on AID-mediated mutagenesis. The results indicated that a DSB downstream of a marker gene on chromosome V increased the frequency of mutations in the marker gene. Therefore, induction of a DSB in

combination with AID expression is a biologically plausible scenario for AID-mediated SHM.

2. Materials and Methods

2.1 Materials

Synthetic oligodeoxyribonucleotides were from Oligos Etc., Inc. (Wilsonville, OR) and The Midland Certified Reagent Co. (Midland, TX). All other oligonucleotides were from Integrated DNA Technologies Inc. (IDT) (Coralville, IA). [γ - ^{32} P]ATP (7000 Ci/mmol) was from Biomedicals (Irvine, CA). Optikinase was from USB Corp. (Cleveland, OH). Protease inhibitor complete (EDTA-free) was from Roche Molecular Diagnostics (Pleasanton, CA). Uracil-DNA glycosylase (UDG) with 84 amino acids deleted from the amino-terminus was purified as described previously (Slupphaug et al., 1995).

2.2 Yeast strains and plasmids

The strains used in this study were created from a yeast strain BY4741 (Invitrogen, Carlsbad, CA) that was designated as wild-type. The CANM (yVP238) strain was obtained by insertion of the *delito perfecto* sequence downstream of the *CAN1* locus. The *CORE-I-SceI* (hygB) cassette was amplified with primers canmhuf 5'-tatacatgttccgataatgtctgagttagtgagtattctaaattagaaaacaaagagcattcgtacgctgcaggtcgac-3' and canmhur 5'-cgttatcagttgtgcctggaaaaggaaaattgtcaattcaaactccgttctaaggataacagggtaacccgcgcgttgccgattcat-3' (seq) and introduced into the genome via homologous recombination. The insertion was confirmed by colony PCR using the following primers: canmf 5'-TATTACCTTTGATCACATTTCCACG-3' ura3.1 5'-TTCAATAGCTCATCAGTCGA-3' sce2 5'-CTGTTTCGATGTTTCAGTTTCGA-3' canmr 5'-CAGCAAGAGTCAAGTTAATGACAAT-3'. The *ung1* and *pol32* derivatives were obtained by complete substitutions of the *UNG1* and *POL32* open reading frames, respectively, with *KanMX* modules. The construct expressing AID with C-terminal FLAG epitope and under the inducible galactose promoter was described previously (Poltoratsky et al., 2004). AID expression was followed by immunoblotting with anti-FLAG antibody (Sigma Aldrich).

2.3 Mutagenesis in the *CAN1* gene

Yeast colonies (~10) were inoculated in a dextrose-containing synthetic yeast medium without leucine and grown overnight. The next day, cells were collected by centrifugation, washed twice with water, resuspended in fresh synthetic medium containing galactose without leucine to an absorbance of ~ 0.05 at 600 nm. Cells were then grown for 48 h at 30 °C. According to the report by Storici et al. 2006, DSB induction is known to occur under these conditions, and we observed a strong reduction in cell survival as a function of this DSB induction. Therefore, to obtain sufficient material for the present experiments, induced DSB-containing cells were grown for 48 h, whereas wild-type cells were grown for 24 h. Additional experiments, not shown, demonstrated that the mutation frequencies were similar at 24 h and 48 h.

To assay for mutagenesis, serial dilutions of cells were plated on canavanine-containing selective and complete (YPDA) media; plates were incubated 2–3 days at 30 °C, and the frequency of mutations and viability were determined. The values shown were from 10 separate measurements in three independent experiments.

Individual colonies were purified by streaking, and the DNA was isolated using the Yeast DNA Purification Kit (EPICENTRE Biotechnologies, Madison, WI) according to the

manufacturer's recommendation. The *CAN1* sequence was amplified with CANF 5'-ATGACAAATTCAAAAGAAGACGCC -3' and CANR 5'-TGCTACAACATTCCAAAATTTGTCC-3' primers. The PCR product of DNA isolated from individual colonies was purified and sequenced with primers CNF2 5'-TTTATGGGTTCTTTGGCATA-3', CNF3 5'-TTATTGGAGAAACCCAGGTG-3', CNF4 5'-ACGTTGGTTCCCGTATTTTA-3', CNR2 5'-TGTGAAGGCAGCGTTAATCA-3', CNR3 5'-TTGTCACCACCAGTAGATGT-3' and CNR4 5'-CGTGGAATGTGATCAAAGGTAAT-3. DNA sequencing was analyzed using the DNASTAR® program.

2.4 Preparation of extracts and immunoblotting

Yeast cells grown to mid-log phase in galactose-containing synthetic medium were collected by centrifugation and resuspended in a lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl and 10 mM β -mercaptoethanol) supplemented with protease inhibitors. Cells were disrupted by vortexing with glass beads (Poltoratsky et al., 2004). Cell debris was removed by centrifugation, and the clear supernatant fraction (cell extract) was stored in aliquots.

Cell extract proteins (20 μ g), as indicated in the Figure Legends, were separated in Nu-PAGE 4–12% Bis-Tris mini gels (Invitrogen), and the proteins were transferred electrophoretically to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.5% (v/v) Tween 20 (TBST) for 1 h, washed once with TBST, and incubated for 2 h with appropriately-diluted affinity-purified monoclonal anti-FLAG M2 primary antibody (Sigma-Aldrich, St. Louis, MO). After washing 3 times with TBST, the membrane was incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The activity of immobilized horseradish peroxidase-conjugate was detected by enhanced chemiluminescence (ECL), as suggested by the manufacturer.

2.5 Uracil-DNA glycosylase assay

The UDG assay was performed using a ^{32} P-labeled 34 bp duplex DNA that contained a G:U mismatch at position 16. The 34 bp substrate was constructed by annealing two oligodeoxynucleotides : 5'-CTGCAGCTGATGCGCUGTACGGATCCCCGGGTAC-3' and 5' GTACCCGGGGATCCGTACGGCGCATCAGCTGCAG-3'. The uracil-containing strand was labeled with [γ - ^{32} P]ATP using T4 polynucleotide kinase and was then annealed to the G-containing strand by heating the solution at 90 °C for 3 min and allowing the solution to slowly cool to 25 °C. Unincorporated [γ - ^{32} P]ATP was removed by using a MicroSpin™ G-25 column (GE HealthCare), according to the manufacturer's protocol. The UDG assay was performed with a 10 μ l reaction mixture containing 50 mM HEPES, pH 7.5, 20 mM KCl, 0.5 mM EDTA, 2 mM DTT, 50 nM ^{32} P-labeled DNA, 10 μ g cell extract and 10 nM purified UDG as indicated in the Figure Legends. The reaction mixtures were incubated at 37 °C for 5 min. AP-sites generated by uracil removal from DNA substrates were hydrolyzed by the addition of 0.1N NaOH and incubating for 5 min at 75 °C. An equal volume of gel-loading dye was then added. After incubation at 75 °C for 2 min, the reaction products were separated by electrophoresis in a TBE-urea gel (Invitrogen, Pre-cast gel) for 30 min at constant voltage (200 V). Imaging and data analysis were performed by Typhoon PhosphorImager and ImageQuant™ software (GE HealthCare).

3. Results

We made use of a yeast-based system as a model to study the effect on AID-mediated mutagenesis of a distal double-strand break. Utilizing the *delito perfecto* system described

by Storici and Resnick (Storici and Resnick, 2006), we constructed the CANM strain in which the cleavage site for the inducible endonuclease I-*SceI* was introduced into the BY4741 strain (yVP238). This allowed us to examine the effect of simultaneously inducing a double-strand break and AID expression on mutagenesis in a marker gene, *CAN1*. The DSB site (I-*SceI*) was positioned 7 kb downstream of the *CAN1* marker gene on chromosome V. The integrated sequence did not contain the marker gene, but did contain several key elements, as illustrated in Fig. 1: The gene encoding the I-*SceI* endonuclease under the control of the *GAL* promoter; two markers (*URA3* and Hygromycin B resistance); and the I-*SceI* double-strand cleavage site. Expression of the I-*SceI* endonuclease is induced when these cells utilize galactose as carbon source and site-specific DSBs are introduced (Storici and Resnick, 2006). We altered the strategy of ectopic AID expression used earlier (Poltoratsky et al., 2004). Here, cells were grown in glucose carbon source medium, washed and then transferred to galactose carbon source medium for growth. The strains used for the analysis were transformed either with vector containing the *AID* gene under the *GAL* promoter or with an empty vector. Therefore, transfer of these cells to galactose medium simultaneously induced AID expression and a DSB, or as a control, a DSB alone. The strains used are summarized in Table I.

3.1 Effect of simultaneous induction of a DSB and AID expression on AID-mediated mutagenesis

To monitor the effect of a DSB in combination with AID expression, we compared the frequency of forward mutations in the genomic *CAN1* gene using two strains, BY4741 (termed wild-type) and CANM (induced DSB by galactose). The results are expressed as median values from 10 separate replicate determinations in each experiment and multiple experiments were conducted. AID induction in the wild-type strain minimally increased the frequency of mutations, from $\sim 6 \times 10^6$ to $\sim 15 \times 10^6$ (Fig. 2A, Tables II and III). These results are consistent with previously published data demonstrating the activity of AID in yeast cells (Gomez-Gonzalez and Aguilera, 2007; Poltoratsky et al., 2004). Introduction of a DSB in the CANM strain increased the frequency of mutations 3- to 4-fold, (from $\sim 6 \times 10^6$ to $\sim 22 \times 10^6$) (Table III); the origin of these DSB-induced mutations was not investigated. In contrast, simultaneous induction of a DSB and AID expression strongly increased the frequency of mutations compared with that in the wild-type strain, i.e., from $\sim 15 \times 10^6$ to $\sim 231 \times 10^6$ (Fig. 2A, Tables II and III). This difference in frequency of mutations was not due to differences in the amount of AID expression, as the levels of AID in all of the induced strains were comparable (Fig. 2B and data not shown). Finally, since we were aware of possible effects of MMR on AID-induced mutagenesis, we examined mutagenesis in our wild-type cells after deletion of the *MSH6* gene. This gene deletion had no effect on the AID-mediated mutagenesis results described above (data not shown).

3.2 Sequences of the DSB-induced and AID-mediated mutations

Because the substrate for AID activity is single-stranded DNA, AID-mediated mutagenesis could have a strand-bias signature (for discussion, see Yang et al. 2007 (Yang et al., 2007); Pham et al., 2003 (Pham et al., 2003)). In the case of bypass synthesis opposite the uracil base, the strand-bias signature would be accumulation of C to T transitions when the deamination targets are in the sense strand. We tested the spectrum of mutations accumulated in the endogenous *CAN1* locus. Individual clones were isolated and the *CAN1* gene was sequenced. The analysis revealed that in the wild-type strain, the ratio between C to T and G to A mutations was about 1:1, indicating the absence of strand bias. In contrast, in the CANM strain expressing AID, the ratio was 6:1, with 18 mutations for C to T and 3 for G to A (Fig. 3 and Table IV). These differences between the wild-type strain and the CANM strain expressing AID were statistically significant, as indicated in the legend of Figure 3. Thus, it appeared in cells with a DSB and AID expression, the sense or transcribed

strand was exposed to the AID-mediated effect. This sequence analysis also revealed that the mutations did not occur in clusters within one molecule of DNA and that there was no strong hotspot (Table IV).

3.3 Effect of a DSB on AID-mediated DNA lesion formation

AID deaminates the cytosine base in single-stranded DNA, producing the uracil base that is viewed as a DNA lesion. This lesion could be repaired by the base excision repair (BER) pathway (Lindahl and Wood, 1999), and similarly, the repair of the induced DSB is required for the cells to remain viable. To explore the effect of uracil-DNA repair, we down-regulated uracil-DNA glycosylase, the enzyme that initiates the uracil-DNA BER pathway. For disruption of the *UNG1* gene, we first confirmed that the galactose-containing cell extract from the *UNG1*-deficient strain had minimal UDG activity (Fig. 2C, lane 4). In contrast, the extract from the CANM strain had strong activity (lane 3), indicating that endogenous UDG was active in processing the substrate. The *UNG1*-deficiency was completely complemented by the addition of purified UDG (lane 5). The frequency of mutations at the *CAN1* locus in the *ung1* strains was strongly increased; this was equivalent to an increase of 52-fold in the *ung1* strain and 234-fold in the CANM *ung1* strain expressing AID, respectively, as compared to that of wild-type (Fig. 2A, Tables II). In this latter case, mutation frequency (1376×10^6) was greater than additive, compared with the CANM strain (231×10^6) and *ung1* strain (305×10^6) expressing AID. These differences were statistically significant (Table III). Therefore, initiation of the uracil-DNA repair pathway was not required for the mutagenesis, as expected, and uracil-DNA repair was functional in the wild-type cells. In addition, it is likely that uracil lesions formed by AID deamination in the *ung1*-deficient strains were not excised and were mutagenic by virtue of lesion bypass replication. These results also were consistent with the idea that the effect of a DSB on mutagenesis in the AID-expressing strain was at the level of promoting uracil lesion formation in a stretch of single-stranded DNA, i.e., in light of the substrate specificity of AID.

Next, we examined the topic of replicative polymerase lesion bypass. Polymerase delta (Pol δ) is a replicative DNA polymerase, and in *S. cerevisiae* it consists of three subunits Pol3, Pol31, and Pol32 (Johansson et al., 2001). The Pol32 subunit is believed to be a processivity factor for the DNA polymerase and is required for lesion bypass (Jain et al., 2009; Lydeard et al., 2007). While the *pol3* and *pol31* deletion mutations are lethal, the *pol32* deletion mutation is not. To test for a role of this replicative DNA polymerase in the mutagenesis observed here, we disrupted the *pol32* gene in the CANM strain. The frequency of the AID-mediated mutations in cells lacking *POL32* was diminished 10-fold compared to the isogenic *POL32* strain, i.e., from 22×10^6 to 231×10^6 (Fig. 2A, Tables II and III). The frequency of mutations was restored in cells lacking *POL32* (CANM *pol32*) when this strain was transformed with a wild-type *POL32* gene (Fig. 2A).

Finally, the repair of DSBs is a complex multi-step process including 5'-resection at the DSB forming a long ssDNA segment, DNA synthesis to restore the double-strand, and DNA ligation to restore the integrity of the DNA. In yeast cells, the 5'-resection is initiated by the MRX complex (Mre11, Rad50 and Xr2), and the long-range resection depends on the activity of Exo1 (Lewis et al., 2004; Nakada et al., 2004). To evaluate a role of this pathway in the mutagenesis described here, we deleted the *EXO1* gene in the CANM strain. The frequency of AID-mediated mutations in this CANM Exo1 minus strain was found to be lower than that of the Exo1 proficient strain, i.e., from 231×10^6 to 50×10^6 (Fig. 2A). These results are consistent with the idea that Exo1 resection played a role in the DSB-dependent AID-mediated mutagenesis.

4. Discussion

Expression of human AID elevates the frequency of point mutations in yeast (Gomez-Gonzalez and Aguilera, 2007; Poltoratsky et al., 2004), and such mutations occur in inverse correlation with the uracil-DNA repair capacity of the cell (i.e., lower repair correlates with higher mutations). In human B cells, AID deaminates cytosine residues in DNA producing uracil, and this C to U change is associated with a variety of changes that are integral to immunoglobulin gene maturation. First, there is an increase in the frequency of the point mutation C/G to T/A within the SHM hotspot sequence (RGWY/RWCY). In addition to the role of AID in mediating localized mutagenesis such as this, double-strand breaks are known to stimulate general genome-wide mutagenesis and could potentially be involved in the localized mutagenesis of SHM (Ratray and Strathern, 2003; Ratray and Strathern, 2005). Next, immunoglobulin class switch recombination is initiated by AID. In addition, Ma and co-workers (Ma et al., 2009) recently introduced a model system for study of DSB-induced mutagenesis involving error-prone lesion bypass of chemically-induced base damage in single-strand DNA (Ma et al., 2009; Yang et al., 2008). Our results have shown that a DSB in the presence of AID expression can be associated with lesion bypass mutagenesis. This appeared to have been through a single-stranded DNA intermediate, in light of the specificity of AID and the strand biased pattern of the mutations observed.

It is worth discussing the relationship between MMR, BER and the AID-mediated DSB-dependent mutagenesis described here. MMR and BER play roles in SHM, but down-regulation of genes involved in these repair pathways had different effects on frequency and spectrum of mutations (Xue et al., 2006). In mice, down-regulation of the BER gene *ung* resulted in SHM increases in G/C base pair mutagenesis and decreases in A/T base pair mutagenesis (Rada et al., 2004). This type of mutagenesis effect was also observed in *E. coli* and *S. cerevisiae* deficient in uracil-DNA glycosylase (Gomez-Gonzalez and Aguilera, 2007; Petersen-Mahrt et al., 2002). Down-regulation of the MMR genes MSH2 and MSH6 in mice resulted in a decrease of mutations in A/T base pairs without changing the frequency of mutations in G/C base pairs (Rada et al., 2004; Wiesendanger et al., 2000). Because the majority of mutations observed here were in G/C base pairs, we did not test the effect of MMR on the AID-mediated DSB-dependent mutagenesis. Nevertheless, our results preliminary results demonstrated that deletion of the MMR gene MSH6 had no effect on the frequency of mutagenesis in the wild-type strain (data not shown).

A working model for the role of AID in SHM of Ig genes was proposed previously (Petersen-Mahrt et al., 2002; Poltoratsky et al., 2000), as follows: The AID-induced uracil-DNA lesion is subject to either mutagenic lesion bypass, or to normal repair in an accurate fashion by the base excision repair (BER) pathway. Although accurate repair is the case in most cell types, including mouse fibroblasts, in immune-competent human B cells, the error-free BER process is compromised due to down-regulation of the key BER enzyme, DNA polymerase beta (Pol β) (Poltoratsky et al., 2007). This deficiency may make way for an error-prone repair process resulting in increased rates of mutagenesis during a form of error-prone BER repair (Poltoratsky et al., 2005; Sobol et al., 2003; Sobol et al., 2002).

In the present study, an alternate mechanism involving a role for DSBs in SHM was reinforced in a yeast model system. The results suggest that single-stranded DNA produced secondary to long-range resection at a DSB can participate in AID-mediated mutagenesis involving uracil lesion bypass by the replicative DNA polymerase, Pol δ . The requirements for Exo1 and for *POL32* in the AID-induced mutagenesis is quite intriguing. The Pol32 protein interacts with PCNA, and it had been shown previously that PCNA is required for AID mediated SHM. In general, the increase in mutation frequencies observed for simultaneous DSB induction and AID expression may be the result of a balance among

several parameters, including formation of the DNA lesions (uracil-DNA and DSBs) and the efficiency of repair of the lesion. Nevertheless, the precise mechanism linking a DBS and lesion bypass mutagenesis is unknown and was not investigated here.

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Fig. 1. System for study of DSB-induced mutagenesis in yeast

A schematic representation of genes within the CANM strain. *KIURA3*, *Hyg-R*, and *I-SceI* ORF correspond to ORF of orotidine-5'-phosphate decarboxylase, hygromycin B phosphotransferase and intron-encoded endonuclease *I-SecI*, respectively. The *P Gal1/10* denotes to galactose inducible divergent promoter and *I-SceI* site corresponds to the double-strand break site (DSB site). The CANM (yVP238) strain was obtained by insertion of the *delito perfecto* sequence into chromosome V downstream the *CAN1* locus, as described under Materials and Methods.

Fig. 2. Effect of AID expression and UDG expression on DSB-induced mutagenesis

(A), *CAN1* mutation frequency was evaluated as a function of AID expression in wild type cells, DSB-induced cells (CANM, CANM *ung1*, and CANM *pol32*), and *ung1* cells. The method of Maritz and Jarrett (Maritz and Jarrett, 1978) was used to determine confidence intervals for the median values of mutation frequency (Table II). Mann-Whitney test (Conover, 1976) was performed to calculate the P-values (Table III). The measurements shown are from 3 independent experiments, involving a total of ten replicates for each value. Standard errors are shown (see Tables II and III). (B), Immunoblotting analysis of cell extracts (20 μ g), as noted in the Figure using anti-AID antibody. After SDS-PAGE and transfer of separated proteins to a membrane, the membrane was blotted with antibody to AID. Experiments were conducted as described under Materials and Methods. An arrow shows the region of the blot corresponding to the 24-kDa AID monomer. (C), Assays of two cell extracts (CANM and CANM *ung1*) noted in the Figure for uracil-DNA glycosylase enzymatic activity. Photograph of a phosphorimage is shown, after reaction mixtures were subjected to denaturing gel electrophoresis. Experiments were conducted as described under Materials and Methods. Arrows indicate the migration positions of the ³²P-labeled uracil-containing DNA substrate and the DNA product after uracil removal. The reaction mixtures were incubated without or with purified UDG (lanes 1 and 2, respectively), with cell extracts for wild type *UNG1* or deleted *UNG1* (CANM or CANM *ung1*, lanes 3 and 4, respectively), or with cell extracts in lane 4 with purified UDG (CANM *ung1* + UDG, lane 5); reaction mixtures analyzed in lanes 1 to 5 were incubated for 5 min. Representative results are shown.

WT					CANM AID				
	G	A	T	C		G	A	T	C
G	X	8			G	X	3		
A		X			A		X		3
T			X		T		1	X	1
C	2		7	X	C	1	2	18	X

Fig. 3. Summary of mutational spectrum analysis in wild-type and CANM AID strains

Mutant cells were selected and the CAN1 gene was sequenced, as described under Materials and Methods. The mutation frequencies obtained in the experiment are shown. The sequence information is summarized as the number of base substitutions from the base in the left column to the base shown at the top, e.g., 8 G to A mutations were found in the wild-type (WT) strain. A Fisher's exact test on these data was conducted. A significant difference in the proportion of changes that are G → A in WT compared to CANM AID was found; likewise, the proportion of changes that are C → T differs significantly between WT and CANM AID. The one-sided Fisher's exact p-value corresponded to 0.0161 and the two-sided p-value corresponded to 0.0255.

Table I

List of strains

Cells	Strain	Genotype	Expression	Source
WT	BY4741	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ</i>	pESC-Leu	Invitrogen
WT AID	BY4741	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ</i>	pESC-AID	This study
<i>ung1</i>	yPP232	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; ung1::KanMX4</i>	pESC-Leu	This study
<i>ung1</i> AID	yVP232	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; ung1::KanMX4</i>	pESC-AID	This study
CANM	yVP238	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1</i>	pESC-Leu	This study
CANM AID	yVP238	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1</i>	pESC-AID	This study
CANM <i>ung1</i>	yVP308	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1 ung1::KanMX4</i>	pESC-Leu	This study
CANM <i>ung1</i> AID	yVP308	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1 ung1::KanMX4</i>	pESC-AID	This study
CANM <i>pol32</i>	yVP311	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1; pol32::KanMX4</i>	pESC-Leu	This study
CANM <i>pol32</i> AID	yVP311	<i>MATa; his3Δ; leu2Δ; met15Δ; ura3Δ; CAN1::CORE-I-Sce1; pol32::KanMX4</i>	pESC-AID	This study

Table II

Mutation frequencies as a function of AID express in yeast

Median $\pm$ s.e. of median ($\times 10^6$)			
Genotype	Minus AID [*]	Plus AID [*]	Mann-Whitney p-value ^a
Wild-type	5.87 $\pm$ 2.15	15.35 $\pm$ 5.05	0.0034
CANM	21.73 $\pm$ 17.32	231.28 $\pm$ 86.33	<0.0001
<i>ung1</i>	19.88 $\pm$ 6.95	305.24 $\pm$ 112.44	<0.0001
CANM <i>ung1</i>	33.49 $\pm$ 12.83	1376 $\pm$ 479	<0.0001
CANM <i>pol32</i>	8.75 $\pm$ 3.49	21.84 $\pm$ 3.36	0.0002

^{*}The method of Maritz and Jarrett (Maritz and Jarrett, 1978) was used to estimate confidence intervals for the median mutation frequency.

^aP-values were calculated using Mann-Whitney test (Conover, 1976).

Table III

Comparison of P-values with or without AID expression in yeast

Comparison		Mann-Whitney p-value ^a	
		– AID	+ AID
Wild-type vs.	CANM	<0.0001	<0.0001
	<i>ung1</i>	0.0002	<0.0001
	CANM <i>ung1</i>	<0.0001	<0.0001
	CANM <i>pol32</i>	0.062	0.023
CANM vs.	<i>ung1</i>	0.684	0.089
	CANM <i>ung1</i>	0.248	<0.0001
	CANM <i>pol32</i>	0.0029	<0.0001
<i>ung1</i> vs.	CANM <i>ung1</i>	0.019	0.0002
	CANM <i>pol32</i>	0.0039	<0.0001
CANM <i>ung1</i> vs.	CANM <i>pol32</i>	<0.0001	<0.0001

^aP-values were calculated using Mann-Whitney test (Conover, 1976).

Table IV

Sequence of *CAN1* mutations in CANM plus AID strain^a

No. Mutation Transitions	Flanking sequence	Copies
226 C to T	GAAGGAGAAAGTA <u>T</u> AGAACGCTGAAGT	
300 C to T	GTACTATTGGTA <u>T</u> AGGTCTTTTCATT	
1614 C to T	AGCTGTTTGGAT <u>T</u> TTATTTCAATGCA	2
1571 C to T	GTGTTAGCTTTG <u>T</u> TGCCGCCTATATC	3
1665 C to T	GATTGGAGATGT <u>T</u> GACATCGATTCC	
926 C to T	AAGCTGCAAACCTCAGAAAAATCCGTT	
1621 C to T	TGGATCTTATTT <u>T</u> AATGCATATTCAG	
967 C to T	AAAGTTGTTTCT <u>T</u> GTATCTTAACCTT	
992 C to T	TCTACATTGGCT <u>T</u> TCTATTATTCATT	2
1019 C to T	GACTTTTAGTTC <u>T</u> ATACAATGACCCCT	
1042 C to T	CCTAAACTAACAT <u>A</u> AATCTACTTCCTA	2
1181 C to T	TTTACGTTGGTT <u>T</u> CCGTATTTTATTT	
1414 C to T	ATCAGATTTATGT <u>A</u> AGCTTTGAAATA	
928 G to A	CTGCAAACCCCA <u>A</u> AAAAATCCGTTCCA	
675 G to A	ATTTCGAGTTCTG <u>A</u> TCGCTTCCATCA	
1184 G to A	ACGTTGGTTCCC <u>A</u> TATTTTATTTGGT	
Transversions		
494 A to C	TTGGTGCGGCCA <u>C</u> TGGTTACATGTAT	
1034 A to C	ACAATGACCCTA <u>C</u> ACTAACACAATCT	
1132 A to C	TTCAACGCTGTT <u>C</u> TCTTAACAACCAT	
1488 T to A	CGGCTTGGCTTA <u>A</u> TATGCGGCCACAT	
530 T to C	GGGCAATCACTT <u>C</u> TGCCCTGGAACCT	
1571 C to G	GTGTTAGCTTTG <u>G</u> TGCCGCCTATATC	
440 C to A	TCCCTGTTACAT <u>A</u> CTCTTTCACAGTT	
579 C to A	ATTTTGGACGTAA <u>A</u> AAAGTTCCACTGG	

^aMutated nucleotides are underlined.