Highly mutagenic and severely imbalanced dNTP pools can escape detection by the S-phase checkpoint

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Received January 27, 2010; Accepted February 12, 2010

ABSTRACT

A balanced supply of deoxyribonucleoside triphosphates (dNTPs) is one of the key prerequisites for faithful genome duplication. Both the overall concentration and the balance among the individual dNTPs (dATP, dTTP, dGTP, and dCTP) are tightly regulated, primarily by the enzyme ribonucleotide reductase (RNR). We asked whether dNTP pool imbalances interfere with cell cycle progression and are detected by the S-phase checkpoint, a genome surveillance mechanism activated in response to DNA damage or replication blocks. By introducing single amino acid substitutions in loop 2 of the allosteric specificity site of Saccharomyces cerevisiae RNR, we obtained a collection of strains with various dNTP pool imbalances. Even mild dNTP pool imbalances were mutagenic, but the mutagenic potential of different dNTP pool imbalances did not directly correlate with their severity. The S-phase checkpoint was activated by the depletion of one or several dNTPs. In contrast, when none of the dNTPs was limiting for DNA replication, even extreme and mutagenic dNTP pool imbalances did not activate the S-phase checkpoint and did not interfere with the cell cycle progression.

INTRODUCTION

Accurate duplication of DNA is indispensable for the maintenance of genome integrity. The four deoxyribonucleoside triphosphates (dNTPs) are the precursors for DNA synthesis. Because dNTP pool imbalances are mutagenic, the concentration of dNTPs is tightly controlled (1,2). Most of dNTP pool imbalances are detected by the cell. An increase in the concentration of one dNTP usually results in depletion of another dNTP, which in turn leads to the inhibition of DNA replication and to activation of the S-phase checkpoint, a genome surveillance mechanism. The activated S-phase checkpoint arrests cell cycle progression, stabilizes replication forks and activates DNA repair (3). In higher eukaryotes the activated checkpoint can lead to apoptosis. However, it is not known whether the dNTP pool imbalances that do not result in a depletion of one or several dNTPs interfere with DNA replication or lead to the activation of the S-phase checkpoint. If undetected, such dNTP pool imbalances may lead to higher mutation rates, genomic instability and development of cancer.

dNTP pool imbalances can be brought about by mutations affecting the allosteric regulation of the enzymes involved in dNTP biosynthesis: CTP synthetase (4), dCMP deaminase (5,6), or ribonucleotide reductase (RNR) (6). Mutations in these enzymes were obtained in cultured Chinese Hamster ovary cells or S49 mouse lymphosarcoma cells after lengthy selections for resistance to inhibitory concentrations of various nucleosides or their analogues. It is therefore conceivable that the S-phase checkpoint or other putative surveillance mechanisms that could be involved in monitoring of the dNTP pool quality are defective in such cells.

To investigate how dNTP pool imbalances affect cell cycle progression and checkpoint activation in a checkpoint-proficient eukaryotic cell, we decided to perturb the dNTP pool in yeast Saccharomyces cerevisiae by introducing mutations in the allosteric specificity site of RNR. RNR catalyses the rate-limiting step in the production of all four dNTPs required for the synthesis of nuclear and mitochondrial DNA (1,7). Eukaryotic RNRs reduce NDPs to corresponding dNDPs, which are then phosphorylated to dNTPs. Yeast RNR is encoded by four genes. RNR1 and RNR3 encode the large subunit (8,9). RNR3 is a non-essential paralogue of RNR1, and is normally expressed at very low levels, but is highly induced after DNA damage due to the activation of the S-phase checkpoint (8). The Rnr3 protein has a low catalytic activity, can form a heterodimer with Rnr1 (10),
The allosteric specificity site in all crystallized RNRs is proposed to be organized by two loops, called loop 1 and loop 2 (Figure 1A), which are ordered only in the presence of a specificity effector (19, 21–23). The crystal structures of RNR in complex with four cognate allosteric specificity effector–substrate pairs suggest the molecular mechanism of substrate specificity regulation (19, 23). Loop 1 interacts only with effectors bound at the specificity site, while loop 2 interacts with the bases of both effectors and substrates and provides specificity by adopting unique conformations for each effector–substrate pair.

Each species has its own balance of the four dNTPs. In logarithmically growing wild-type yeast cells, the S-phase concentration of dTTP is ~2-fold higher than the concentrations of dCTP and dATP, and ~4-fold higher than the concentration of dGTP (24). The amounts of individual dNTPs present in a cell are not proportional to the total amount of the corresponding bases in the yeast genome (Figure 1B). dNTP production is tightly coupled to utilization, and the minute amounts of individual dNTPs present in S-phase at any given moment are only enough for the synthesis of a small fraction of the genome (Figure 1B). For example, the amount of dGTP present in S-phase of a yeast cell is enough for the synthesis of only ~4% of genome’s G bases.

Here, we demonstrate that different single amino acid substitutions in loop 2 of yeast RNR lead to defined and different dNTP pool imbalances in vivo. We take advantage of this fact to investigate the extent to which systematic variations in dNTP levels affect cellular rates of proliferation and mutagenesis. The results reveal a fine balance between the cellular dNTP concentrations, the S-phase checkpoint response and DNA replication fidelity.

**MATERIALS AND METHODS**

**Plasmids and yeast strains**

To facilitate RNRI mutagenesis, we flanked loop 2 in the pESC-URA-RNRI plasmid by unique endonuclease restriction sites for BbsIII and KasI without changing the Rnr1 amino acid sequence (Supplementary Figure S1). The TRP1 selection marker was introduced after the RNRI gene. The wild-type loop 2 sequence was excised with BbsIII and KasI and the digested vector was ligated with chemically synthesized loop 2 containing the desired mutations (Supplementary Table S1). The presence of mutations was confirmed by sequencing. The pESC-URA-rnr1-D287N and pESC-URA-rnr1-D287A plasmids were constructed by the modification of pESC-URA-RNRI plasmid using QuikChange™ Site-directed mutagenesis kit (Stratagene) and the corresponding primers (Supplementary Table S2).

All yeast strains used in this study are isogenic to W4069-4C (24). Supplemental Table S3 gives only the allele(s) that differ from the W4069-4C genotype. To replace the wild-type RNRI gene with the mutant alleles, the 2μ origin in the pESC-URA-rnr1 plasmids was removed as described before (25), the plasmids were linearized with BstEII and integrated by homologous
recombination into the RNR1 locus of the W4069-4C strain. BsuRII cuts RNR1 738 bp before the loop 2 sequence. The correct integration was confirmed by sequencing. The resulting strains contain an rnr1 mutant allele under the wild-type RNR1 promoter, the pESC-URA backbone sequence and the wild-type RNR1 gene under GAL1 promoter, which is repressed in glucose-containing media.

Strains with the plasmids expressing wild-type or mutant RNR1 were grown in synthetic complete media without uracil (SC-URA) with 2% dextrose or galactose. Strains with the integrated mutant RNR1 alleles were grown in YPAD media (1% yeast extract, 2% bacto-peptone, 250 mg/l adenine, 2% dextrose, 2% agar for plates). Flow cytometry analysis was done as described (26).

**Determination of dNTP and NTP pools**

At a density from 0.4 × 10^7 to 0.5 × 10^7 cells/ml, ~3.70 × 10^8 (as determined by OD_{600}) cells were harvested by filtration through 25 mm White AAWP nitrocellulose filters (0.8 μm, Millipore AB, Solna, Sweden). To determine the exact number of cells, the cells were also counted in hemocytometer. The filters were immersed in 700 μl of ice-cold extraction solution (12% w/v trichloroacetic acid, 15 mM MgCl₂) in Eppendorf tubes. The following steps were carried out at 4°C. The tubes were vortexed for 30 s, incubated for 15 min and vortexed again for 30 s. The filters were removed and the 700 μl supernatants were collected after centrifugation at 20000 g for 1 min and added to 800 μl of ice-cold Freon–trioctylamine mixture [10 ml of Freon (1,1,2-trichlorotrifluoroethane), Aldrich, Sigma-Aldrich Sweden AB, Stockholm, Sweden, 99% and 2.8 ml of trioctylamine, Fluka, Sigma-Aldrich Sweden AB, Stockholm, Sweden, >99%]. The samples were vortexed and centrifuged for 1 min at 20000 g. The aqueous phase was collected and added to 700 μl of ice-cold Freon–trioctylamine mixture, 475 and 47.5 μl of the aqueous phase were collected. The 475 μl aliquots of the aqueous phase were pH adjusted with 1 M NH₄HCO₃ (pH 8.9), loaded on boronate columns [Affi-Gel 601 (Bio-Rad)] and eluted with 50 mM NH₄HCO₃, pH 8.9, 15 mM MgCl₂ to separate dNTPs and NTPs. The eluates with purified dNTPs were adjusted to pH 3.4 with 6 M HCl, separated on a Partisphere SAX-5 HPLC column (4.6 × 12.5 cm, PolyLC Inc., Columbia, MD, USA) and quantified using a UV-2075 Plus detector (Jasco, Möndal, Sweden). Nucleotides were isocratically eluted using 0.36 M ammonium phosphate buffer (pH 3.4, 2.5% v/v acetonitrile). The 47.5 μl aliquots of the aqueous phase were adjusted to pH 3.4 and used to quantify NTPs by HPLC in the same way as dNTPs.

**Calculation of the dNTP molecules**

Saccharomyces cerevisiae genome contains 12.1 × 10^6 bp and requires 24.2 × 10^6 dNTPs for its duplication. The percentages of A, T, G and C in the yeast genome are 31, 31, 19 and 19%, respectively. Therefore, the number of dATP, dTTP, dGTP and dCTP molecules required for one round of replication is 7.5 × 10^6, 7.5 × 10^6, 4.6 × 10^6 and 4.6 × 10^6, respectively. There is ~35 pmol of dATP present in 10^6 logarithmically growing cells (Figure 3B), or 35 × 10^-20 mol of dATP per cell, which corresponds to 0.21 × 10^6 molecules per cell. Because the concentration of dNTPs in S phase is ~2-fold higher than the concentration of dNTPs in logarithmically growing cells, an estimated number of dATP molecules present at any given moment in an S-phase cell is 0.4 × 10^6. The numbers of dTTP, dGTP and dCTP molecules present in an S-phase cell (1.0 × 10^6, 0.2 × 10^6 and 0.5 × 10^6, respectively) were calculated in the same way as for dATP. These numbers were used to calculate the percentages of dNTP shown in Figure 1B.

**Measurement of mutation rates**

Eight independent cultures from two different isolates of each strain were grown in YPAD complete media until saturation. Cells were then washed twice with sterile water and the appropriate dilutions were plated on synthetic complete medium lacking arginine and containing 60 μg/ml of L-canavanine (SC-Arg + Can) to score the Can^r colonies and on complete media (YPAD) plates to count viable cells. The plates were incubated for 3–5 days at 30°C before counting. The frequency of forward mutation to Can^r at the CAN1 locus was calculated by dividing the number of Can^r colonies by the viable cell count. Mutation rates were calculated from the frequencies as described previously (27). Statistical analyses to determine the 95% confidence limits for the median and the significance of the differences between the mutation rates (Wilcoxon–Mann–Whitney non-parametric test) were done as described in Ref. (28).

**Western blotting**

The protein extracts were made as described (29). For Rnr2 and Rnr3 detection, affinity purified polyclonal anti-Rnr2 and anti-Rnr3 antibodies were used at 1 : 500 dilutions. For Rnr1, anti-Rnr2 and anti-Rnr3 antibodies were used at 1 : 500. For Western blotting, anti-Rnr2 and anti-Rnr3 antibodies were used at 1 : 500.

**RESULTS**

**Mutations in loop 2 of Rnr1 lead to imbalanced dNTP pools**

The amino acid sequence of loop 2 is conserved among species (Figure 2A). It was possible that all changes in loop 2 would abolish RNR activity or completely disrupt the allosteric specificity regulation, and thereby have similar effects on cellular dNTP levels. To initially determine whether different mutations in loop 2 lead to different dNTP pool imbalances, we overexpressed Rnr1 proteins with substitutions in residues Y285, D287, Q288 and R293 of loop 2, and measured the resulting dNTP pools. We chose these residues either because they are conserved and/or because structural studies showed that they are involved in important interactions with the effectors or substrates in yeast RNR as demonstrated by Dealwis and co-workers (19). Overexpression of Rnr1...
alleles with mutations in loop 2 using an inducible GAL1 promoter (Figure 2B) resulted in unique new dNTP pool balances (Figure 2C). These were different from the dNTP pool balance observed in the cells with an overexpressed wild-type RNR1 gene, which resulted in a $\approx$9–10-fold increase in all 4 dNTPs (25). The results in Figure 2C indicate that each substitution in loop 2 changed the allosteric specificity regulation in a distinctive way. In all strains with overexpressed $rnr1$ mutants, the individual dNTP concentrations were higher than in the wild-type cells, except for dCTP in the $rnr1$-Q288A-overexpressing strain, which was $\approx$20% below wild-type levels. The largest increase for pyrimidine dNTPs was observed in the $rnr1$-Y285A strain (55-fold for dCTP and 33-fold for dTTP) and the largest increase for purine dNTPs was in the $rnr1$-Q288A strain (14-fold for dATP and 63-fold for dGTP).

Continuous expression of RNR1 from the GAL1 promoter induces the increased dNTP concentrations outside of S-phase, which might interfere with cell cycle progression and DNA damage checkpoint activation (25). Also, overproduction of Rnr1 protein (Figure 2B) could...
affect cells independently of the changes in the dNTP pool. Therefore, we replaced the endogenous RNR1 by the rnr1 mutant alleles and used the resulting strains in all subsequent studies. We were unable to obtain viable haploid yeast cells harbouring rnr1-D287N, rnr1-D287A and rnr1-Q288E alleles, perhaps because the activity of the corresponding Rnr1 proteins was too low. In all viable strains with the integrated rnr1 mutants, the dNTP pools were imbalanced (Figure 3B). In the strains harbouring the rnr1-Q288A and rnr1-R293A alleles, the concentration...
of one or several dNTPs was below normal, indicating that utilization of these dNTPs was faster than their production. The rnr1-Y285A strain had only ~8% of the normal dCTP concentration and the rnr1-R293A strain had 36, 66 and 30% of the normal dCTP, dTTP, and dGTP concentrations, respectively. These results suggest that the Rnr1-Q288A and Rnr1-R293A proteins can provide sufficient RNR activity only when overexpressed from the GAL1 promoter (Figure 2C). The rnr1-Y285A strain had extremely high pyrimidine dNTP concentrations (20-fold increased dCTP and 17-fold increased dTTP), whereas the concentrations of the purine dNTPs were close to normal (Figure 3B). These results demonstrate for the first time that it is possible to obtain yeast strains with different imbalanced dNTP pools by introducing mutations in loop 2 of Rnr1.

Effects of dNTP pool imbalances on cell cycle progression

The strains with the integrated rnr1-Q288A and rnr1-R293A alleles had dNTP pools below their normal level. In these two strains, the S-phase was significantly prolonged (Figure 3C) and the proliferation rate was decreased (Figure 3D and E). We also observed a large proportion of dead cells (Figure 3C).

The rnr1-R293A mutant was synthetic lethal with the deletion of RNR3, encoding the alternative large RNR subunit (Figure 3F). We were also not able to obtain viable colonies after sporulating rnr1-Q288A rnr3A diploids. These results suggest that the rnr1-R293A and rnr1-Q288A strains rely on the non-essential Rnr3 protein for survival because the Rnr1-Q288A and Rnr1-R293A proteins by themselves are not able to support the production of sufficient amounts of one or several dNTPs.

Interestingly, the rnr1-Y285F and rnr1-Y285A strains did not demonstrate any cell cycle defects (Figure 3C), despite highly imbalanced dNTP pools in rnr1-Q288A (20- and 17-fold increased dCTP and dTTP, respectively). The proliferation rates of the rnr1-Y285F and rnr1-Y285A strains were similar to wild-type (Figure 3E). Furthermore, the rnr1-Y285F rnr3A and rnr1-Y285A rnr3A strains were viable and grew normally (data not shown). These data demonstrate that when none of the dNTPs is limiting for DNA replication, even highly imbalanced dNTP pools do not interfere with the cell cycle progression.

Effects of dNTP pool imbalances on Can r mutation rates

All substitutions in loop 2 of Rnr1 increased the Can r mutation rates (Table 1). A separate study (Kumar, D., Abdulovic, A., Kunkel, T.A. and Chabes, A., submitted for publication) strongly suggests that this is due to decreased DNA replication fidelity resulting from the dNTP pool imbalances. Interestingly, even a mild dNTP pool imbalance in rnr1-Y285F resulted in a ~2.5-fold increase in the mutation rate. The mutation rates were not directly proportional to the degree of the dNTP pool imbalance. For example, the severe imbalance in rnr1-Y285A resulted in the highest increase in the mutation rate, while the severe imbalance in rnr1-Q288A (the relative excess of dGTP over dCTP is ~200-fold, and the relative excess of dATP over dCTP is ~80-fold) resulted in a moderate, 4-fold increase in the mutation rate. Thus, different dNTP pool imbalances vary in their mutagenic potential. However, it should be noted that the slow cell cycle progression and/or activated S-phase checkpoint (see below) could influence the mutation rates in rnr1-Q288A.

Severely imbalanced dNTP pools can escape the S-phase checkpoint

It is well established that stalling of replication forks due to the depletion of one or several dNTPs leads to the activation of the S-phase checkpoint. The key checkpoint protein kinases in budding yeast are Mec1 (homologue of human ATR) and Rad53 (homologue of CHK2 and functional homologue of CHK1 in humans). Rad53 activates a non-essential kinase Dun1, which is responsible for the activation of RNR (3, 31). In human cells, ATR and CHK1 are upstream regulators of p53 and 253 are mutated in many cancers. It has been proposed that, in mammalian cells, the ATR–CHK1–p53 S-phase checkpoint is activated by the MMR system responding to the mis-incorporation of deoxyribonucleotides from imbalanced pools, and not due to the stalling of DNA synthesis (32). Therefore, it seemed possible that the S-phase checkpoint might be activated in the rnr1 mutant strains, either due to depletion of dNTPs in rnr1-Q288A and rnr1-R293A, or to increased mis-incorporation of dNTPs and activation of MMR in the rnr1-Y285F and rnr1-Y285A mutant strains.

Table 1. Mutation rates in the wild-type and integrated rnr1 mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation rate (× 10⁻⁶)</th>
<th>95% CI (× 10⁻⁶)</th>
<th>Fold-increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4.2</td>
<td>1.6–4.4</td>
<td>1</td>
</tr>
<tr>
<td>rnr1-Y285F</td>
<td>10.6</td>
<td>9.4–11.6</td>
<td>2.5</td>
</tr>
<tr>
<td>rnr1-Y285A</td>
<td>57</td>
<td>43–103</td>
<td>13.6</td>
</tr>
<tr>
<td>rnr1-Q288A</td>
<td>17</td>
<td>12–24</td>
<td>4.0</td>
</tr>
<tr>
<td>rnr1-R293A</td>
<td>15</td>
<td>9.8–17</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Strains indicating that the S-phase checkpoint (see below) could influence the mutation rates in rnr1-Q288A.
Rnr2, Rnr3 and Rnr4 levels are not elevated in rnr1-Y285F. Methane sulphonate (MMS) depends on a functional S-phase checkpoint (presence of checkpoint activation. Western blot analysis demonstrating that the increase of Rnr3 and Rnr4 levels in response to DNA damage by methyl methanethiosulphonate (MMTS) to the similar level as after treatment by DNA damaging [4-nitroquinoline oxide (4-NQO) and MMS] and replication blocking [hydroxyurea (HU)] agents. (C) Additionally, rnr1-Y285F is not synthetically lethal with either rnr3A or dun1A. Therefore, dNTP pools that are severely imbalanced but not limiting for replication are not detected by the S-phase checkpoint in yeast.

**DISCUSSION**

The present study reveals that even highly imbalanced and highly mutagenic dNTP pools are well tolerated and do not activate S-phase surveillance mechanisms in yeast as long as none of the four dNTPs is limiting for replication. An imbalanced but not limiting for replication dNTP pool could lead to decreased proliferation and activation of the S-phase checkpoint by at least two mechanisms. One possibility is that the DNA polymerase could idle due to the repeated cycles of incorrect nucleotide incorporation and exonucleolytic proofreading. Another is activation of the S-phase checkpoint by the mismatch repair system in response to the mismatch independently of the mismatch repair. The activation of checkpoint by the mismatch repair system in response to the S_{N1}-type alkylating agents involved recognition of a damaged base, O6-me-G. It will be interesting to investigate whether the elevation of pyrimidine dNTP pools and/or whether the mismatches between the undamaged bases also will trigger a checkpoint response in higher eukaryotes.

Another conclusion of this study is that different substitutions in loop 2 lead to different dNTP pool imbalances in vivo. The crystal structure of Rnr1 in complex with four cognate allosteric specificity effector–substrate pairs from Dealwis and co-workers provides some clues to the observed changes in the dNTP pools. dGTP is the only effector that hydrogen-bonds the side chain of D287 to the observed changes in the dNTP pools. dGTP is the only effector that hydrogen-bonds the side chain of D287. Mutation of D287 to alanine or asparagine may, therefore, weaken the binding of dGTP, the positive effector for ADP reduction, effectively leading to a relatively low increase in dATP in the D287A and D287N mutants (Figure 2C). The Q288 residue directly contacts all substrates except GDP (19). Thus, it is plausible that mutating Q288 will negatively affect binding of all substrates except GDP, leading to a high dGTP concentration, which is exactly what we observe (Figures 2C and 3B). dGTP is in turn a positive effector for ADP.
reduction and a negative effector for UDP and CDP reduction, which may explain the relatively high dATP levels and the relatively low dCTP and dTTP levels in the Q288 mutants. R293 interacts with the base of the substrate in the ADP- and GDP-bound structures only (19). This interaction does not explain why the levels dCTP and dTTP are affected more than dATP. In the dGTP-ADP complex, the guanine base hydrogen bonds to the bottom of loop 2, involving main-chain atoms of Y285 (19). It is possible that mutation of Y285 weakens the binding of dGTP, a negative effector for the reduction of CDP and UDP and a positive effector for the reduction of ADP, in turn leading to high pyrimidine dNTPs.

A collection of yeast strains with various defined dNTP pool imbalances will be highly useful for the in vivo studies of, for example, the fidelity of nuclear and mitochondrial DNA polymerases, the contribution of translesion polymerases to the bypass of spontaneous DNA lesions, and the specificity and efficiency of the mismatch repair system. *Saccharomyces cerevisiae* is a good model system for studying the effects of mutations in RNR on the dNTP pools balance and the dNTP-dependent processes. This is partly because, in contrast to many bacteria and mammalian cells, wild-type *S. cerevisiae* lack nucleoside kinases, and therefore are unable to uptake nucleosides from the media (34). Furthermore, *S. cerevisiae* has no 5'-nucleotidases that degrade nucleoside monophosphates to nucleotides (1). In the absence of deoxyribonucleotidase degradation and excretion, and of deoxyribonucleoside uptake, a yeast cell presents a convenient, closed 'microreactor' for the simultaneous assay of RNR activity towards all four substrates.

Considering complete conservation of loop 2 between yeast and mammalian RNRs (Figure 2A), it is likely that mutations investigated in this study will lead to similar dNTP pool imbalances in mammalian cells. Stable, defined dNTP pool imbalances produced by mutations in the enzymes involved in dNTP biosynthesis are more useful for studying replication fidelity, mismatch repair and other dNTP-dependent processes than imbalances obtained by the addition of nucleosides, their analogues or inhibitors of the dNTP synthesis. The exogenously supplied nucleosides and drugs quickly change their concentration and are metabolized to a number of unknown compounds, making it difficult to investigate their exact effects.

We were surprised to observe only a modest, 4-fold increase in the mutation rate in the *rnr1-Q288A* strain. The relative dNTP pool imbalance in this strain is larger than in the *rnr1-Y285A* strain, which has a more significant, 13.5-fold increase in the mutation rate. There are several possible explanations to this observation: (i) a more efficient misincorporation of pyrimidine dNTPs compared to purine dNTPs; (ii) a more efficient proofreading in *rnr1-Q288A* due to the slow DNA replication and inhibition of proofreading in *rnr1-Y285A* due to the excessive dNTP pools; (iii) an increased correction of mismatches in *rnr1-Q288A* due to the activated S-phase checkpoint; and (iv) a better recognition of misincorporated purines by the mismatch repair system.

Further studies will be required to explain the differences in the *rnr1-Q288A* and *rnr1-Y285A* mutations rates.

The first reports implicating mutations in human RNR in genetic disorders were published very recently. Mutations in the human *RRM2B* gene encoding an alternative small RNR subunit were demonstrated to be associated with mitochondrial (mt) DNA depletion syndrome (MDS) leading to muscular dystrophy (35), autosomal-dominant progressive external ophthalmoplegia (adPEO) leading to weakness of the external eye muscles and exercise intolerance (36) and mitochondrial neurogastrointestinal encephalopathy (MNGIE) (37). The other causes of these disorders involve mutations in the mtDNA polymerase γ, mtDNA helicase Twinkle or in the enzymes of the mitochondrial nucleotide salvage and transport. However, a number of clinical cases associated with these disorders remain unexplained. It will be interesting to investigate whether mutations in loop 2 of the human large RNR subunit are involved in these and other genetic disorders or are early events in the evolution of cancer cells.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank Rodney Rothstein and Olga Tsaponina for yeast strains, Olga Tsaponina for technical help with Western blotting, Amy Abdulovic, Thomas Kunkel and Lars Thelander for critical reading of the manuscript, and members of the Chabes group for valuable advice and discussion.

**FUNDING**

Swedish Foundation for Strategic Research; Swedish Research Council; Swedish Cancer Society (to A.C.); Stiftelsen J.C. Kempe's Minnesstipendiefond (to D.K.). Funding for open access charge: Swedish Research Council.

Conflict of interest statement. None declared.

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