Ribonucleotides incorporated by the yeast mitochondrial DNA polymerase are not repaired

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Edited by Philip C. Hanawalt, Stanford University, Stanford, CA, and approved October 17, 2017 (received for review July 25, 2017)

Incorporation of ribonucleotides into DNA during genome replication is a significant source of genomic instability. The frequency of ribonucleotides in DNA is determined by deoxyribonucleoside triphosphate/ribonucleoside triphosphate (dNTP/rNTP) ratios, by the ability of DNA polymerases to discriminate against ribonucleotides, and by the capacity of repair mechanisms to remove incorporated ribonucleotides. To simultaneously compare how the nuclear and mitochondrial genomes incorporate and remove ribonucleotides, we challenged these processes by changing the balance of cellular dNTPs. Using a collection of yeast strains with altered dNTP pools, we discovered an inverse relationship between the concentration of individual dNTPs and the amount of the corresponding ribonucleotides incorporated in mitochondrial DNA, while in nuclear DNA the ribonucleotide pattern was only altered in the absence of ribonucleotide excision repair. Our analysis uncovers major differences in ribonucleotide repair between the two genomes and provides concrete evidence that yeast mitochondria lack mechanisms for removal of ribonucleotides incorporated by the mtDNA polymerase. Furthermore, as cytosolic dNTP pool imbalances were transmitted equally well into the nucleus and the mitochondria, our results support a view of the cytosolic and mitochondrial dNTP pools in frequent exchange.

DNA replication | dNTP | mitochondrial DNA | ribonucleotide incorporation | ribonucleotide excision repair

A key factor contributing to genomic stability is an adequate supply of the deoxyribonucleoside triphosphate (dNTPs) that are required for replication and repair of DNA. Increases or decreases in cellular dNTP pools, as well as imbalances between the individual dNTPs, have deleterious consequences for the organism (1–4) and can lead to increased mutagenesis despite the presence of functional repair pathways (5–9). Insufficient dNTP pools cause replication stress in the form of replication fork stalling, the accumulation of single-stranded DNA, and chromosomal rearrangements. Accordingly, alterations in the dNTP pool have been suggested to contribute to the increased mutation rate and genomic instability during cancer development (10, 11). These findings underscore the critical importance of maintaining a sufficient and balanced dNTP pool.

A central regulator of the absolute and relative dNTP levels in the cell is the enzyme ribonucleotide reductase (RNR) that catalyses the reduction of ribonucleoside diphosphates (rNDPs) to deoxyribonucleoside diphosphates (dNDPs) in the cytosol. RNR is tightly controlled at multiple levels to ensure an adequate supply of dNTPs for genome replication during S phase and for repair in response to DNA damage. Allosteric regulation of RNR determines both the overall dNTP concentration in the cell and the dNTP pool balance: that is, the relative amounts of the four individual dNTPs (dCTP, dTTP, dATP, and dGTP) (12). Point mutations in loop 2 of the allosteric specificity site of budding yeast RNR result in distinct dNTP pool imbalances, some of which are highly mutagenic (6, 13). In addition to allosteric regulation, the Mec1/Rad53 genome integrity checkpoint regulates yeast RNR activity through several different mechanisms (14).

The incorporation of ribonucleotides (rNMPs) into the genome during DNA replication has become recognized as a significant source of genomic instability. Given that the physiological concentrations of ribonucleoside triphosphates (rNTPs), the building blocks of RNA, are one to two orders-of-magnitude higher than those of dNTPs, rNMPs are frequently incorporated into DNA during replication (15, 16). rNMPs embedded in the genome are efficiently removed by ribonucleotide excision repair (RER), a dedicated repair pathway that is initiated by cleavage at the rNMP by RNase H2 (17, 18). However, in the absence of RER, the yeast and mouse genomes have been estimated to contain over 10,000 and over 1 million incorporated rNMPs, respectively (16, 19), making rNMPs by far the most frequent noncanonical nucleotide incorporated during DNA replication. Embedded rNMPs are a potential source of genomic instability due to their reactive 2′-hydroxyl group that puts the DNA backbone at risk for cleavage. Accordingly, RER-deficient mice exhibit chromosomal rearrangements and micronuclei, and die during embryonic development (19). rNMPs are better tolerated in yeast, and RER-deficient yeast exhibit normal growth, albeit with signs of genomic instability

Significance

Mitochondria are essential for energy production. However, a number of defects that affect the cellular levels of deoxyribonucleoside triphosphates (dNTPs), the building blocks of DNA, threaten the proper maintenance of mitochondrial DNA (mtDNA) and lead to human disease. We show that imbalances in the total cellular dNTP pool are transmitted into the mitochondria and that they alter the frequency of ribonucleotides, the building blocks of RNA, that are incorporated into mtDNA in yeast. We further show that yeast mitochondria lack repair pathways that remove embedded ribonucleotides. Because ribonucleotides are a known cause of genome instability, our findings may explain why altered cellular dNTP pools lead to defects in mtDNA but not in nuclear DNA in certain human diseases.

Author contributions: P.H.W., J.M.E.F., A.R.C., and A.C. designed research; P.H.W., M.K.M.E., J.M.E.F., C.N., A.K.N., and A.R.C. performed research; J.S. and S.W. contributed new reagents/analytic tools; P.H.W., M.K.M.E., J.M.E.F., S.W., A.R.C., and A.C. analyzed data; and P.H.W. and A.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE100352).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1713085114/-/DCSupplemental.

and mild genome integrity checkpoint activation associated with a slight increase in dNTP pools (16, 20).

The presence of ribonucleotides in mature mammalian mitochondrial DNA was established over 40 y ago (21–23). These ribonucleotides have more recently been shown to be present in stretches of one to three rNMPs, as indicated by the sensitivity of mouse mtDNA to cleavage by RNase H2 (19). This finding implies that RNase H2 is absent from mammalian mitochondria. Ribonucleotides in human mitochondrial DNA were recently mapped, and their absolute and relative levels were shown to be affected by changes in the mitochondrial dNTP pool (24). Yeast mtDNA also contains rNMPs (25, 26), but to what extent these stem from unrepaired residues of RNA primers or from incorporation by the mitochondrial DNA polymerase remains unclear. To compare the incorporation and repair of rNMPs between the nuclear and mitochondrial genomes, we utilized a collection of yeast strains with altered dNTP pools.

We show that even mild dNTP pool imbalances result in changes in the relative rNMP frequencies in the mtDNA, demonstrating that yeast mitochondria lack mechanisms for the repair of rNMPs incorporated during replication. In contrast to mtDNA, the relative rNMP frequencies in nuclear DNA (nDNA) are altered by dNTP pool imbalances only when RER is eliminated, which suggests that RER is extremely efficient in the nucleus. We observe a strong correlation between mitochondrial and nuclear rNMP incorporation patterns in RER-deficient cells, suggesting that cytosolic dNTP pool imbalances are transmitted equally well into the nuclear and mitochondrial compartments. Finally, we demonstrate that the overall dNTP levels determine the amount of rNMPs in mtDNA.

Results

dNTP Pool Imbalances Alter the rNMP Incorporation Pattern in nDNA of RER-Deficient Strains, but Not WT Strains. To examine the effect of dNTP pool alterations on the pattern of rNMP incorporation in the genome, we made use of *Saccharomyces cerevisiae* strains with amino acid substitutions in the allosteric specificity site of Rnr1 that give rise to distinct and stable dNTP pool imbalances. For example, the *rnr1-Q288A* strain is characterized by extremely low dCTP and increased dATP and dGTP, while *rnr1-Y285A* and *rnr1-Y285F* exhibit increased levels of dCTP and dTTP (~20-fold in *rnr1-Y285A* and ~3-fold in *rnr1-Y285F*) (6) (Fig. 1*A*). We constructed additional strains with dNTP pool imbalances: *rnr1-Q288E,* with an ~7-fold increase in dGTP, and *rnr1-Y285A,Q288E*, with over 20-fold increases in dCTP, dTTP, and dGTP, but only a 2-fold increase in dATP (Fig. 1*A*). Our panel of strains also included the *rnr1-D57N* mutant that is unresponsive to dATP feedback inhibition (7) and therefore has increased, but balanced, dNTP pools. Finally, the *rnr1-D57N,Q288E* double-mutation conferred a dNTP pool imbalance similar to the *rnr1-Q288E* single-mutant but with higher overall dNTP levels (Fig. 1*A*). To detect all rNMPs incorporated into nDNA during replication, the RNR mutant strains were also crossed into the *rnh201*Δ background that is deficient for RER. The deletion of *RNH201* did not alter the dNTP pool imbalances in any of the strains (Fig. 1*B*). The *rnr1* mutations had only a slight effect on rNTP pools (Fig. S1); therefore, the dNTP:rNTP ratios in these strains are principally determined by the level of each individual dNTP.

To compare how dNTP pool imbalances affect rNMP incorporation in the nuclear and mitochondrial genomes, we used the recently described HydEn-seq method that maps the identity and location of incorporated rNMPs genome-wide (25). Even extreme dNTP pool imbalances (leading to, for example, a dCTP:rCTP ratio of 1:6,340 in *rnr1-Q288A* compared with 1:37 in WT) did not affect the relative frequency of rNMP incorporation into nDNA in strains that were proficient for RER (*RNH201*$^+$ strains) (Fig. 1 *A* and *C* and Fig. S2), demonstrating a very high efficiency of RER in the nucleus. Because the observed profile in Fig. 1*C* is very similar for all of the strains, we assume that the observed signal represents low-level background not derived from rNMPs.

In contrast, when RER was eliminated by deletion of *RNH201*, the relative frequency of the incorporated ribonucleotides in nDNA was inversely related to the levels of individual dNTPs (Fig. 1 *B* and *D*). For example, while rCMP constituted 35% of the ribonucleotides incorporated into the nuclear genome of a strain with normal dNTP pools, it increased to 66% in the *rnr1-Q288A rnh201*Δ strain (Fig. 1*D*; compare the blue bars for WT and *rnr1-Q288A*). Note that the ribonucleotide incorporation

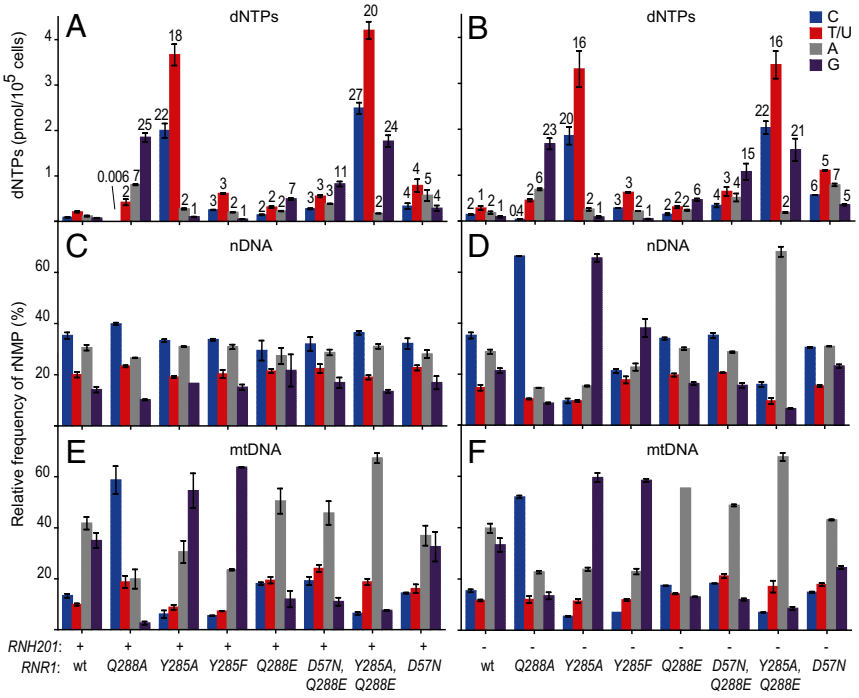

Fig. 1. rNMP incorporation profiles in mtDNA and nDNA of yeast strains with altered dNTP pools. (*A* and *B*) dNTP pool levels of the strains used for the mapping of rNMPs. The number above each bar indicates the fold-change in dNTP level relative to WT. (*A*) *RNH201*$^+$ strains and (*B*) *RNH201*$^-$ strains. (*C* and *D*) Relative frequencies of each rNMP incorporated into nuclear DNA of (*C*) *RNH201*$^+$ strains and (*D*) *RNH201*$^-$ strains. (*E* and *F*) Relative frequencies of each rNMP incorporated into mitochondrial DNA of (*E*) *RNH201*$^+$ and (*F*) *RNH201*$^-$ strains. Data in all panels are presented as mean ± SEM of at least two independent isolates of the same genotype. Mutations in *RNR1* are indicated below the graph; "*rnr1*" is omitted for clarity. wt, wild type. See also Figs. S1 and S2 and Table S1.

frequency of each ribonucleotide is presented as a relative value (percent of total rNMPs incorporated), whereby the increase in relative rCMP frequency in *rnr1-Q288A rnh201*Δ causes a decrease in relative frequency of the other three ribonucleotides (rUMP, rAMP, and rGMP). Similarly, examination of the *rnr1-Y285A rnh201*Δ and *rnr1-Y285F rnh201*Δ strains that exhibit an ∼20-fold and ∼3-fold increase in dCTP levels, respectively (Fig. 1*B*), shows a relative decrease in rCMP frequency to 10% and 21% in *rnr1-Y285A rnh201*Δ and *rnr1-Y285F rnh201*Δ, respectively (Fig. 1*D*). Therefore, the relative frequency of rCMP negatively correlates with the dCTP levels. Interestingly, in the *rnr1-Y285F rnh201*Δ strain, the relative frequency of rUMP did not decrease in response to a threefold increase in dTTP level, while a threefold increase in dCTP level in the same strain decreased the relative frequency of rCMP from 35 to 21%. This observation suggests that either DNA polymerases discriminate against rUTP better than rCTP or that rUMP is repaired by other mechanisms in addition to RER. The relative frequencies of rNMPs in the *rnr1-D57N rnh201*Δ strain, which has a balanced increase in dNTP pools, did not change appreciably (Fig. 1*D*; compare *rnr1-D57N* to WT).

Overall, in our panel of *rnh201*Δ strains with imbalanced dNTP pools, the relative frequencies of rNMPs in nDNA correlated inversely with the levels of the corresponding dNTPs (Fig. 1 *B* and *D*). Taken together, these results demonstrate that incorporation of rNMPs is highly dependent on the supply of individual dNTPs and that incorporated rNMPs are removed from nDNA by RER with an extremely high efficiency.

rNMPs Incorporated by the mtDNA Polymerase Are Not Repaired. In striking contrast to the nDNA where dNTP pool imbalances only altered the relative frequency of rNMPs in RER-deficient strains, the relative frequencies of rNMPs in mtDNA were equally affected in RER-proficient and RER-deficient backgrounds (Fig. 1, compare *E* and *F*). Furthermore, even mild dNTP pool imbalances led to altered frequencies of individual rNMPs in mtDNA in RER-proficient strains (Fig. 1 *A* and *E*, *cf rnr1-Y285F* and WT; see also Fig. S2). These observations suggest the absence of RNase H2 or any other rNMP repair mechanism in the mitochondria.

As was the case with nDNA, the changes in relative frequencies of rNMPs in mtDNA could be rationalized based on the size and balance of the dNTP pool. As expected, the most drastic changes were seen in the strains with the highest dNTP pool imbalances, such as *rnr1-Q288A* (rCMP frequency increased from 13 to 59%) and *rnr1-Y285A,Q288E* (rAMP frequency increased from 42 to 67%). However, in contrast to the nuclear genome where the dNTP pool imbalances of *rnr1-Y285F rnh201*Δ resulted in rNMP frequencies intermediate between WT and *rnr1-Y285A rnh201*Δ, the mitochondrial rNMP frequencies of *rnr1-Y285F rnh201*Δ and *rnr1-Y285A rnh201*Δ were virtually identical. One possible explanation for this observation is that the threefold increase in the dCTP pool in *rnr1-Y285F rnh201*Δ is sufficient to reduce the incorporation of rCMP in mtDNA to a minimum, whereby any further dCTP pool increase in *rnr1-Y285A rnh201*Δ has no additional effect. In line with the findings for nDNA, the balanced four- to fivefold increase in dNTPs in *rnr1-D57N* (Fig. 1*A*) did not lead to any significant difference in the pattern of mitochondrial rNMP incorporation (Fig. 1*E*). Therefore, the relative frequencies of the four rNMPs embedded in mtDNA are governed by the balance of the overall cellular dNTP pool and are not affected by the absence or presence of RNase H2. Together, these observations demonstrate that yeast mitochondria lack efficient mechanisms for the removal of rNMPs incorporated during replication.

In the Absence of RER, dNTP Pool Imbalances Cause Similar Changes in Incorporation of rNMPs in nDNA and mtDNA. Our experimental approach allowed us to compare the consequences of dNTP pool imbalances on incorporation of rNMPs into nuclear and mitochondrial DNA within the same population of cells. A positive correlation was observed for the relative frequencies of rCMP, rAMP, and rGMP in the nuclear and mitochondrial genomes in the RER-deficient background ($P < 0.001$) (*rnh201*Δ strains) (Fig. 2, *Lower*). No correlation was detected for rUMP, as the relative frequency of rUMP varied very little between strains (range from 7.3 to 25% in mtDNA and from 9.5 to 23% in nDNA). In contrast, no positive correlation was found for any of the four rNMPs in the RER-proficient background because of the efficient RER-mediated repair in the nuclear genome (*RNH201* strains) (Fig. 2, *Upper*).

The strong positive correlation between relative mitochondrial and nuclear rCMP, rAMP, and rGMP frequencies in the *rnh201*Δ background suggests that mutations in RNR affected dNTP pools both in the nucleus and in the mitochondria to a similar degree. Therefore, these results demonstrate that dNTP pool imbalances arising in the cytosol are transmitted equally well into the nuclear and mitochondrial compartments.

Overall dNTP Levels Determine the Amount of rNMPs in mtDNA. The relative rNMP frequencies in mtDNA were not changed in the *rnr1-D57N* strain, which has a proportional elevation in the levels of all four dNTPs (Fig. 1). This could be interpreted to mean that a balanced increase in all dNTPs does not affect rNMP incorporation because in such a strain none of the dNTPs is in relative shortage that could provoke an increase in rNMP incorporation. Alternatively, there might be less incorporation of all rNMPs in *rnr1-D57N*, but because all rNMPs are incorporated with the same relative frequency as in WT, no change is observed in the assay. Because mtDNA contains no defined ends, it is not possible to normalize mitochondrial rNMPs against chromosome ends to obtain the quantitative data required to distinguish between the above-mentioned possibilities. We therefore treated DNA preparations with PmeI that linearizes yeast mtDNA, creating DNA ends for quantification of mitochondrial rNMPs.

This approach was used to compare the level of rNMP incorporation in WT and *rnr1-D57N crt1*Δ *sml1*Δ, which we expected to have highly elevated dNTP pools because Crt1 and Sml1 are the transcriptional repressor and the protein inhibitor, respectively, of RNR. Indeed, we found that this triple mutant strain had a 10- to 15-fold increase in all dNTPs (Fig. 3*A*) and that this dNTP pool increase led to a reduction in the number of rNMPs in the mitochondrial genome (Fig. 3*B*, compare WT and *rnr1-D57N crt1*Δ *sml1*Δ). Furthermore, in accordance with the lack of RNase H2 function in mitochondria, incorporated rNMPs did not increase in mtDNA upon deletion of *RNH201*. In fact, we observed slightly fewer rNMPs in mtDNA of *rnh201*Δ cells, which is consistent with the mild increase in dNTP pools

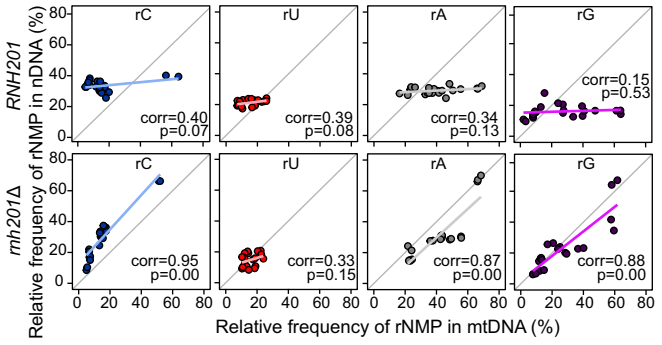

Fig. 2. The rNMP incorporation frequencies in mtDNA and nDNA correlate in strains deficient for RER. Scatter plots of the relative rNMP frequencies for each individual rNMP in the nuclear DNA (*y* axis) and mitochondrial DNA (*x* axis). (*Upper*) *RNH201*$^{+}$ strains. (*Lower*) *rnh201*Δ strains. Correlation coefficients and *P* values are indicated.

(Fig. 3*A*) caused by the modest checkpoint activation reported earlier in this strain (20). In support of the notion that the rNMP decrease in *rnh201Δ* is due to increased dNTP pools, the deletion of *RNH201* in the *rnr1-D57N crt1Δ sml1Δ* strain with already high dNTP pools did not further decrease the frequency of mitochondrial rNMPs.

In contrast, the inactivation of RER led to the expected increase in incorporated rNMPs in the nuclear genome (Fig. 3*B*, *Right*; compare *RNH201*$^{+}$ and *RNH201*$^{-}$ in the WT background) as previously reported (25). The increased dNTP pools in the *rnr1-D57N crt1Δ sml1Δ* strain decreased nuclear rNMP incorporation only in the *RNH201*$^{-}$ background (Fig. 3*B*, *Right*; compare WT to *rnr1-D57N crt1Δ sml1Δ* in the *RNH201*$^{+}$ vs. *RNH201*$^{-}$ background), which suggests that RER removes rNMPs from nDNA with very high efficiency. Therefore, in contrast to what we observed for mtDNA, nuclear rNMP incorporation could not be further reduced by increased dNTP pools in cells proficient in RER.

To visualize the quantitative HydEn-seq data using an independent approach, we performed Southern blot analysis of mtDNA after alkaline hydrolysis or incubation with recombinant RNase H2, treatments that cause strand cleavage at incorporated rNMPs. The average fragment size of alkali-treated mtDNA in strains with increased dNTP pools (*rnr1-D57N crt1Δ sml1Δ* and *rnr1-D57N crt1Δ sml1Δ rnh201Δ*) was markedly longer than in the control strains (Fig. 3*C*, left-hand side). Treatment with RNase H2 led to an identical outcome, verifying that the strand cleavage is indeed due to rNMPs (Fig. 3*C*, right-hand side). The fragment sizes after alkali- or RNase H2-treatment are consistent with a lower frequency of embedded rNMPs when dNTP pools are increased (1 rNMP per approximately 30,000 bases incorporated in *rnr1-D57N crt1Δ sml1Δ* and *rnr1-D57N crt1Δ sml1Δ rnh201Δ* vs. 1 rNMP per 4,500 bases in WT) (Fig. 3*D*). Taken together, the data presented in Fig. 3 *A*–*D* confirm that RNase H2 does not remove ribonucleotides incorporated in mtDNA during replication and that the amount of rNMPs incorporated into mtDNA is strongly reduced when the cellular dNTP pools increase.

Finally, we analyzed petite frequency as a readout of mtDNA stability in strains with normal or increased dNTP pools. The petite frequency of the *rnr1-D57N crt1Δ sml1Δ* and *rnr1-D57N crt1Δ sml1Δ rnh201Δ* strains with high dNTP pools was decreased compared with WT (Fig. 3*E*) and correlated well with the rNMP frequency of mtDNA (Fig. 3*B*). The observed reduction in petite frequency indicates that mtDNA stability is improved in yeast strains with increased dNTP pools and a reduced frequency of mtDNA rNMPs.

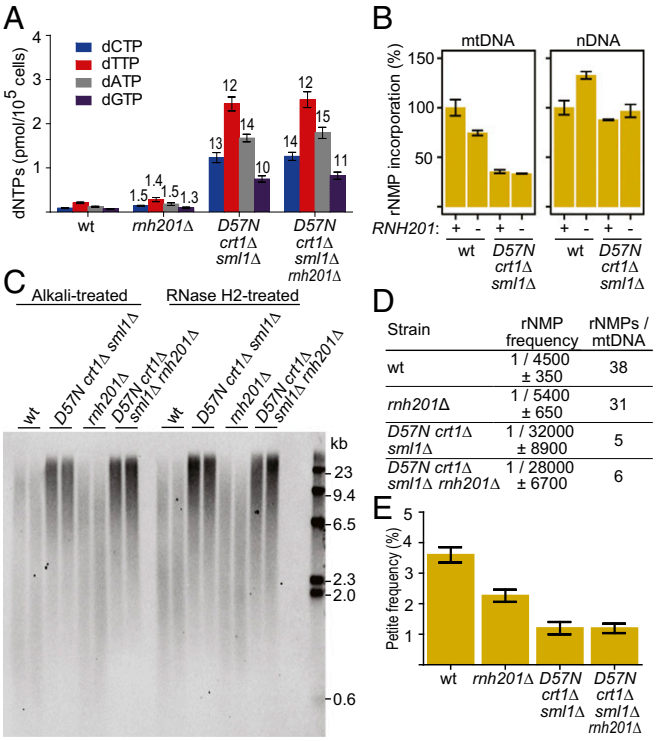

Fig. 3. The size of the cellular dNTP pool dictates the frequency of rNMP incorporation into mtDNA. (*A*) dNTP pools of the strains used for the experiment, presented as mean ± SEM ($n \geq 2$). The numbers above the bars indicate the fold-change in dNTP levels relative to WT. *D57N* refers to *rnr1-D57N*. See Fig. S1 for rNTP pools. (*B*) PmeI-digested total DNA from strains with normal (WT) or increased (*D57N crt1Δ sml1Δ*) dNTP pools was subjected to HydEn-seq to quantify rNMPs in the mtDNA and nDNA. The rNMP frequency of WT was set to 100%. The *RNH201* status is indicated by a + (*RNH201*$^{+}$) or − (*rnh201Δ*); $n \geq 2$. (*C*) Southern blot analysis of the *COX3* gene in mtDNA in strains with normal (WT) or increased (*D57N crt1Δ sml1Δ*) dNTP pools. The DNA from two independent isolates of each strain was cleaved at incorporated rNMPs using alkaline treatment or digestion with RNase H2. The size of DNA marker bands is indicated. (*D*) The average rNMP frequency was determined from the Southern blot in *C*, as described in *SI Materials and Methods*, and used to calculate the approximate number of rNMPs per 85-kb double-stranded mtDNA unit. The average rNMP frequency from alkali- and RNase H2-treated samples is given ±SD. (*E*) Petite frequency of strains with normal (WT) or increased dNTP pools (*D57N crt1Δ sml1Δ*) in backgrounds that are either proficient or deficient (*rnh201Δ*) for RER. Bars indicate the mean ± SEM; $n = 2$.

The mtDNA Polymerase Frequently Incorporates rNMPs. Inspection of the relative incorporation frequencies of the four rNMPs in the RER-deficient strain with a normal dNTP pool balance revealed differences between the two genomes: while nDNA contained mostly rCMP and rAMP, the most frequently embedded rNMPs in mtDNA were rAMP and rGMP (WT in Fig. 1 *D* and *F*). The rNMP frequency in mtDNA differed from that of nDNA even after normalization of the rNMP frequencies by the base composition of each respective genome, with rGMP overrepresented in mtDNA while rCMP was the most frequent rNMP in nDNA (Fig. 4*A*). The dissimilarity of the mtDNA and nDNA rNMP profiles is expected to derive either from differences in dNTP pool balance in the nucleus vs. the mitochondria or from a differential ability of the replicative polymerases in each compartment to discriminate against specific ribonucleotides. Because accurate measurement of the mitochondrial dNTP pool balance is challenging with current methods, we focused our attention on the properties of the DNA polymerases. We set out to characterize the rNTP discrimination ability of the yeast mtDNA polymerase Mip1.

The WT and exonuclease-deficient (D171A, E173A; exo^{-}) variants of Mip1 were overexpressed in *Escherichia coli* and purified to near homogeneity (Fig. 4*B* and Fig. S3*A*). We first tested the overall frequency of rNMP incorporation by Mip1 during replication of a primed single-stranded 3-kb substrate at physiological dNTP concentrations (15) and in the presence or absence of rNTPs. The products of these reactions were of similar length (Fig. S3*B*). Alkaline treatment of reaction products from rNTP-containing reactions resulted in a marked reduction in product size, indicating frequent rNMP incorporation by Mip1, while products of dNTP-only reactions were not alkali-sensitive (Fig. 4*C*). The median length of alkali-treated products was determined and used to calculate the rNMP incorporation frequency as previously described (17) (Fig. S3*C*). Both the WT and the exo^{-} variants of Mip1 incorporated rNMPs at an average frequency of approximately one rNMP per 600 nt, indicating that the proofreading activity does not significantly contribute to removing rNMPs incorporated by Mip1 (Table S2). This is in agreement with reports of inefficient or absent proofreading of rNMPs by other replicative DNA polymerases (24, 27, 28). The obtained rNMP incorporation frequency for Mip1 was comparable to that reported

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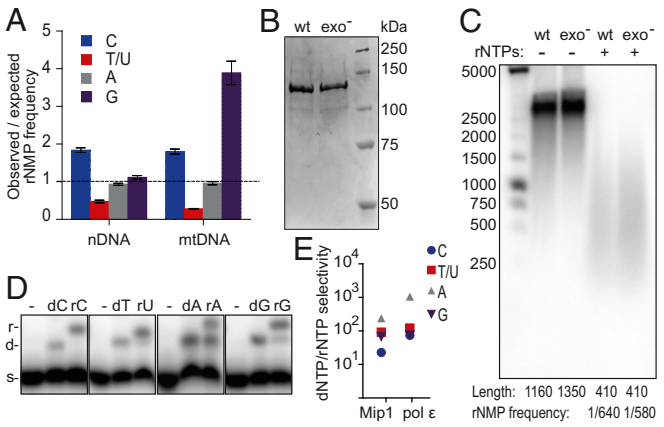

Fig. 4. Biochemical analysis of the rNMP incorporation properties of the yeast mtDNA polymerase Mip1. (*A*) The observed rNMP frequencies from Fig. 1 *D* and *F* were divided by the proportion of each base in nDNA and mtDNA, respectively. The base compositions used were: 19.2% C/30.9% T/30.9% A/19.2% G, and 8.6% C/41.5% T/41.5% A/8.6% G for nDNA and mtDNA, respectively. (*B*) SDS/PAGE analysis of the purified recombinant WT and exo⁻ Mip1. See also Fig. S3. (*C*) Replication assays were carried out on a 3-kb primed ssDNA template using WT or exo⁻ Mip1 in the presence or absence of rNTPs. Nucleotide concentrations corresponded to those measured from logarithmically growing yeast cells. Alkaline hydrolyzed products were separated on an alkaline agarose gel. The median lengths and the frequency of rNMP incorporation is indicated; see also Fig. S3 and Table S2. (*D*) Discrimination assay with exo⁻ Mip1. The rNMP product (r) has reduced mobility compared with the dNMP product (d). The substrate is marked with "s". (*E*) Average discrimination factors were calculated for Mip1 and pol ε based on a minimum of three incorporation reactions for each nucleotide.

for yeast pol δ and pol ε using a similar assay (1 rNMP per 720 and 640 nt, respectively) (17).

Next, we studied the ability of Mip1 to discriminate dNTPs from rNTPs during the insertion of a single nucleotide across its cognate base. Primer extension assays were carried out with exo⁻ Mip1 on a 15-mer primer annealed to a 34-mer template strand, and the reactions contained a single dNTP or rNTP at the concentration measured in unsynchronized, logarithmically growing yeast cells (15). When resolved on a denaturing polyacrylamide gel, products containing a single incorporated dNMP migrate faster than those with a single incorporated rNMP (15). Band intensities were quantified and used to calculate the discrimination factor for each dNTP/rNTP pair. Mip1 was found to discriminate most efficiently against rATP and least efficiently against rCTP (discrimination factors were 3.0×10^2 and 4.5×10^1, respectively) (Fig. 4 *D* and *E*). For comparison, we performed an identical analysis using the exo⁻ catalytic core of pol ε (29) and found it to be most selective against insertion of rATP (discrimination factor 1.0×10^3), while the other three dNTP/rNTP pairs had discrimination factors in the range of $1.2–1.8 \times 10^2$. The obtained values for pol ε were in good agreement with previously reported values (16).

The nucleotide selectivity profile of Mip1 shown in Fig. 4*E* does not explain the high relative frequency of rGMP in mtDNA in vivo (Fig. 4*A*) because the mitochondrial DNA polymerase did not appear to be especially poor at discriminating against rGMP in our assay conditions. This observation could reflect limitations of the discrimination assay, which could, for example, be affected by sequence context. Alternatively, the disparity between the in vivo and in vitro rNMP frequencies might indicate that the mitochondrial dGTP pool in vivo is actually lower than the measured total cellular dGTP pool, giving rise to an unexpectedly high frequency of rGMP in the mitochondrial genome.

Discussion

Mutations in enzymes involved in cellular dNTP metabolism are implicated in a number of mitochondrial diseases, including mitochondrial neurogastrointestinal encephalopathy, forms of mitochondrial depletion syndrome, and progressive external ophthalmoplegia (30). Some of the implicated enzymes, such as deoxyguanosine kinase and thymidine kinase 2, regulate only the intramitochondrial dNTP pool, while others [e.g., thymidine phosphorylase (TP) or the alternative small subunit of RNR (RRM2B)] regulate total cellular dNTP pools (1–4). At present, our understanding of the pathological mechanisms underlying these diseases is incomplete. For example, it is difficult to explain why defects in certain enzymes of dNTP metabolism affect some tissues but not others, or why disturbances in total dNTP pools caused by, for example, mutations in TP or RRM2B manifest as mitochondrial diseases without affecting nDNA. Although the defects in TP or RRM2B cause symptoms in terminally differentiated tissues not undergoing nDNA replication, dNTPs are required not only for mtDNA synthesis but also for repair of the estimated 10^4–10^5 DNA-damaging events that target the nuclear genome of each of our cells every day (31).

The majority of the dNTPs in dividing cells are synthesized in the cytosol by RNR as part of the de novo pathway, and in actively dividing human cells the mitochondrial dNTPs are primarily derived from the RNR-driven pathway with relatively minor contribution from the salvage pathway (32, 33). The situation is even more clear-cut in *S. cerevisiae*, which lacks the enzymes of the salvage pathway, making yeast mitochondria entirely dependent on de novo dNTP synthesis by RNR in the cytosol. This dependence is illustrated by the fact that increased RNR activity, obtained either by overexpressing RNR1 or by deleting its inhibitor Sml1, has been linked to an increase in the stability of mtDNA (34, 35), while hypomorphic mutations in RNR or overexpression of Sml1 decrease mtDNA stability (34, 36, 37).

In this study, we used a panel of yeast strains with distinct and permanent dNTP pool imbalances to simultaneously compare how the efficiencies of rNMP incorporation and repair differ between nuclear and mitochondrial DNA. We discovered that various dNTP pool imbalances result in robust changes in the rNMP incorporation pattern in mtDNA (Fig. 1 *E* and *F*) and that the frequency of rNMPs in the mtDNA is dictated by the size of the total cellular dNTP pool (Fig. 3). These results demonstrate that yeast mitochondria lack efficient mechanisms for the removal of rNMPs that are incorporated during replication. Furthermore, the changes in the relative levels of embedded rNMPs in the nDNA and mtDNA showed a strong positive correlation in the *mh201*Δ background, demonstrating that dNTP pool imbalances exert comparable effects on both genomes in the absence of RER (Fig. 2). In contrast, in the presence of functional RER, the rNMP incorporation pattern in nDNA was unresponsive to dNTP pool changes, indicating virtually complete removal of rNMPs embedded during replication of nDNA (Fig. 1*C*).

At the same time, our results indicate that dNTP pool imbalances that arise in *S. cerevisiae* during dNTP synthesis by RNR mutants in the cytosol are transmitted into the mitochondria and do not appear to be "filtered out" during mitochondrial import to maintain a balanced intramitochondrial dNTP pool. These findings support a view of unregulated nucleotide transport across the mitochondrial membrane and are in agreement with the rapid interchange of mitochondrial and cytosolic nucleotide pools in mammalian cultured cells put forward by Bianchi and coworkers (33, 38). However, the efficiency of import may vary for different dNTPs depending on the properties of their transporters, which could result in an intramitochondrial dNTP pool that differs in balance and concentration from that in the cytosol. It remains to be established whether the dGTP pool is relatively lower inside the mitochondria, as might be expected based on the high relative frequency of rGMP in the mtDNA (Fig. 4*A*). A further implication of the striking effect of dNTP pool alterations on mitochondrial rNMP incorporation is that it confirms that the frequent rNMPs found in the mitochondrial genome are incorporated during

replication and not derived from other processes, such as annealing of RNA transcripts to mature mtDNA.

The presented results also suggest that even mild alterations in the levels of individual dNTPs can modify the insertion frequency of the corresponding rNMP by both the nuclear and mitochondrial DNA polymerases (Fig. 1 *D–F*). In fact, comparison of *rnr1-Y285F* with the *rnr1-Y285A* strain that bears a similar but more extreme dNTP pool imbalance suggests that the threefold increase in dCTP in *rnr1-Y285F* is sufficient to reduce mitochondrial rCMP frequency to a minimum, whereby the further increase in dCTP levels in *rnr1-Y285A* has no apparent impact on the levels of rCMP in mtDNA. If the same applies to the other dNTP/rNTP pairs, the rNMPs incorporated in mtDNA can likely be reduced to a very low level by a more modest overall increase in dNTP pools than the 10- to 15-fold increase in the *rnr1-D57N crt1*Δ *sml1*Δ strain used in Fig. 3. It is worth noting that the relative frequency of incorporated rUMP did not decrease appreciably despite significant increases in the level of dTTP in strains, such as *rnr1-Y285A* or *rnr1-Y285A,Q288E* (Fig. 1 *D–F*). Because Mip1 was not better at discriminating against rUTP than the other rNTPs (Fig. 4 *D* and *E*), additional factors might contribute to the clearance of rUMP incorporated in yeast mtDNA.

It is tempting to speculate that the lack of rNMP repair in mitochondria, if conserved in humans, could help explain why some pathogenic mutations in enzymes of cellular dNTP metabolism lead specifically to mitochondrial disease. In theory, moderate reductions in levels of single or multiple dNTPs in the total cellular nucleotide pool could lead to the increased frequency of mitochondrial rNMPs and thereby instability of the mitochondrial genome, while nuclear rNMPs would be promptly removed by RER. Although single rNMPs are efficiently bypassed by the human mitochondrial DNA polymerase pol γ (39) and might therefore not pose a direct problem for mtDNA replication, their reactive 2′-hydroxyl groups put the DNA backbone at increased risk of cleavage.

Materials and Methods

See *SI Materials and Methods* for a more detailed discussion of materials and methods used.

Genome-Wide Mapping of Ribonucleotides in Vivo. Ribonucleotides in genomic DNA were mapped by alkaline hydrolysis and subsequent high-throughput sequencing of the 5′ ends (HydEn-seq), as previously described (25).

Data Availability. The sequencing data has been deposited in the Gene Expression Omnibus database under accession no. GSE100352.

ACKNOWLEDGMENTS. We thank Sushma Sharma for yeast strains; Erik Johansson and Pia Osterman for purified pol ε; and members of the A.C. laboratory for valuable discussions and comments. This project was supported by a grant from the Swedish Cancer Society (to A.C.); the Swedish Research Council Grants 2014-2262 (to A.C.) and 2014-6466 (to A.R.C.); the Swedish Foundation for Strategic Research Grant ICA14-0060 (to A.R.C.); and a grant from the Wallenberg Foundation (to S.W.). P.H.W. was supported by postdoctoral fellowships from the Swedish Society for Medical Research and the Swedish Cancer Society.

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