Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases

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Measurements of nucleoside triphosphate levels in ***Saccharomyces cerevisiae*** reveal that the four rNTPs are in 36- to 190-fold molar excess over their corresponding dNTPs. During DNA synthesis in vitro using the physiological nucleoside triphosphate concentrations, yeast DNA polymerase ***ε***, which is implicated in leading strand replication, incorporates one rNMP for every 1,250 dNMPs. Pol ***δ*** and Pol α, which conduct lagging strand replication, incorporate one rNMP for every 5,000 or 625 dNMPs, respectively. Discrimination against rNMP incorporation varies widely, in some cases by more than 100-fold, depending on the identity of the base and the template sequence context in which it is located. Given estimates of the amount of replication catalyzed by Pols α, ***δ***, and ***ε***, the results are consistent with the possibility that more than 10,000 rNMPs may be incorporated into the nuclear genome during each round of replication in yeast. Thus, rNMPs may be the most common noncanonical nucleotides introduced into the eukaryotic genome. Potential beneficial and negative consequences of abundant ribonucleotide incorporation into DNA are discussed, including the possibility that unrepaired rNMPs in DNA could be problematic because yeast DNA polymerase ***ε*** has difficulty bypassing a single rNMP present within a DNA template.

DNA replication | nucleotide precursors | nucleotide selectivity

The integrity of the eukaryotic genome is ensured in part by the chemical nature of the storage medium—DNA. Compared to RNA, DNA is inherently more resistant to strand cleavage due to the absence of a reactive 2' hydroxyl on the ribose ring. The active sites of most DNA polymerases are evolved to efficiently exclude ribonucleoside triphosphates (rNTPs) from being incorporated during DNA synthesis (reviewed in (1)). However, rNTP exclusion is not absolute. Early studies (reviewed in (1, 2)) revealed that DNA polymerases do incorporate rNMPs during DNA synthesis. Kinetic studies (3–13) have further demonstrated that selectivity for insertion of dNMPs into DNA rather than rNMPs varies from 10-fold to $>10^6$-fold, depending on the DNA polymerase and the dNTP/rNTP pair examined. rNMP incorporation during DNA synthesis is potentially made more probable by the fact that the concentrations of rNTPs in vivo are higher than are the concentrations of dNTPs (e.g., see refs. 2, 14 and results of this study). Thus some rNMPs are likely to be stably incorporated into DNA during replication, and possibly during DNA repair, e.g., nonhomologous end joining (NHEJ) of double strand breaks in DNA (9, 15). This possibility is supported by biochemical studies implicating RNase H2 and FEN1 in the repair of single ribonucleotides in DNA (16, 17). It is therefore of interest to know just how frequently rNMPs are incorporated into DNA by the DNA polymerases that synthesize the most DNA in a eukaryotic cell, namely DNA polymerases α, δ, and ε. Here we investigate this by first measuring the rNTP and dNTP concentrations in budding yeast. We then use these concentrations in DNA synthesis reactions in vitro to determine how frequently yeast Pols α, δ, and ε incorporate ribonucleotides into DNA. The results suggest that ribonucleotides may be incorporated into DNA in much higher amounts than previously appreciated, a possibility that has several implications.

Results and Discussion

dNTP and rNTP Pools. We first measured the amount of the four dNTPs and rNTPs in extracts prepared from logarithmically growing wild-type *S. cerevisiae* (18), and used the data to calculate (19) their concentrations in vivo. The results (Table 1) suggest that the concentrations of the dNTPs range from 12 to 30 μM. These results are similar to those reported in earlier studies of *S. cerevisiae*, albeit reported earlier in picomoles/cell number (18, 20). In comparison to dNTP concentrations, rNTP concentrations are much higher, ranging from 500 to 3,000 μM (Table 1). Thus, the rNTP:dNTP ratios range from 36:1 for cytosine to 190:1 for adenine. The amounts of rNTPs also greatly exceed those of dNTPs in mammalian cells (e.g., see ref. 14).

Discrimination Against rNMP Insertion. The selectivity with which Pols α, δ, and ε incorporate dNMPs as compared to rNMPs during DNA synthesis has not previously been reported. To survey all four rNTP:dNTP combinations with all three polymerases, we designed an assay (Fig. 1*A*) to examine the first step required for stable incorporation, insertion opposite a template base. Primer extension reactions were performed containing one correct dNTP or rNTP present at the concentration in Table 1. A typical result is shown in Fig. 1*B* using Pol δ, which inserts each of the four dNMPs and each of the four rNMPs, with the latter products having reduced mobility. Band intensities were used to calculate (see legend to Fig. 1*C*) the degree to which the polymerases prefer to insert dNMPs as compared to rNMPs. The results (Fig. 1*C*) indicate that all three polymerases strongly prefer to incorporate dNMPs. Selectivity varies by more than 1,000-fold, depending on the polymerase and the identity of the nucleotide. For Pol δ, selectivity ranges from 1.7×10^6 for dT/rU to 1×10^4 for dG/rG and dC/rC. The selectivity of Pol α (catalytic subunit only, to avoid rNMP insertion by its associated RNA primase) is slightly lower for three of the four nucleotides, and 220-fold lower for dT/rU. The selectivity of Pol ε is the lowest of the three enzymes, with only a 500-fold preference for inserting dC rather than rC.

The survey was validated for Pol δ by steady-state kinetics, the previous method used to determine selectivity for inserting dNMPs over rNMPs. The results (Table 2) yield selectivity values

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Table 1. Nucleotide concentrations in ***Saccharomyces cerevisiae***

dNTP	Concentration (μM)	rNTP	Concentration (μM)
dA	16	rA	3000
dC	14	rC	500
dG	12	rG	700
dT	30	rU	1700

The analysis was performed as described in Methods. The nucleotide concentrations were calculated based on a estimated value of 45 μm^3 for the volume of the soluble fraction of a haploid yeast cell, as described in (19). These measurements are for logarithmically growing cells. A previous study (18) indicated that the concentrations of dNTPs in S-phase cells are about twofold higher than the average concentration in logarithmically growing cells.

of 1.6×10^4 for dA/rA and 1.3×10^4 for dC/rC, in agreement with the selectivity obtained in the survey (Fig. 1*C*). The selectivity of the yeast polymerases was also compared to kinetic values (Fig. 1*C*) for two other B family polymerases, RB69 Pol (8) and ϕ29 Pol (6). These insertion data illustrate that discrimination against rNMP insertion is high, but not absolute.

Stable Incorporation of rNMPs into DNA. The above measurements are for reactions containing one nucleotide being inserted at one template position, and the approach only considers initial insertion. To determine if Pols α, δ,and ε stably incorporate rNMPs into duplex DNA, reactions were performed to extend a 40-mer primer hybridized to a 70-mer template (Fig. S1*A*). DNA products were then subjected to alkaline hydrolysis under conditions that completely hydrolyze the DNA backbone at positions where a ribonucleotide is present (Fig. S1*B* and *C*). Three reactions were performed (Fig. S1*D*). The first contained only the four dNTPs, at the concentrations in Table 1. The second contained the four dNTPs plus the four rNTPs, again at the concentrations in Table 1. The third reaction was like the second except that the concentration of the dNTPs was increased 10-fold. Primer extension generated a large proportion of full-length products (e.g., for Pol α, see first lane in Fig. S1*D*). These were separated from shorter products by PAGE, excised from the gel and recovered. Equivalent amounts of the purified products were untreated or treated with alkali, and the resulting products were separated by PAGE (Fig. S1*D*).

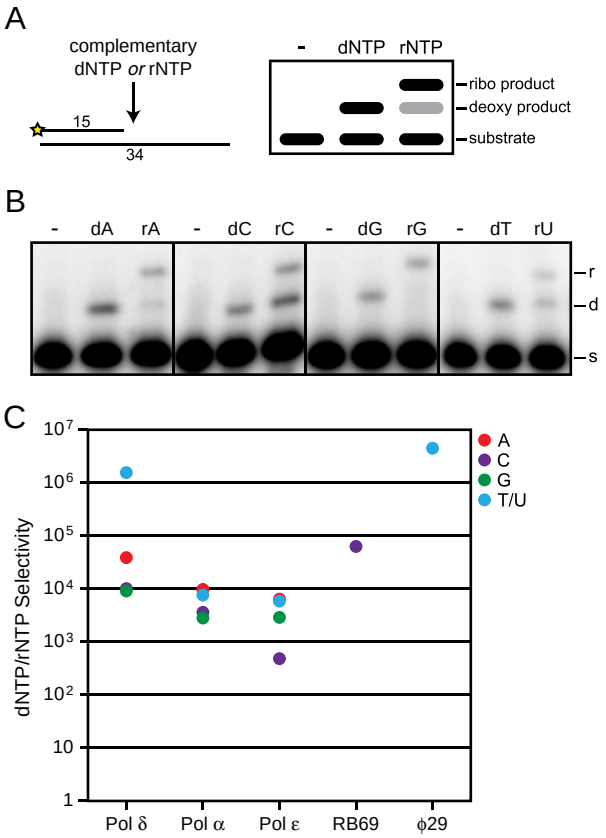

Fig. 1. Discrimination against rNMP insertion by yeast DNA polymerases. (A) Schematic of discrimination assay. The ribonucleotide product has reduced mobility compared to the deoxynucleotide product. Reactions containing rNTPs have trace amounts of dNTPs that are incorporated (faint gray band in lane 3). (B) Results with exonuclease-deficient Pol δ. (s), substrate, (d), deoxy product, (r), ribo product. (C) Discrimination against rNMP insertion. Discrimination factors were calculated by dividing the percentage of dNTP product by the percentage of rNTP product, and then multiplying by the ratios of nucleotide concentrations and differences in enzyme concentrations and reaction times. For Pol ε, the previously characterized N-terminal catalytic fragment (49) was used. For comparison, the selectivity of RB69 (8) and ϕ29 (6), two B family polymerases are shown.

Table 2. Steady-state kinetic analysis of dNTP:rNTP discrimination by yeast Pol *δ*

d/rNTP	K_{cat} (s^{-1})	K_m (μM)	Catalytic efficiency	Discrimination factor
dATP	0.25	71	3.5×10^{-3}	16,000
rATP	0.00032	1400	2.2×10^{-7}	
dCTP	0.090	38	2.4×10^{-3}	13,000
rCTP	0.0011	6100	1.9×10^{-7}	

The analysis was performed as described in Methods, using exonuclease-deficient, three-subunit Pol δ.

As expected, the full-length products of the Pol α reaction that contained only dNTPs were not sensitive to alkali (Fig. S1*D*). In contrast, when the products of reactions containing both dNTPs and rNTPs were treated with alkali, 4% of the total products were hydrolyzed (Fig. S1*D*), and bands of varying intensities were observed at 25 positions along the template sequence. Thus, Pol α inserts rNMPs and then extends the resulting termini to incorporate rNMPs into complete DNA chains, and it does so at numerous positions but with variable efficiency. That the dNTPs and rNTPs compete with each other for incorporation is demonstrated by the weaker band intensities observed upon alkali treatment of products of reactions containing a 10-fold higher concentration of the dNTPs (Fig. S1*D*). Similar results were obtained for reactions catalyzed by Pols δ and ε (Fig. S2).

The results for reactions containing all eight nucleotides are shown for all three polymerases in Fig. 2*A*. Quantification reveals that 0.5% of the products generated in the Pol δ reaction were hydrolyzed by alkali (Fig. S2). Over the 25 template positions examined, the average number of rNMPs incorporated per template base copied is therefore 0.02% (*green* value below second lane in Fig. 2*A*), i.e., Pol δ incorporates one rNMP for every 5,000 dNMPs. In the reaction containing 10-fold higher dNTP concentrations, Pol δ incorporated one rNMP for every 75,000 dNMPs. This demonstrates that the rNTPs are effectively competing with the dNTPs for incorporation. On this basis, we calculated the selectivity of Pol δ for dNMP incorporation if all eight nucleoside triphosphates were present at equimolar nucleotide concentrations. Taking into account the ~100-fold higher concentration of rNTPs in the reactions containing all eight nucleotides, the selectivity of Pol δ would be 5×10^5. This value is largely in agreement with the insertion selectivity measured in the survey (Fig. 1*C*) and kinetically (Table 2), especially when one takes into account the rNMP incorporation variability observed over 25 template positions. By comparison, total rNMP incorporation by Pol ε was 2% (and 0.2% with 10X dNTPs), equating to one rNMP for every 1,250 dNMPs incorporated (0.08 value in *blue* in Fig. 2*A*). Pol α exhibited the lowest selectivity, with 4% total rNMP incorporation (0.7% with 10X dNTPs), an average

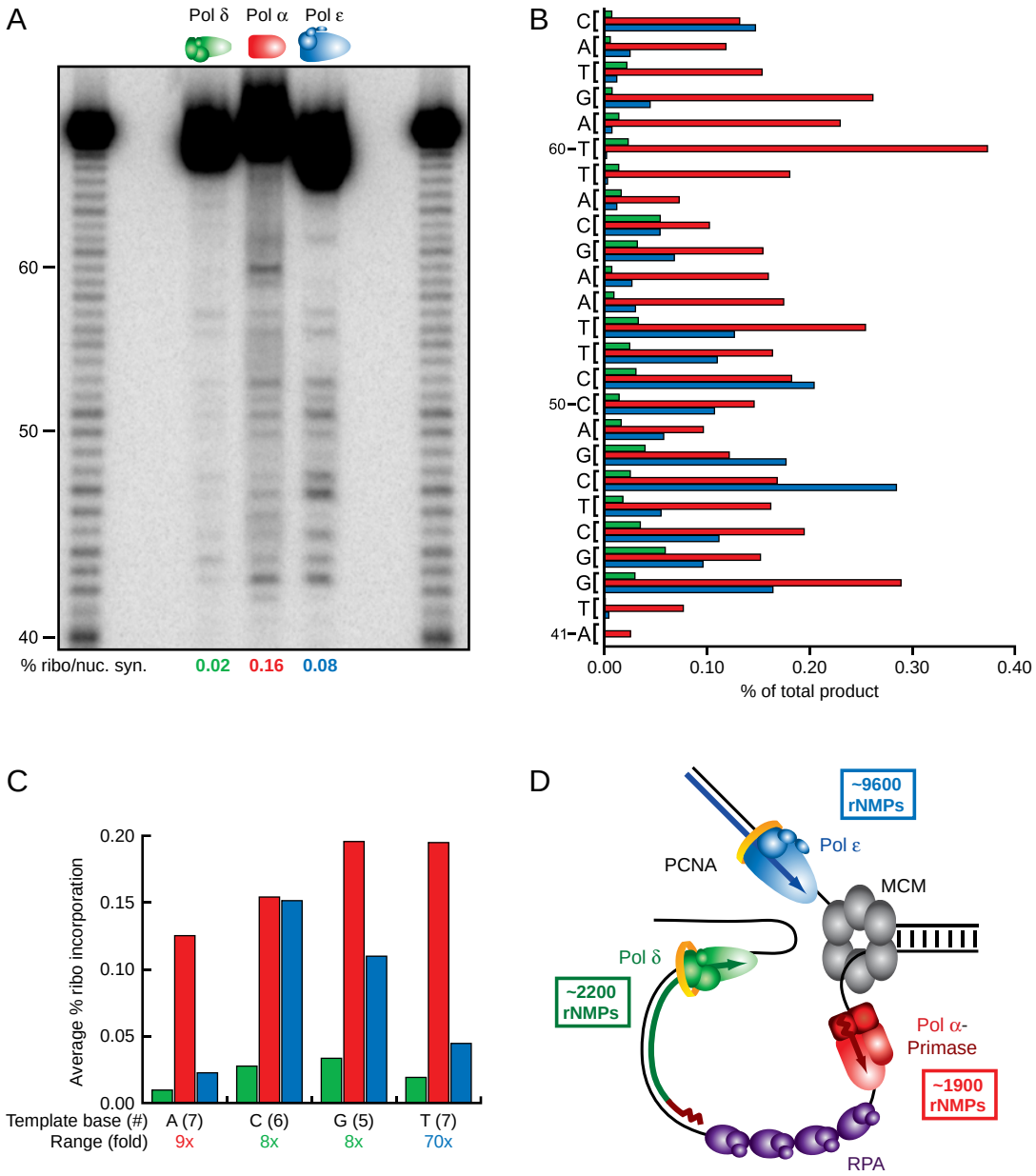

Fig. 2. Stable incorporation of rNMPs into DNA by yeast DNA pols. (A) Alkali cleavage products of reactions with all eight NTPs at cellular concentrations are shown for Pol δ, Pol α, and Pol ε. The frequency of rNMP incorporation per nucleotide synthesized is indicated below each lane. Marker lanes on either side allow determination of the template position for rNMP incorporation. (B) Frequency of rNMP incorporation by Pol δ (green bars), Pol α (red bars), and Pol ε (blue bars) at each of 25 template positions. (C) Average frequency of rNMP incorporation by Pol δ (green bars), Pol α (red bars), and Pol ε (blue bars) according to template base identity. The largest range in rNMP incorporation frequency is shown below each template base, color-coded according to polymerase. (D) Model of a replication fork with the potential number of rNMPs incorporated by each polymerase.

of one rNMP incorporated for every 625 dNMPs incorporated (0.16 value in *red* in Fig. 2*A*).

rNMP Incorporation Varies by Nucleotide, Sequence Context, and Polymerase. Studies of rNMP insertion (3–13) have typically examined few template positions (usually one) and a subset (one or two) of the four nucleotides. The results here greatly expand the view of the selectivity of DNA polymerases by providing direct comparisons of rNMP incorporation by all three replicative polymerases at all four template bases, each in several different sequence contexts. The results reveal that rNMP incorporation varies widely along the template (Fig. 2*A*–*C*), as a function of the polymerase, the identity of the template base and the sequence context.

Are rNMPs the Most Common NonCanonical Nucleotide Placed into DNA? rNMPs were stably incorporated by wild-type yeast replicative polymerases, i.e., Pols δ and ε contain their noncatalytic accessory subunits and their 3′ exonucleases are intact. Also, the DNA synthesis reactions contain all eight nucleoside triphosphates at physiological concentrations. Thus it is reasonable to consider the rNMP incorporation data in light of a model for the division of labor among the three polymerases at the replication fork in vivo (21–23). In this model, Pol ε performs the bulk of leading strand replication, i.e., 50% of the yeast genome, or 1.2×10^7 nucleotides. If Pol ε were to incorporate one rNMP for every 1,250 dNMPs in vivo, it would introduce 9,600 rNMPs into the genome during each round of leading strand DNA replication (Fig. 2*D*). Pol δ and Pol α perform approximately 90% and

10% of lagging strand replication, respectively. Given the results in Fig. 2*A*, these polymerases could theoretically introduce 2,200 and 1,900 rNMPs into the genome during each round of lagging strand DNA replication. The total is more than 13,000 rNMPs, about 70% of which would be incorporated during leading strand replication. If homologous mammalian polymerases behave similarly (currently untested), then replication of the mammalian nuclear genome, which is 500 times larger than the *S. cerevisiae* nuclear genome, would introduce several million rNMPs into the genome. Thus rNMPs could be the most common abnormal nucleotides initially placed into nuclear genomes, potentially exceeding the abundance of commonly studied DNA lesions such as abasic sites and 8-oxo-guanine (24).

Bypass of a Single rNMP in a DNA Template. We next asked if an unrepaired rNMP in a DNA template impedes DNA synthesis by a replicative DNA polymerase. While Pol ε efficiently copies a normal DNA primer-template (Fig. 3), it has difficulty copying the equivalent template when it contains a single rG (Fig. 3). Relative to the fully DNA template, incorporation is problematic for insertion opposite the rG, and for four additional incorporations.

Implications. Replication dogma (2) teaches that DNA polymerases cannot perform de novo synthesis, but must start from primers that are often RNA chains synthesized by RNA primases, such as the one that copurifies with Pol α. Numerous studies have investigated how these RNA primers are removed during maturation of Okazaki fragments on the lagging strand (25). The present study suggests that an additional burden may be to remove rNMPs incorporated by DNA polymerases because they have imperfect dNTP selectivity, compounded by the naturally higher abundance of rNTPs as compared to dNTPs. That rNMPs are likely to be incorporated by DNA polymerases in vivo is suggested by biochemical studies implicating a type 2 human RNase H in the repair of single ribonucleotides present in DNA (16, 26). A similar role has been proposed for *S. cerevisiae* RNase H(35), based on the fact that it can incise the DNA backbone on the 5′-side of a single ribonucleotide in duplex DNA (17), and the fact that it prefers a single ribose in DNA as a substrate in comparison to a stretch of riboses. If rNMPs are incorporated at anywhere near the abundance suggested by the present data, then there may be redundant repair pathways for removing rNMPs incorporated by DNA polymerases. This is analogous

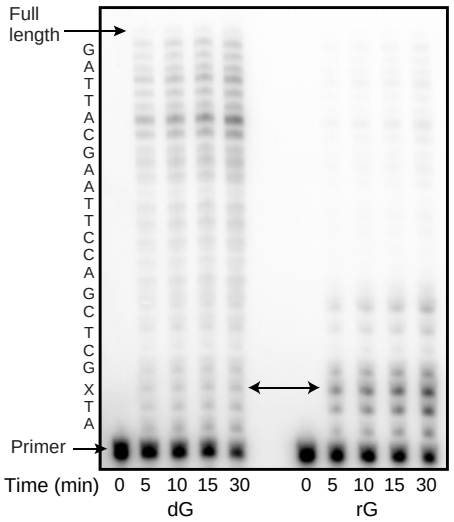

Fig. 3. Bypass of a single rNMP in a DNA template. The analysis was performed as described in Methods. The sequence on the left is that of the template strand. The X marks the location of dG or rG in the template. For the lane marked "0" min, no enzyme was added.

to the multiple ways to process the 5′ ends of Okazaki fragments initiated by RNA primase (25), and the multiple pathways that contribute to the repair of other common lesions in DNA, such as abasic sites and 8-oxo-guanine (24, 27). Multiple eukaryotic ribonucleases H exist to process RNA/DNA hybrids that may form during replication and repair (reviewed in (28)).

DNA polymerases are known to incorporate damaged dNMPs with ambiguous coding potential, as well as undamaged nucleoside triphosphates that retain more normal coding potential. An example of the latter is dUTP, which is present in small amounts in cellular dNTP pools and has been estimated to be incorporated opposite template adenine perhaps 2,000 times per nuclear genome replication in mice (29). Once incorporated into DNA, dUMP is efficiently repaired by base excision repair, and failure to remove it when paired opposite adenine has little consequence because uracil codes like thymine. Theoretically, rNMPs incorporated during replication may also be tolerated well, to the extent they too may retain relatively normal base coding potential. However, unrepaired rNMPs in duplex DNA may not be completely innocuous, because ribonucleotides in duplex DNA can promote a *B*- to *A*-form conversion (30, 31). This could influence DNA replication because efficient and accurate DNA synthesis by replicative DNA polymerases depends on DNA helix geometry. Indeed, the experiment in Fig. 3 reveals that a rNMP in a DNA template strand slows synthesis by a polymerase that participates in leading strand DNA replication (21). This may be related to the finding that deletion of RNase H(35) increases the sensitivity of *S. cerevisiae* to hydroxyurea (32), an inhibitor of replication that reduces dNTP pools and alters the dNTP:rNTP ratio.

An unrepaired rNMP in DNA could also be mutagenic despite the fact that the base of the rNMP may have normal Watson-Crick coding potential. A structural study has shown that a 3′-terminal ribose promotes a *B*- to *A*-form conversion (33), potentially resulting in a primer terminus that is more difficult to extend than normal. We previously proposed a model (34), for which there is considerable evidence (see ref. 35 and references therein), wherein "difficult to extend" termini can rearrange to misaligned intermediates with normal, correct terminal base pairs that, upon further extension, yield insertion/deletion mutations. Consistent with this possibility are studies (36, 37) demonstrating that a yeast *rnh35* strain has a spontaneous mutator phenotype. In those studies, the mutator effect was suggested to result from defective processing of RNA primers at the 5′-ends of Okazaki fragments. However, it may be that at least some of the observed mutagenesis resulted from replication of templates containing unrepaired rNMPs that were incorporated by Pols α, δ, and/or ε. It remains to be determined if rNMPs in DNA might stall replication to the extent needed to induce cellular stress responses or double strand DNA breaks. Another possibility worth investigating is whether unrepaired rNMPs in DNA reduce the efficiency of transcription, since transcription is impaired by lesions in DNA (reviewed in (38)). Inhibition could be more likely if there are sequence contexts in the genome that are particularly prone to rNMP incorporation. Variations exceeding 100-fold are apparent from the current survey of only 25 positions (Fig. 2). This is a very small target compared to the size of nuclear genomes, leading one to wonder if rNMP incorporation is even more prevalent in certain sequence contexts (repetitive sequences, non *B*-DNA), and if so, with what consequences.

The idea that rNMP incorporation into DNA may be more common than previously appreciated leads one to wonder about possible benefits of rNMP incorporation. For example, DNA polymerase μ incorporates rNMPs into DNA to such an extent that it is suggested to use rNTPs as normal precursors during NHEJ of double strand DNA breaks in the G1 phase of the cell cycle, when dNTP concentrations are particularly low (9). Here it may be relevant that ligases involved in NHEJ prefer to seal

strand breaks containing a monoribonucleotide on the 3′-OH end (9, 15). Extending this logic, it is theoretically possible that rNMPs may be incorporated into DNA during repair synthesis performed by either Pol δ or Pol ε, e.g., during mismatch repair, nucleotide excision repair, or break-induced recombination. The present study could also be relevant to mating-type switching in *Schizosaccharomyces pombe*. This switching depends on a Pol α-dependent imprint consisting of two ribonucleotides introduced into the lagging strand DNA template during S phase. The imprint is maintained until the following S phase, where it stalls leading strand replication, thereby inducing recombination that leads to mating-type switching (see ref. 39 and references therein). One possible origin for a ribonucleotide imprint is lagging strand DNA replication, perhaps involving RNA primase or rNMP incorporation by Pol α, which has the highest "per-nucleotide" rNMP incorporation capacity of the three replicative polymerases (Fig. 2*A*).

The average selectivity of Pol δ and Pol ε is somewhat higher than that of Pol α (Fig. 2*A*). Pol ε and Pol δ have intrinsic 3′ exonuclease activities that proofread mismatched primer termini to prevent mutations, and might also excise primer terminal ribonucleotides. One study (40) has demonstrated that the 3′ exonucleases of Klenow fragment, T4 and T7 DNA polymerases can remove an rNMP from a 3′ terminus. If the 3′ exonucleases of Pol δ and Pol ε also excise rNMPs, it would interesting to know if the phenotypes of yeast strains, or the cancer susceptibility (41) of mice that lack the 3′ exonuclease activity of Pol ε and Pol δ, may at least partly reflect failure to excise rNMPs during replication. Also of interest is the fact that wild-type, 4-subunit Pol ε holoenzyme can incorporate an average of one rNMP per 1,250 bases of newly synthesized DNA. Based on the idea that Pol ε has a prime role in leading strand replication (21, 22), most of these rNMPs may be introduced into the nascent leading strand. Even if rNMPs were present only transiently and eventually replaced with dNMPs by DNA repair, this density for a potentially helix-distorting base pair might serve a transient signaling function. Possibilities include signaling for mismatch repair, nucleosome loading behind the replication fork, chromatin remodeling, and gene silencing. The presence of a single ribonucleotide in DNA has been shown to reduce nucleosome formation (42 79), and several studies (reviewed in (43)) have shown that Pol ε is involved in gene silencing and one of its noncatalytic subunits is involved in chromatin remodeling. Moreover, mutations in *S. pombe cdc22*, and *tds1* genes, encoding the large subunit of ribonucleotide reductase and a putative thymidylate synthase, respectively, cause spreading of silencing across heterochromatic barriers in the mating-type switching region (44). These two genes regulate dNTP pools, such that defects in *cdc22* and *tds1* may alter dNTP:rNTP ratios to promote increased incorporation of rNMPs, offering one theoretical mechanism by which epigenetic changes may be modulated.

Materials and Methods

Determination of dNTP and NTP Pools. At a density from 0.4×10^7 to 0.5×10^7 cells/mL, $\sim 3.7 \times 10^8$ cells were harvested by filtration through 25 mm White AAWP nitrocellulose filters (0.8 mm, Millipore AB). The filters were immersed in 700 μL of ice-cold extraction solution (12% w/v trichloroacetic acid, 15 mM $MgCl_2$) 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, 700 μL supernatants were collected after centrifugation at 20,000 x g for 1 min, and added to 800 μL of ice-cold Freon-trioctylamine mixture consisting of 10 mL of Freon (1,1,2-trichlorotrifluoroethane, Aldrich, Sigma-Aldrich Sweden AB >99%) and 2.8 mL of trioctylamine (Fluka, Sigma-Aldrich Sweden AB,>99%). The samples were vortexed and centrifuged for 1 min at 20,000 x g. The aqueous phase was collected and added to 700 μL of ice-cold freon-trioctylamine mixture. 475 μL and 47.5 μL of the aqueous phase were collected. The 475 μL aliquots of the aqueous phase were pH adjusted with 1 M NH_4HCO_3 (pH 8.9) and loaded on boronate columns (Affi-Gel 601, BioRad) to separate dNTPs and NTPs. Purified dNTP samples were adjusted to pH 3.4 with 6M HCl, separated on a Partisphere SAX-5 HPLC column (4.6 × 25 cm, PolyLC Inc.) and quantified using a UV-2075 Plus detector (Jasco). 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.

Discrimination Against NTP Insertion. Insertion of dA/rA and dG/rG were analyzed using a substrate made by annealing a ^{32}P-labeled primer strand (5′- CTGCAGCTGATGCGC) to a template strand (5′-GTACCCGGGGATCCG-TAC(T/C)GCGCATCAGCTGCAG) that either contained a T or a C at the templating position for the incoming nucleotide. Insertion of dC/rC and dT/rU were analyzed using a substrate made by annealing a 5′-^{32}P-labeled primer strand (5′- CTGCAGCTGATGCGA) to a template strand (5′-GTACCCGGG-GATCCGTAC(G/A)TCGCATCAGCTGCAG) that contained a G or an A at the templating position for the incoming nucleotide. Reaction mixtures (10 μL) contained 100 nM DNA substrate. For Pol δ, the reaction buffer contained 20 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, and 8 mM Mg acetate. For Pol α, the reaction buffer contained 20 mM Tris (pH 8.0), 200 μg/mL BSA, 2 mM DTT, and 10 mM $MgCl_2$. For Pol ε, the reaction buffer contained 40 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 100 mM NaCl, and 8 mM Mg acetate. A dNTP or rNTP was included at its measured cellular concentration. Reactions were initiated by adding polymerase and incubation was at 30 °C. Polymerase concentrations varied from 0.2 to 10 nM and incubation times varied from 1–20 min, in all cases resulting in extension of less than 20% of the initial primer. Reactions were terminated by adding an equal volume of formamide loading dye, and analyzed by electrophoresis in a denaturing 15% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software (Molecular Dynamics).

Kinetic Analysis of NTP Insertion. Kinetics of dA/rA and dC/rC insertion by exonuclease-deficient Pol δ were analyzed using the substrates described above for discrimination against rNMP insertion. Reactions (10 μL) were performed with 1,000 femtomoles (100 nM) DNA substrate in reaction buffer containing 20 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, and 8 mM Mg Acetate. Each experiment tested at least six different concentrations of the complementary dNTP or rNTP, with the concentration of polymerase and reaction time varied to obtain steady-state conditions. Reaction products were separated on a 15% denaturing polyacrylamide gel, and radiolabeled products were detected and quantified with a PhosphorImager and ImageQuaNT software (Molecular Dynamics). K_m and k_{cat} values were calculated as described (45).

Stable Incorporation of rNMPs into DNA. Four-subunit Pol ε (46), three subunit Pol δ (47) and the catalytic subunit of Pol α (48) were purified as described. Stable incorporation of rNMPs by the replicative polymerases was analyzed using a substrate made by annealing a 40-mer ^{32}P-labeled primer strand (5′-CCAGTGAATTTCTGCAGGTCGACTCCAAAGGTCAACCCGG) to a 70-mer template strand (5′-ATGACCATGATTACGAATTCCAGCTCGGTACCGGGTT GA-CCTTTGGAGTCGACCTGCAGAAATTCACTGG). Reaction mixtures contained 100 nM DNA substrate and the reaction buffer for each polymerase described above. Nucleotide substrates were added at cellular concentrations (Table 1), and contained only the four dNTPs, all eight nucleotides, or all eight nucleotides with the dNTP concentrations increased 10-fold over the concentrations in Table 1. Reactions were initiated by adding 10 nM Pol α, 2 nM Pol ε, or 40 nM Pol δ. Incubation was at 30 °C. Reactions were terminated after 30 min by adding an equal volume of formamide loading dye, and were separated in a denaturing 8% polyacrylamide gel. Full-length reaction products were identified by exposing the gel on x-ray film, and were excised and purified. Equivalent amounts of recovered products (as determined by scintillation counting) were treated with either 0.3 M KCl or 0.3 M KOH for 2 h at 55 °C. Following addition of an equal volume of formamide loading dye, equivalent amounts of pre- and postexcision samples were analyzed by electrophoresis in a denaturing 8% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software (Molecular Dynamics).

Bypass of a Single rG in a DNA Template. Reactions were performed to copy a 65-mer template, 5′-CATGATTACGAATTCCAGCTCGXTACCGGGTTGACCTTT-GGAGTCGACCTGCAGAA ATTCACTGG (where X = dG or rG) annealed to a 5′-^{32}P-labeled 40-mer primer (3′-GGCCCAACTGGAAA CCTCAGCTGGACGT-CTTTAAGTGACC). Reaction mixtures (30 μL) contained 2.8 picomoles (93 nM) DNA and 1.6 fmol Pol ε in a reaction buffer of 40 mM Tris (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 100 mM NaCl, 8 mM Mg Acetate, and

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10 μM dNTPs. All components except the polymerase were mixed on ice and then incubated at 30 °C for 1 min. The polymerase was added to initiate the reaction and aliquots were removed at 5, 10, 15, and 30 min. An equivalent volume of formamide loading dye was added to terminate the reaction. The products were heated to 95 °C for 3 min and separated by electrophoresis through an 8% denaturing polyacrylamide gel containing 25% formamide. A PhosphorImager (Molecular Dynamics) was used to visualize the products.

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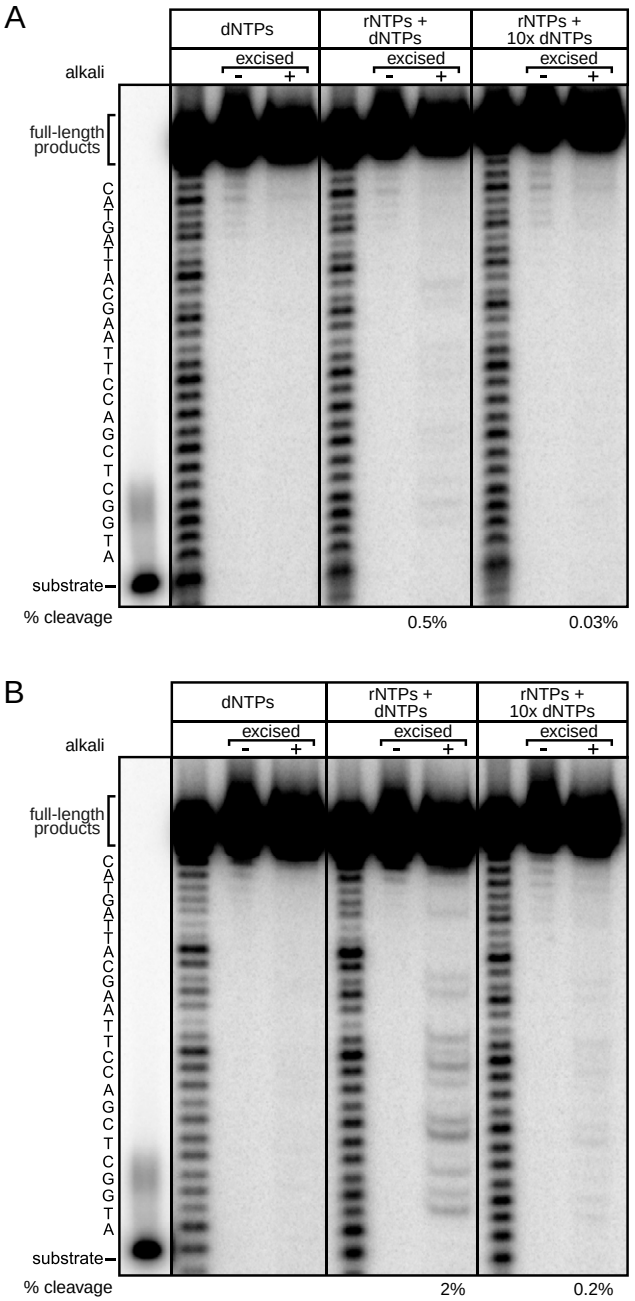

Fig. S2. The extension products of Pol δ (A) and Pol ε (B) were analyzed for ribonucleotide incorporation using the alkali cleavage assay (Fig. S1A), in polymerization reaction mixtures containing either the four dNTPs only, all eight NTPs, or all eight NTPs with the four dNTP concentrations all increased 10-fold over cellular concentrations. The percentage of alkali sensitive product is indicated below each image. For these experiments, full-length, exonuclease proficient polymerases were used (3-subunit Pol δ, 4-subunit Pol ε).