Annals of Mutagenesis

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Austin Publishing Group

Research Article

Thymidylate Depletion Stimulates Homologous Recombination by *UNG1*-Dependent and *UNG1*-Independent Mechanisms in *Saccharomyces cerevisiae*

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Received: November 01, 2017; **Accepted:** November 15, 2017; **Published:** November 22, 2017

Abstract

Folate depletion increases the incidence of birth defects and triggers chromosomal breakage and homologous recombination. Inadequate levels of folates alter nucleotide balance by reducing thymidylate, leading to uracil incorporation into DNA. However, cellular DNA repair mechanisms that trigger homologous recombination in folate depleted cells are not well understood. We determined which types of homologous recombination events increase in budding yeast after wild type and *ung1* mutants, defective in uracil glycosylase, are exposed to Methotrexate (MTX), a chemotherapeutic drug that inhibits dihydrofolate reductase and thus leads to a deficiency of tetrahydrofolate and thymidylate. We measured unequal sister chromatid exchange (SCE) in haploid strains containing tandem *his3* fragments, *his3-Δ5'* and *his3-Δ3'*, and chromosomal translocations in diploid strains containing *his3-Δ5'* and *his3-Δ3'* positioned on chromosomes II and IV, respectively. We measured homolog recombination in diploid strains containing two non-reverting *ade2 alleles*, *ade2-a* and *ade2-n* (heteroallelic recombination). After wild type (*UNG1*) strains were exposed to MTX, we observed five-fold and 10-fold higher rates of SCE and translocations, respectively, but after *ung1* strains were exposed to MTX, we observed a two-fold maximum increase in recombination rates. We observed 14-fold higher rates heteroallelic recombination in either wild type or *ung1* strains after exposure to MTX. These data indicate that *UNG1*-dependent mechanisms participate in MTX-associated reciprocal exchange events, while *UNG1*-independent mechanisms participate in MTX-associated gene conversion events. We suggest that there are multiple pathways by which thymidylate stress stimulates homologous recombination in yeast.

Keywords: Methotrexate; Homologous recombination; Uracil glycosylase; Budding yeast

Introduction

Folate deficiency is detrimental to rapidly growing cells, and is correlated with high incidence of neural tube defects in newborn infants [1,2]. Chemotherapeutic treatment of aggressive cancers may include methotrexate (MTX) (for review, see [3]), a drug that inhibits dihydrofolate reductase, resulting in both thymidylate depletion and an altered ratio of S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) [4]. Nucleotide imbalance and altered SAM/SAH ratios correlate with cellular hypomethylation, micronuclei formation, and chromosomal breaks [5,6]. These observations underscore the importance of understanding genetic changes that correlate with folate deficiency. Genetic instability phenotypes conferred by thymidylate depletion include higher frequencies of gene amplifications, mutations, and gene conversion events (for review, see [7]). These phenotypes correlate with uracil incorporation in DNA, which results from the replicative DNA polymerases using dUTP as a substrate (for review, see [8]). Uracil in DNA increases the probability of G-U mismatches, which leads to both mutation and genetic instability [9]. High levels of dUTPase, an enzyme which converts dUTP todUMP, suppress genetic instability in budding yeast [10] and the yeast gene encodingd UTPase is essential [11].

DNA lesions that initiate recombination and chromosomal rearrangements may result from base excision repair (BER) that excises uracil from DNA (for review, see [8]). Uracil bases in DNA are substrates for uracil glycosylases (Ung), and subsequent abasicsites are then repaired by BER mechanisms [12]. High levels of uracil incorporation during DNA replication could lead to repeated rounds of BER that increase the probability of strand breakage [5]. Thus, particular chromosomal rearrangements associated with MTX exposure may require uracil glycosylase to initiate recombinogenic lesions.

Because there are many uracil glycosylases in higher eukaryotes, it is difficult to determine which one may be required for genetic instability phenotypes that result from folate deficiencies. In *Saccharomyces cerevisiae* (budding yeast), there is only one uracil glycosylase, which is encoded by *UNG1*, rendering it easier to describe the function uracil excision has in genetic instability phenotypes [13]. *Ung1* mutants are sensitive to bisulfite and yield higher levels of petites, due to loss of mitochondrial function [14]. In

Citation: Fasullo M. Thymidylate Depletion Stimulates Homologous Recombination by *UNG1*-Dependent and *UNG1*-Independent Mechanisms in *Saccharomyces cerevisiae*. Ann Mutagen. 2017; 1(1): 1005.

Table 1: Yeast Strains.

Strain	Genotype	Source
YA102	*MAT***a** *ura3-52 his3-Δ200 ade2-101 lys2-801 trp1-Δ1 gal3*	This Laboratory
YB135	*MAT***a** *ura3-52 his3-Δ200 ade2-a lys2-801 trp1-Δ1 gal3*	Derived from YA102
YB391	*MAT***a** *ura3-52 his3-Δ200 ade2-n lys2-801 trp1-Δ1 gal3*	Derived from YA102
YB392	*MATα ura3-52 his3-Δ200 ade2-a lys2-801 trp1-Δ1gal3*	Mating-type switched in YB391
YB393	*MATα ura3-52 his3-Δ200 ade2-n lys2-801 trp1-Δ1gal3*	Mating-type switched in YB315
YA223	*MAT***a** *his3 Δ l leu2 Δ0 metl5 Δ0 ura3 Δung1:KanMX*	Res Gene (6067)
YB394	*MAT***a** *ura3 his3-Δ 200 ade2-a lys2-801 trp1-Δ1 gal3 ung1:kanMX*	Meiotic segregant derived from cross of YB393 and YA223
YB395	*MATα ura3 his3-Δ 200 ade2-n lys2-801 trp1-Δ 1 gal3 ung1:kanMX*	Meiotic segregant derived from YB392 and YA223
Strains to measure sister chromatid exchange		
YB163	*MAT***a**-*inc ura3-52 his3- Δ200 ade2-101 lys2-801 trp1-Δ1 gal3-trp1::[his3- Δ3'::HOcs, his3- Δ5']*	This Laboratory
YB204	YB204 *MATα ura3-52 his3-Δ200 leu2-Δ1 ade2-101 lys2-801 trp Δ1 trp1::his3-Δ5' his3- Δ3'::HOcs*	This Laboratory
YB205	YB205 *MATα ura3-52 his3-Δ200 leu2-Δ1 ade2-101 lys2-801 trp Δ1 trp1::his3-Δ5' his3-Δ3'::Hocs rad51::URA3*	This Laboratory
YB396	*MATα ura3 his3-200 leu2 lys2-801 trp1 Δ1 trp1::his3-Δ5'his3'-Δ3::HOcs ung1:kanMX*	Meiotic segregant derived from cross of YA223 and YB204
Strains to measure heteroallelic recombination		
YB397	YB315 x YB393	This Laboratory
YB398	YB394 x YB395	This Laboratory
Strains to measure translocations		
YB109	*MATα ura3-52 his3-Δ200 ade2-101 trp1-Δ1 gal3-leu2-3,112 GAL1::his3-Δ5' trp1::his3- Δ3'::HOcs lys2- (leaky)*	This Laboratory
YB318	*MATα ura3-52 his3- Δ200 ade2-n trp1- Δ1 gal3- leu2-3, 112 GAL1::his3- Δ5' trp1::his3- Δ3'::HOcs lys2- (leaky*	This Laboratory
YB110	YA102 x YB109	This Laboratory
YB399	*MATα his3-Δ200 leu2 met15 delta0 ura3 ade2-n trp1-Δ1ung1:kanMX GAL1:his3- Δ5'trp1:his3-Δ3'::HOcs*	Meiotic segregant derived from cross of YB318 and YA223
YB400	YB398 x YB393	This Laboratory

this manuscript, we measured different types of recombination events that are stimulated after MTX exposure. We observed that sister chromatid exchange (SCE), homolog (heteroallelic) recombination, and non-homologous recombination events were stimulated by MTX exposure. Both rates of MTX-associated translocation and SCE were reduced in *ung1* mutants, while heteroallelic recombination events were not. We suggest that both *UNG1*-dependent and independent events are important in stimulating recombination that results from MTX-associated nucleotide imbalance.

Materials and Methods

Media and yeast strains

Standard media for the culture of yeast, SC (synthetic complete, dextrose), SC-HIS(SC lacking histidine), and YPD (YP, dextrose), are described by Burke *et al.* (2000, [15]). YPD-Kan (G418) plates contain YPD+50μg/ml G418 (Sigma). YP(A)Dcontains YPD with 80mg/L adenine. YPD-MTX plates and YP(A)D-MTX contains sulfanilamide (final concentration 5 mg/ml) and the indicted concentration of MTX. Yeast strains are listed in Table 1, and were derived from S288c. Strains used to measure SCE contain two overlapping *his3* fragments, positioned in tandem at *trp1*, and were derived from YB163 and YB204 [16-18]. To measure SCE in *ung1* mutant, we obtained a meiotic segregant from a genetic cross of YB204 and the *ung1*::*KanMX*strain (YA223), which is available in the yeast deletion collection. The *ung1*::*KanMX*marker was confirmed by Kan^R and by Polymerase Chain Reaction (PCR), using primers suggested by the *Saccharomyces cerevisiae* genome data base (http://www.yeastgenome.org/).

Heteroallelic recombination was measured by selecting for Ade^+ recombinants between two non-reverting *ade2 alleles*, *ade2-a* and *ade2-n* [19]. We replaced the *ade2-101* allele in YA102 with *ade2-n* (YB391) and *ade2-a* (YB315) by two-step gene replacement [20] using the plasmid pKH9 [19]. We switched the mating type of YB391 and YB315 to *MATα* using the galactose-inducible HO endonuclease present in pGHOT *GAL3* [21] to make YB392 and YB393, respectively. To measure heteroallelic recombination in a *ung1* homozygous diploid, both YB392 and YB393 were crossed with the *ung1*:*KanMX*strain (YA223), and haploids, YB394 and YB395, containing either *ade2-a* or *ade2-n* and *ung1*::*KanMX* were obtained by tetrad dissection. The *ung1* diploidis a cross of YB394 and YB395.

Diploid strains were used to measure translocations and were derived from a cross of one haploid (YB109) that contains the *his3* fragments on one copy of chromosomes II and IV, and another which did not contain the *his3* fragments (YA102) [21]. To measure translocations in *ung1* strains, YB318, a derivative of YB109, was first crossed with *ung1*::*KanMX*(YA223), and a meiotic segregant (YB399) containing *ung1*::*KanMX* and the *his3* fragments on chromosomes II and IV was obtained. YB400 is a homozygous ung1 diploid obtained from a cross of YB399 and YB394.

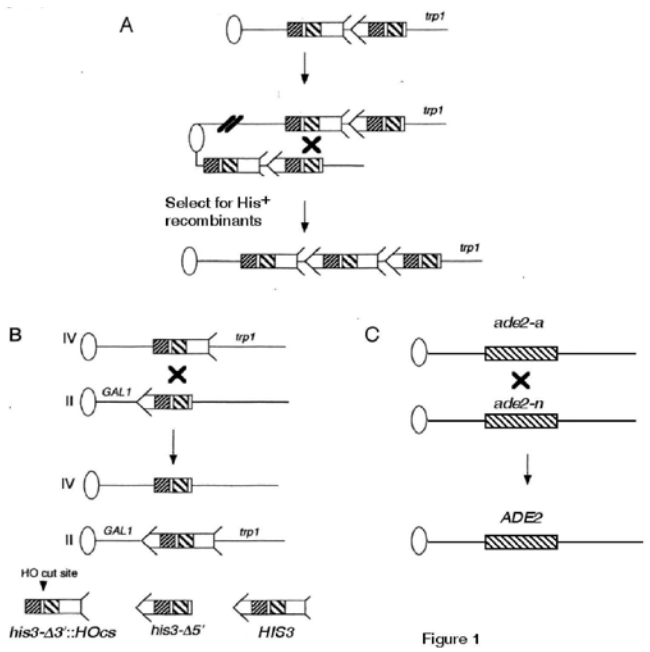

Figure 1

Figure 1: Unequal SCE, translocation, and heteroallelic recombination assays used in this study. Ovals represent centromeres and lines represent chromosomes. For simplicity, the left arms of chromosomes are not included. An arrow and feathers together denote *HIS3*. As indicated in the bottom left of the figure, the 5' deletion lacks the feather and the 3' deletion lacks the arrow. The two regions of the sequence identity shared by the *his3* fragments are indicated by decorated boxes; closely-spaced diagonal-filled boxes indicate a region of 167 bp, and the broadly-spaced diagonal line-filled boxes indicate a region of ~300bp. The 117-bp HO cut site (*HOcs*), as indicated by an arrow head, is located between these sequences within the *his3-Δ3'::HOcs* fragment, but not used in this study. (A) The *his3*-truncated fragments are integrated into the *trp1* locus to measure unequal SCE events. (B) Translocation events result from recombination between the same *his3* fragments located each on chromosomes II and IV. Positions of the *GAL1* and *trp1* are shown on chromosomes II, IV, and the reciprocal translocation. (C) Heteroallelic recombination between *ade2-a* and *ade2-n* generates *ADE2*. *ADE2* and *ade2* alleles are represented as boxes; *ade2-a* and *ade2-n* are separated by approximately 1kb.

Determining rates of spontaneous and MTX-associated recombination

The rates (events per cell division) of spontaneous and MTX-associated SCE, heteroallelic recombination, and translocations, were determined by the method of the median [22], as previously performed [17,18]. Rates of spontaneous heteroallelic recombination were determined on cells inoculated on YP(A)D, on which there is no growth advantage for Ade$^+$ recombinants. Rates of MTX-associated recombination were determined after cells were inoculated on YPD or YP(A)D solid medium supplemented with sulfanilamide and the appropriate MTX concentration and exposed for a week. We determined the statistical significance by the Mann-Whitney U-test [23].

Characterizing heteroallelic recombination events as gene conversion events

Reciprocal exchange between *ade2-a* and *ade2-n* generates *ADE2* and *ade2-a,n alleles*, while gene conversion between the two alleles would generate *ADE2* and either *ade2-a* or *ade2-n* [19]. To genotype the *ade2 allele* in Ade$^+$ recombinants, Ade$^+$ diploids were sporulated, and *ade2* meiotic segregants were obtained after tetrad dissection. The *ade2 allele* was then amplified using primers 5'CGCTATCCTCGGTTCTGCAT3' and 5'TAACGCCGTATCGTGATTAA3' and the PCR product was digested with either AatII or NdeI. Meiotic segregrants containing only *ade2-a* or *ade2-n* are indicative of gene conversion, while meiotic segregants containing only *ade2-a,n* are indicative of reciprocal exchange.

Chromosomal DNA gels

Undigested yeast chromosomal DNA was resolved on contour-clamped homogeneous electric field (CHEF) gels containing 1% agarose [24]. The gels were runat 220 V (6 V/cm) for 26 hr at a 90-sec pulse time [17]. Chromosomal DNA was transferred to nylon after exposure to 60J/m^2 of UV radiation for Southern blot analysis [17,25]. The 1.7-kb *Bam*HI *HIS3* fragment was used as a probe.

Results

Recombination assays

Thymidylate depletion is postulated to stimulate recombination and mutation as aconsequence of uracil incorporation in DNA [5]. We investigated whether rates of specific recombination events would increase after MTX exposure, which decreases dihydrofolate reductase activity. We then identified which types of MTX-associated homologous recombination events require *UNG1*, which encodes uracil glycosylase. The combination assays (Figure 1) to measure sister chromatid recombination, heteroallelic recombination, and homology-directed translocations have been previously described [16,17,26].

MTX-associated *SCE* requires *UNG1* and *RAD51*

To determine whether recombination correlated with thymidylate depletion requires BER mechanisms to generate a basic sites in DNA [5,12], we measured rates of unequal SCE recombination in wild type, *rad51*, and *ung1* haploid mutants after exposure to MTX. In the absence of MTX, we observed that spontaneous rates of unequal SCE were slightly decreased in *ung1* mutants, compared to wild type (Table 1). Slightly elevated rates of spontaneous unequal SCE in the *rad51* mutant is consistent with results obtained from other studies [27].

To determine whether MTX exposure would increase SCE, we exposed yeast to MTX either in liquid medium or on plates (Table 2). We did not observe an increase in SCE frequencies after log phase cells were exposed for 3-5 hrs in liquid medium containing MTX (data not shown). We did observe that cells exposed to a continuous exposure to 2μg/ml of MTX in solid medium leads to 5-fold stimulation of

Table 2: Rates of Sister Chromatid Exchange (SCE) after exposure to methotrexate.

Strain	Genotype[a]	Rate of Recombination(x10^6)		Fold Difference[b]
		Spontaneous	MTX-associated (2μg/ml)	
YB163	Wild type	1 $\pm$ 0.2	5.2 $\pm$ 0.1	5
YB205	*rad51*	1.5 $\pm$ 0.2	2.6 $\pm$ 0.8	<2
YB396	*ung1*	0.6 $\pm$ 0.1	1.2 $\pm$ 0.8	2

[a]For full genotype, see Table 1. N>2

[b]Fold difference = rate obtained after MTX exposure/rate obtained with no MTX exposure

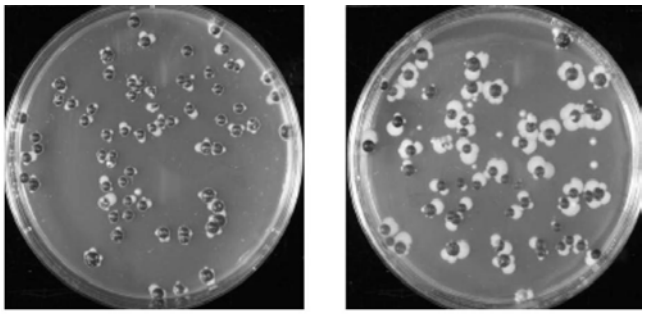

Figure 2: Heteroallelic recombination between *ade2-a* and *ade2-n* can be visualized by a colony sector phenotype. Diploid cells of YB397 were plated on YPD and YPD containing 1μg/ml MTX. Plates were incubated for two-week period.

Table 3: Rates of heteroallelic recombination after exposure to methotrexate.

Strain	Genotype[a]	Rate of Recombination (x10^{7})		
		Spontaneous 1μg/ml	MTX-associated[b]	
			1μg/ml	6μg/ml
YB397	Wild type	5.8 ± 0.6	50.5 ± 32 (8)	87 ± 9 (14)
YB398	*ung1*	6 ± 3	25 ± 9 (4)	88 ± 9 (14)

[a]For full genotype, see Table 1. N>2

[b]Fold difference = rate obtained after MTX exposure/rate obtained with no MTX exposure.

unequal SCE rates in wild type (YB163). No further increase in rates of SCE was observed after increasing the MTX concentration to 5μg/ml. However, 2μg/ml MTX exposure generated only a two-fold increase in SCE rates in *ung1* and in *rad51* mutants. Because the *rad51* mutant take longer to form colonies on YPD supplemented with MTX, we also determined whether exposure to lower concentrations of MTX, 0.5μg/ml and 1μg/ml, increased SCE rates, but we observed less than two-fold increases in rates (data not shown). These data indicate that the MTX-associated SCE requires both *RAD51* and *UNG1*. Because *RAD51* is required for DNA damage-associated SCE [17], our results are consistent with a model that suggests that uracil incorporation leads to breaks in DNA.

Rates of *MTX*-associated heteroallelic recombination are the same in *UNG1* and *ung1*::*KanMX* homozygous diploids

We visually observed Ade^{+} recombinants after diploid cells containing *ade2-a* and *ade2-n* were inoculated on YPD medium containing 2μg/ml MTX. Since *ade2* mutations confer red colony pigment and *ADE2* confers white colony pigment, white colony sectors appearing indicate recombination events (Figure 2). We could not distinguish color phenotypes for the ung1 diploid since it grows poorly and accumulates petite mutations on medium containing MTX [14]. This result indicates that MTX-associated heteroalllelic recombination occurs in *ung1* diploids.

We then measured rates of heteroallelic recombination between two *ade2* heteroalleles, *ade2-a* and *ade2-n*, after both wild type and *ung1* diploid cells were exposed to 0, 1, and 6μg/ml of MTX (Table 3). Since YPD is limiting in adenine, we measured rates of recombination in media that was supplemented with four times the normal amount of adenine. Rates of spontaneous recombination were 6x10^{-7} for both wild type and the *ung1* diploid mutant. After exposure to 1μg/ml MTX, rates of recombination were at least four-fold higher for both wild type and *ung1*, but not significantly different (P>0.05). We observed a 14-fold increase in heteroallelic recombination in both *ung1* and wild type strains after exposure to 6μg/ml (Table 3). These results indicate that spontaneous and MTX-associated heteroallelic recombination does not require *UNG1*.

To determine whether the recombination resulted from gene conversion or reciprocal exchange between *ade2* heteroalleles, we determined which *ade2* alleles were present in five MTX-associated Ade^{+} recombinants. We expected that reciprocal recombination between *ade2-a* and *ade2-n* would generate *ADE2* and *ade2-a, n*, containing both *ade2* mutations [26]. To determine the *ade2* genotype, five MTX-associated Ade^{+} diploids were sporulated and tetrads were dissected. We observed 2Ade^{+}/2Ade^{-} among meiotic segregants, indicating Mendelian segregation. By restriction enzyme digestion of PCR products, we determined the *ade2* genotype for two *ade2* meiotic segregants from each of the five Ade^{+} recombinants, and found that all recombinants still contained the *ade2-n* but not *ade2-a allele*. We conclude that the five MTX-associated Ade^{+} recombinants resulted from gene conversion but not reciprocal exchange between the *ade2-n* and *ade2-a alleles*; however we cannot rule out that cross-overs of flanking markers did not occur.

However, among five independent *ung1* Ade^{+} diploid recombinants we did not obtain four *viable* spores from tetrad dissections but we did observe that the mating type alleles, *MATa* and *MATα* segregated 2:2 by random spore analysis. For three of the five Ade^{+} recombinants, all the meiotic segregants (10/10) were Ade^{+}, indicating that the *ade2 allele* in the Ade^{+} diploid was lost by deletion, chromosome loss, or an additional gene conversion event. Sporulation of the two other Ade^{+} recombinants generated Ade^{-} meiotic segregants that contained the *ade2-aallele*. We conclude that these two recombinants likely resulted from gene conversion.

UNG1 is required for *MTX*-associated recombination between *his3* fragments on non-homologous chromosomes

We measured rates of MTX-associated recombination between *his3* fragments on chromosomes II and IV in *UNG1* (YB110, [21]) and *ung1*::*kanMX*(YB400) homozygous diploids. The spontaneous rates for both *UNG1* and *ung1*::*kanMX* homozygous diploids were 3x10^{-8}, consistent with rates obtained in previous experiments for diploid strains. After exposure to 0.5 and 1μg/ml MTX we observed a 16-fold and 20-fold increase in MTX-associated recombination. However, after exposure to 0.5μg/ml MTX we only observed a maximum two-fold (5x10^{-8}/3x10^{-8}, N =2) increase in recombination in the *ung1* diploid strain. These results indicate that *UNG1* is required for MTX-associated translocations generated by ectopic recombination between *his3* fragments. We then characterized the electrophoretic karyotype of the MTX-associated His^{+} recombinants. In previous studies we found that DNA damage-associated events in wild type cells primarily result in reciprocal translocations, as indicated by the appearance of two novel chromosomal bands on CHEF gels [17,21]. In eight MTX-associated recombinants, we observed four recombinants containing reciprocal rearrangements (Figure 3D, Figure 3F-H), typical of spontaneous recombinants.

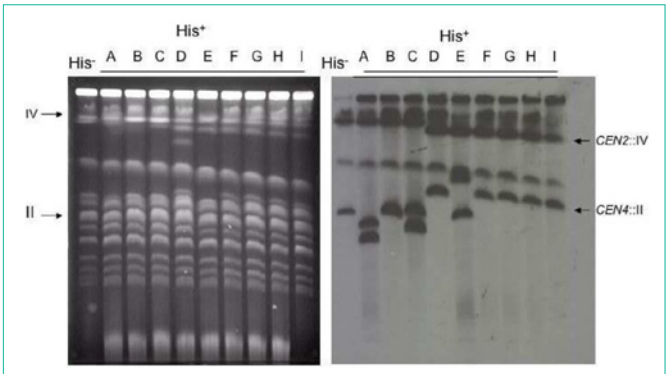

Figure 3: Electrophoretic karyotype of MTX-associated His+ recombinants. Panel on the left is a photograph of a ethidium bromide-stained CHEF gel. Panel on the right is a Southern blot probed with the 1.7 kb *Bam*H1 *HIS3* fragment. Arrows point to chromosomes II and IV, and the translocation chromosomes *CEN2*::IV and *CEN4*::II. The first lane is the His- strain YB110 containing *his3* fragments on chromosomes II and IV. Lanes A-H are individual MTX-associated His+ recombinants. Lane I is a spontaneous His+ recombinant showing position of *CEN2*::IV and *CEN4*::II.

In four recombinants, we observed either chromosome IV or II remain intact but novel chromosomal bands appear, indicating complex rearrangements (Figure 3A-C, Figure 3E). Non-reciprocal translocations and complex rearrangements may result from break-induced replication events [28], in which either chromosome II or IV chromosomes containing *his3* fragments remain intact, but novel chromosome fragments appear. We conclude that MTX exposure stimulates both reciprocal and non-reciprocal rearrangements between *his3* fragments on chromosomes II and IV.

Discussion

Uracil incorporation in DNA increases mutagenesis, homologous recombination, and genomic instability (for review, see [7]). One model suggests that repeated rounds of BER generate more single-strand and double-strand breaks that initiate recombination. Cells exposed to MTX, a chemotherapeutic agent that inhibits dihydrofolate reductase [3], incorporate more uracil into DNA due to thymidylate depletion. In budding yeast, BER mechanisms that excise uracil require *UNG1*, which encodes the only uracil glycosylase [13]. Here, we identified types of MTX-associated homologous recombination that require *UNG1* and derived the following conclusions. First, MTX-associated recombination involving *his3* recombinational substrates requires *UNG1*, while heteroallelic recombination between *ade2* heteroalleles is *UNG1*-independent. Second, MTX exposure leads to high frequencies of complex chromosomal rearrangements when homologous recombination occurs between repeated sequences on non-homologous chromosomes. Third, *UNG1*-independent recombination events may correlate with high frequencies of deletion or aneuploidy in diploids. Our study indicates that there are multiple genetic mechanisms for stimulating recombination after exposure to MTX.

Rates of homologous recombination increase in colonies grown on solid medium supplemented with methotrexate

Our observations were based on measuring rates of recombination using colonies growing on MTX-containing solid medium. We did not observe significant increases after log phase cells were grown for 3-5 hrs in liquid medium containing equivalent concentrations of MTX. These data suggest that repeated cycles of uracil incorporation into DNA may be required to stimulate recombination. Thus, it would be interesting to determine the changes in deoxynucleotide pools and the correlations between uracil incorporation and increase levels of recombination. We observed that low concentrations of MTX (0.5μg/ml) were sufficient to stimulate heteroallelic and ectopic recombination, compared to unequal SCE. We observed that low concentrations of MTX in solid medium were sufficient to increase rates of chromosomal translocations by ten-fold, while much higher concentrations were required to increase rates of SCE. One explanation is that the rates of spontaneous translocations and heteroallelic recombination are lower than SCE, and therefore higher levels of stimulated recombination are easier to detect. Alternatively, heteroallelic recombination and translocations were measured in *MAT***a**/*MATα* diploids, which exhibit higher levels of DNA damage-associated recombination events, compared to haploids or diploids that only express *MAT***a** or *MATα* [29].

UNG1-dependent mechanisms are required for *MTX*-associated SCE and translocations

One mechanism for how MTX exposure stimulates recombination, is that BER mechanisms excise uracil and make single-strand nicks in DNA that may either lead to replication fork collapse or double-strand breaks. The idea that BER stimulate recombination by making DNA lesions was supported by observations that both *UNG1* and *RAD51* were required for MTX-associated SCE. One mammalian uracil glycosylase gene, *smUNG1*, may associate with replication origins, and can complement yeast *ung1* phenotypes [30]. Thus an attractive model is that DNA replication or single stranded DNA leads to uracil incorporation into DNA, and *ung1* creates nicks that are processed into recombinogenic lesions in S phase or G2. Considering that MTX exposure also stimulates SCE is mammalian cells [31], it would be interesting to know which mammalian uracil glycosylase is required for MTX-associated SCE.

Since BER repair mechanisms are efficient, we do not know how much uracil incorporation is required to induce recombination. Considering that we observed complicated chromosomal rearrangements in some MTX-associated recombinants, it ispossible that multiple breaks occur in cells undergoing recombination.

UNG1-independent mechanisms contribute to *MTX*-associated heteroallelic recombination and may be associated with chromosomal loss

We speculate that, besides BER, there are additional *UNG1*-independent DNA repair pathways that can form recombinogenic lesions at DNA sites where uracil is incorporated. Considering that G-U mismatches are also substrates for mismatch repair enzymes [9], one possibility is that single-strand gaps made during mismatch repair are recombinogenic or more labile to become double-strand breaks. The idea that mismatch repair enzymes can form lesions that initiate recombination is supported by the observations that *MSH2* is required for immunoglobulin class switching in mammalian cells [32]. *MSH2* also functions in stabilizing recombination intermediates in mating type switching [33]. Additional experiments are necessary to determine the role of mismatch repair mechanisms in MTX-associated recombination.

Interestingly, *ung1* diploid recombinants exhibited poor spore viability and for some Ade$^+$ recombinants no *ade2* meiotic segregants were observed. Multiple aspects of DNA metabolism are likely affected in *ungl* mutants; for example, RNA polymerase may stall more frequently at sites of nucleotide misincorporation [34]. We speculate that these changes lead to additional genetic instability among *UNG1*-independent recombination events.

Conclusion

In conclusion, MTX exposure can stimulate homologous recombination between sister chromatids, heteroalleles, and repeated sequences on non-homologs in buddingyeast. Our data suggest that there are multiple mechanisms by which cellular response to thymidylate depletion can generate recombinogenic lesions.

Acknowledgements

We are indebted to Hongmei Chen for her excellent technical assistance in this project.

This work was supported by No. 1-FY01-629 from the March of Dimes.

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