Please cite this article in press as: Sparks et al., RNase H2-Initiated Ribonucleotide Excision Repair, Molecular Cell (2012), http://dx.doi.org/10.1016/j.molcel.2012.06.035

Molecular Cell
Short Article

RNase H2-Initiated Ribonucleotide Excision Repair

Justin L. Sparks,[1] Hyongi Chon,[2] Susana M. Cerritelli,[2] Thomas A. Kunkel,[3] Erik Johansson,[4] Robert J. Crouch,[2] and Peter M. Burgers[1,*]
[1]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA
[2]Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD 20892, USA
[3]Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA
[4]Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87, Umeå, Sweden
*Correspondence: burgers@biochem.wustl.edu
http://dx.doi.org/10.1016/j.molcel.2012.06.035

SUMMARY

Ribonucleotides are incorporated into DNA by the replicative DNA polymerases at frequencies of about 2 per kb, which makes them by far the most abundant form of potential DNA damage in the cell. Their removal is essential for restoring a stable intact chromosome. Here, we present a complete biochemical reconstitution of the ribonucleotide excision repair (RER) pathway with enzymes purified from *Saccharomyces cerevisiae*. RER is most efficient when the ribonucleotide is incised by RNase H2, and further excised by the flap endonuclease FEN1 with strand displacement synthesis carried out by DNA polymerase δ, the PCNA clamp, its loader RFC, and completed by DNA ligase I. We observed partial redundancy for several of the enzymes in this pathway. Exo1 substitutes for FEN1 and Pol ε for Pol δ with reasonable efficiency. However, RNase H1 fails to substitute for RNase H2 in the incision step of RER.

INTRODUCTION

DNA polymerases show an extraordinary ability to distinguish deoxyribonucleotide from ribonucleotide precursors for DNA replication (reviewed in Joyce [1997]). Replicative DNA polymerases typically show a specificity ratio of $\sim 10^{-4}$-10^{-5} (rNTPs incorporated per dNTP incorporated). However, because the cellular concentrations of rNTPs in yeast are estimated to be 10- to 100-fold higher than those of dNTPs (Nick McElhinny et al., 2010b), the actual misincorporation of ribonucleotides into genomic DNA is likely to be much higher as well and could reach ratios on the order of 10^{-3}. Compared to a deoxyribonucleotide, a ribonucleotide has a reactive 2′ hydroxyl on the sugar moiety that renders the DNA backbone more susceptible to strand cleavage. This could potentially reduce genome stability and affect its replication during a subsequent cell cycle (Nick McElhinny et al., 2010a; Watt et al., 2011). Therefore, repair mechanisms exist to deal with ribo-damage. The one initiated by ribonuclease H2 (RNase H2), called *r*ibonucleotide excision *r*epair (RER), is considered to be the most common repair pathway.

Ribonuclease H enzymes cut RNA/DNA hybrids. RNase H1 is most active on RNA/DNA hybrids containing several consecutive ribonucleotides, and no incision activity has been observed on a DNA substrate with a single embedded ribonucleotide (Cerritelli and Crouch, 2009). Consecutive RNA residues are not expected to result from the occasional, stochastic insertion of ribonucleotides by DNA polymerases, or at least these occurrences should be extremely rare. On the other hand, RNase H2 shows prominent activity on a single ribonucleotide embedded within double-stranded DNA (Eder and Walder, 1991; Rychlik et al., 2010). RNase H2 incises the DNA 5′ of the ribonucleotide, generating DNA containing 3′-hydroxyl and 5′-phospho-ribonucleotide ends (see Figure 4). This product is expected to be an ideal substrate for the Okazaki fragment maturation system that normally deals with 5′-RNA-terminated DNA fragments (Burgers, 2009), however, whether repair actually proceeds by this or a similar pathway remains to be determined. Ribonucleotide-containing DNA can also be incised by Topoisomerase I (Kim et al., 2011; Sekiguchi and Shuman, 1997), whose reaction mechanism proceeds through a 3′-phospho-tyrosyl intermediate. If this covalent intermediate is localized at the ribonucleotide, it can be resolved aberrantly through nucleophilic attack by the vicinal 2′-OH group resulting in the generation of a 2′-3′-cyclic phosphate with concomitant release of Topo I generating a single-strand break. Currently, it is not clear whether or how these cyclic phosphate intermediates are processed. However, based on genetic analysis in yeast, Topo I-mediated cleavage is likely a minor pathway for ribo-repair (Kim et al., 2011).

Mutations in RNase H2 are associated with Aicardi-Goutières syndrome, an autosomal recessive disorder. Patients have progressive microencephaly and show complex neurological defects in motor function and altered interferon response (Crow et al., 2006). However, defects in other enzymes, such as the 3′-exonuclease TREX1 (Stetson et al., 2008), and SAMHD1, a dNTP triphosphatase (Powell et al., 2011), also cause Aicardi-Goutières syndrome, making a direct connection between the syndrome and ribonucleotide excision repair less straightforward. A homozygous null mouse for RNase H2 shows embryonic lethality at the gastrulation stage of development (Reijns et al., 2012). On the other hand, yeast RNase H2 null mutants are viable. Nonetheless, growth defects and even lethality result

Please cite this article in press as: Sparks et al., RNase H2-Initiated Ribonucleotide Excision Repair, Molecular Cell (2012), http://dx.doi.org/10.1016/j.molcel.2012.06.035

when a *rnh201Δ* mutation is combined with other defects in DNA metabolism (Budd et al., 2005; Lazzaro et al., 2012).

Ribonucleotide excision repair (RER) initiated by RNase H2 has so far not been studied in a reconstituted, purified system. Earlier studies in crude yeast extracts already implicated RNase H2 and the flap endonuclease FEN1 in RER. Reduced incision at a ribonucleotide in DNA was observed in extracts from strains with a deletion of *RNH201*, encoding the catalytic subunit of RNase H2, or a deletion of *RAD27*, encoding FEN1 nuclease that is involved in Okazaki fragment maturation (Rydberg and Game, 2002). However, these studies did not identify additional components of the repair pathway. Here, we present a complete biochemical analysis of the RER pathway with recombinant proteins. We used, as substrate for RER, DNA replicated by the replicative DNA polymerases in reactions containing physiological concentrations of dNTPs and rNTPs, rather than using preformed DNA substrates with embedded ribonucleotides. In this fashion, context effects due to the use of a specific oligonucleotide substrate are eliminated, and events that are most likely to occur during DNA replication are substrates for RER. Our data show (1) that ribonucleotide misincorporation during leading and lagging strand DNA replication in vitro is comparable; (2) that RNase H2-initiated RER can be efficiently reconstituted with FEN1, Pol δ, PCNA, and DNA ligase I; and (3) that RER shows redundancy for both the nuclease and the DNA polymerase involved in the repair pathway.

RESULTS

The cellular concentrations of each of the dNTP and rNTPs under normal growth conditions have been determined (Nick McElhinny et al., 2010b). The rNTP/dNTP ratios range from 35 for [CTP]/[dCTP] up to almost 200 for [ATP]/[dATP]. When we examined the probability of stable ribonucleotide incorporation by replicative DNA polymerases at these physiologically relevant rNTP/dNTP ratios using defined oligonucleotide template primer as substrates, we found that sequence contexts greatly affected rNMP incorporation probabilities at individual nucleotide positions (Nick McElhinny et al., 2010a). Therefore, in this study, to examine RER of true ribonucleotide replication errors, we first determined the frequency of ribonucleotide incorporation during the replication of a 7.3 kb long single-stranded (ss) viral DNA template (ssM13mp18) under physiological rNTP and dNTP concentrations. Under these conditions, many sequence contexts should be sampled, including those most likely to be made during replication, and a mean distribution of ribonucleotide incorporation can be obtained. We carried out this analysis with both the leading strand polymerase, Pol ε, and the lagging strand polymerase, Pol δ, under conditions of processive DNA replication by interaction of the polymerase with the replication clamp PCNA (proliferating cell nuclear antigen). The replicated DNA was treated with 0.3M NaOH at 55°C, which cleaves DNA at the ribonucleotide positions, and sized by electrophoresis on a denaturing agarose gel.

Replication of the viral ssM13mp18 DNA with or without rNTPs was compared. The ssDNA was coated with yeast RPA in all experiments. Replication assays without rNTPs actually contained 100 μM ATP, in order to effect efficient loading of PCNA by its clamp loader *r*eplication *f*actor *C* (RFC), which is an ATP-dependent process. Replication assays with rNTPs contained a total of 5.9 mM rNTPs, and additional magnesium was added so that the free magnesium concentration in the assay remained the same. Inclusion of rNTPs in the replication assay reduced the rate of processive replication by Pol δ or by Pol ε to about 40% (Figure S1A). However, complete replication of the entire 7.3 kb M13mp18 circle was accomplished after 6 min by Pol δ, and after 15 min by Pol ε (Figure S1B). Alkaline treatment of M13mp18 circles replicated by either Pol δ or by Pol ε without rNTPs showed no sensitivity to alkali as indicated by the presence of predominantly full-length products (Figure 1A). In sharp contrast, M13mp18 circles replicated in the presence of physiological rNTPs were sensitive to treatment with alkali, resulting in a broad distribution of products. In order to convert the radioactivity distribution into product length distribution, the radioactivity distribution was divided by the length distribution in kb. From this normalized distribution, the median length of alkali-stable DNA produced by Pol δ was determined at 0.61 kb and was not significantly different from that produced by Pol ε (0.54 kb, Figure 1B). The average frequency of ribonucleotide incorporation can be obtained by also taking into consideration that the maximum linear product length is 7.3 kb and, second, the

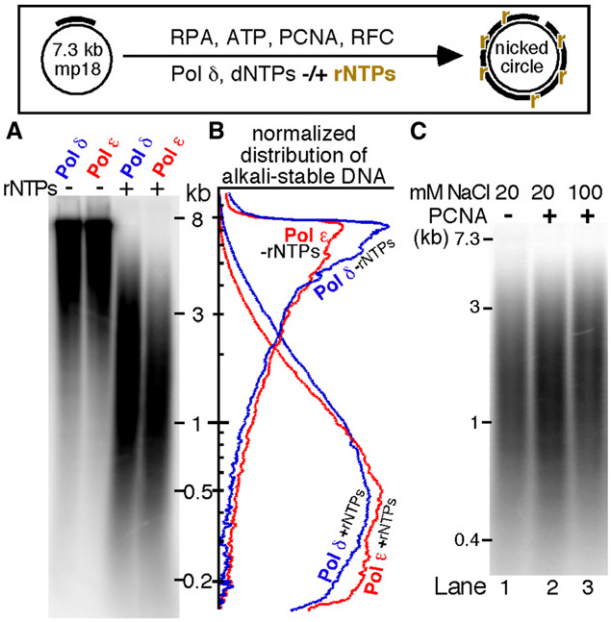

Figure 1. Ribonucleotide Incorporation during DNA Synthesis

(A) Primed RPA-coated SS-M13mp18 DNA was fully replicated by either Pol δ or Pol ε with PCNA under standard conditions, with or without rNTPs (Experimental Procedures). Replication products were alkali treated and separated on an alkaline agarose gel.

(B) The radioactivity distribution was scanned and divided by the DNA size distribution to obtain a normalized molar product distribution (see Supplemental Information for details).

(C) The standard assay (lane 3) was modified in lane 1 to omit PCNA and RFC, reduce salt to 20 mM NaCl, increase Pol δ from 3 nM to 500 nM, and increase the incubation time from 8 min to 3 hr. In lane 2 the standard assay was modified to reduce salt to 20 mM. See also Figure S1.

presence of incompletely replicated molecules that skew the distribution to a lower size. After taking these factors into consideration (see Supplemental Information), the resulting average rNMP insertion frequency is 1 per 720 nt for Pol δ and 1 per 640 nt for Pol ε. In several experiments, this average rNMP insertion frequency varied from 1 per ~0.6–0.9 kb.

These misincorporation values by the two replicative DNA polymerases are significantly higher than previously determined on oligonucleotide templates (Nick McElhinny et al., 2010b). In our previous oligonucleotide system, replication was carried out by the polymerase without accessory factors present. In the current system, the ssDNA is coated with the yeast single-stranded DNA binding protein RPA, and processive replication is performed by either the PCNA-Pol δ or PCNA-Pol ε complex. To assess whether PCNA affected ribonucleotide misincorporation frequencies, we carried out replication by Pol δ without PCNA (Figure 1C). In order to get full-length synthesis of the M13mp18 template by Pol δ alone, we had to reduce the salt concentration from 100 to 20 mM NaCl and increase enzyme concentration and incubation time (Figure S1C). No significant differences in rNTP misincorporation were observed between Pol δ alone and the processive PCNA-Pol δ complex at 20 mM NaCl, when compared to the standard assay at 100 mM NaCl (Figure 1C).

RER Requires RNase H2 and FEN1

A previous study showed defects in the incision of ribonucleotide-containing DNA in extracts from strains deleted for *RNH201* and *RAD27* (Rydberg and Game, 2002). Specifically, the incision step at the ribonucleotide position was severely inhibited in *rnh201Δ* extracts, while the subsequent liberation of this ribonucleotide was attenuated in *rad27Δ* extracts. However, actual repair of the DNA was not demonstrated in this study. To establish a reconstituted RER assay with purified proteins, we set up a two-stage repair assay (Figure 2). In the first step, ss-M13mp18 DNA was replicated by the PCNA-Pol δ complex at physiological dNTP and rNTP concentrations resulting in the average incorporation of ~10 ribonucleotides per circle. Identical controls were carried out without rNTPs. In the second step, RNase H2, FEN1, and DNA ligase I were added. The products were analyzed by neutral agarose electrophoresis in the presence of ethidium bromide, which detects completely replicated covalently closed circles (top panel), and by alkaline agarose gel electrophoresis, after alkaline digestion of the replication products (bottom panel).

Replication of M13mp18 DNA with or without rNTPs yielded full-length products (Figure 2, top, lanes 1 and 6), that matured into covalently closed circular molecules upon addition of FEN1 and DNA ligase I, indicating that the nicked product was matured and sealed with high efficiency (top, lanes 2 and 7). When challenged with alkali, only the DNA replicated in the presence of rNTPs was digested to smaller size products (bottom, compare lanes 2 and 7). Complete repair was achieved by adding RNase H2, which yielded full-length alkali-stable products comparable to those made in the absence of rNTPs (compare lane 10 with 5). Addition of RNase H2 had no effect on the alkali stability of replication products made without rNTPs (compare lanes 2 and 5). Additional controls show that all three enzymes,

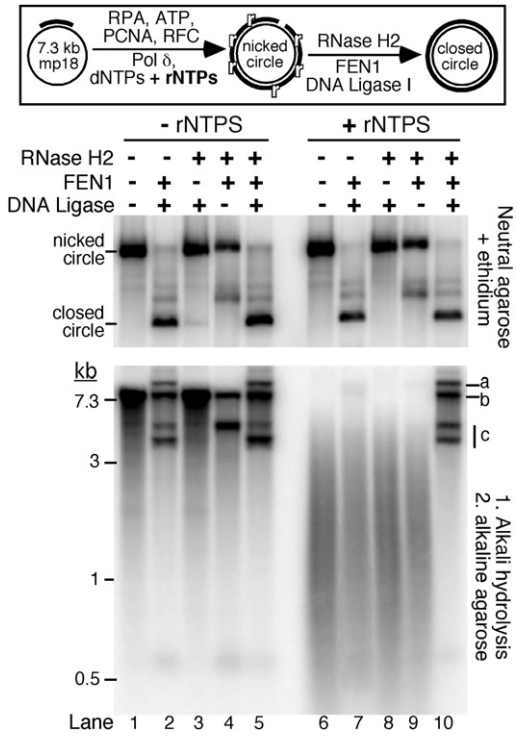

Figure 2. Ribonucleotide Excision Repair Assay

Primed RPA-coated SS-M13mp18 DNA was fully replicated by Pol δ with PCNA under standard conditions with or without rNTPs. After 8 min at 30°C, RNase H2, FEN1, and DNA ligase I were added as indicated, and incubation continued for 4 min. Each reaction was split in two, and one half was separated on a 1% agarose gel in the presence of 0.5 μg/ml of ethidium bromide; the other half was treated with 0.3 M NaOH. The products were recovered by ethanol precipitation and separated on a 1% alkaline agarose gel. Migration positions of nicked circles and covalently closed circles on the neutral agarose gel (top panel) are indicated. Migration positions of circular ssDNA (a), linear ssDNA (b), and denatured forms of dsDNA (c) on the alkaline agarose gel (bottom panel) are indicated. See also Figure S2.

RNase H2, FEN1, and DNA ligase are required in order for any detectable repair. A mutant form of RNase H2, containing the catalytically dead Rnh201-D39A mutation, was completely defective for RER (Figure S2). Therefore, each of the ~10 ribonucleotides present in fully replicated M13mp18 DNA required the action of RNase H2, FEN1, and DNA ligase I in order to incise the ribo-containing DNA, remove the ribonucleotide by nick translation with FEN1 and Pol δ, and finally seal the nick by DNA ligase.

Role of PCNA in RER

All four enzymes involved in RER show interactions with PCNA. PCNA mediates processive DNA synthesis by Pol δ and increases the nuclease efficiency of FEN1 (Li et al., 1995). On the other hand, DNA ligase I binds PCNA, but ligation efficiency is not significantly enhanced by PCNA (Vijayakumar et al., 2007). Rather, the PCNA-binding domain of DNA ligase aids in localizing the enzyme to replication foci (Montecucco et al., 1998). RNase H2 is a three-subunit enzyme. The largest subunit is the catalytic subunit, and the second subunit has a PCNA-binding

Please cite this article in press as: Sparks et al., RNase H2-Initiated Ribonucleotide Excision Repair, Molecular Cell (2012), http://dx.doi.org/10.1016/j.molcel.2012.06.035

domain with unknown function (Chon et al., 2009). The PCNA-binding domain of RNase H2 is essential for localizing it to replication foci in mammalian cells (Bubeck et al., 2011). The activity of an archaeal form of RNase H2 that also contains a PCNA-interaction motif is stimulated by PCNA (Bubeck et al., 2011). However, in model studies with oligonucleotides, we failed to see such stimulation for either the yeast or the human enzyme by its cognate PCNA (Chon et al., 2009).

In order to test the potential importance of PCNA in RER, we synthesized ribonucleotide-containing DNA as described above, isolated the DNA, and then used this substrate in a subsequent assay with Pol δ, RNase H2, FEN1, DNA ligase, either with or without PCNA (and its loader RFC) present. In parallel, the same assay was carried out with RNase H2 lacking the PCNA-interacting motif (ΔPIP). While PCNA greatly stimulated RER, we observed no dependence on the PCNA-interacting motif of RNase H2 (Figure 3A). Sometimes, a role for PCNA in stabilizing the factor under investigation can be revealed by carrying out the assay under suboptimal conditions, e.g., at low concentrations of the factor and/or at high salt. However, under limiting conditions for RNase H2, at which all other factors were saturating and the rate of DNA repair was dependent on the RNase H2 concentration, both mutant and wild-type RNase H2 showed a comparable inhibition of the rate of DNA repair at increasingly higher concentrations of NaCl (Figure S3). We conclude that either RNase H2 can access the ribonucleotide substrate efficiently without prior interaction with PCNA, or that our studies have failed to reveal a functional interaction in vitro.

RNase H1 Does Not Substitute for RNase H2 in RER

Both RNase H1 and RNase H2 incise RNA-containing DNA; however, while RNase H2 incises efficiently at single ribonucleotide positions, RNase H1 is only active on double-stranded DNA containing three of more consecutive ribonucleotides (Tadokoro and Kanaya, 2009). Potentially, interactions of RNase H1 with other repair factors might aid in promoting incision at single ribonucleotides. Therefore, we compared the efficiency of RNase H1 and RNase H2 in RER. RNase H1 was completely defective in promoting RER, even at the highest concentrations tested (Figure 3B).

FEN1 and Exo1, and Pol δ and Pol ε Show Redundancy for RER

In our proposed scheme for RER, nick translation is an important step in removing the incised ribonucleotide residue (Figure 4). Exo1 can partially substitute for FEN1 during Okazaki fragment maturation, as both nucleases can promote nick translation by Pol δ (Stith et al., 2008; Tran et al., 2002). Indeed, substituting FEN1 by Exo1 in the RER reaction allowed complete repair of misincorporated ribonucleotides (Figure 3C). However, the rate of repair promoted by FEN1 was about 2- to 3-fold that of Exo1. In contrast, Dna2 nuclease, which only acts on long 5′-flaps, is completely defective for RER. This agrees with our understanding of Dna2 function during Okazaki fragment maturation, wherein long flaps trimmed by Dna2 still require further processing by FEN1 (Burgers, 2009). Similarly, Pol ε can also function in Okazaki fragment maturation, albeit less efficiently than Pol δ (Garg et al., 2004). In a reconstituted reaction, Pol ε carried out RER at a rate about half of that of Pol δ (Figure 3D).

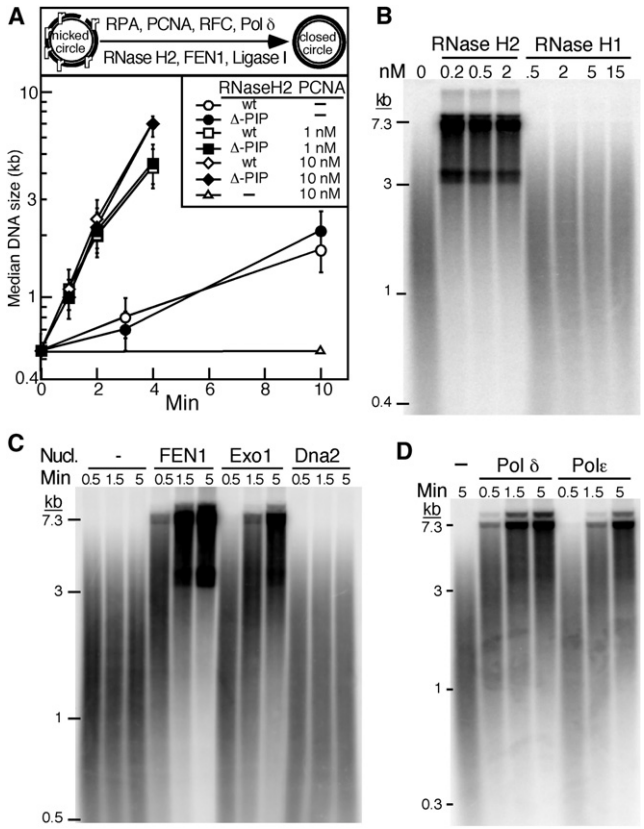

Figure 3. Factor Requirements and Redundancies in RER

(A–D) Primed RPA-coated SS-M13mp18 DNA was fully replicated by Pol δ with PCNA under standard conditions with rNTPs for 8 min. In panels (A) and (D), the replicated DNA was isolated and purified, and the DNA incubated in a second reaction with: (A) RPA, RFC, Pol δ, RNase H2 or RNase H2-ΔPIP, FEN1, DNA ligase I, and the indicated concentrations of PCNA for the times indicated; (D) RPA, PCNA, RFC, RNase H2, FEN1, DNA ligase I, and either Pol δ or Pol ε for the indicated times. In panels (B) and (C), the replication reaction was supplemented with: (B) FEN1, DNA ligase I, and the indicated concentrations of either RNase H1 or RNase H2 for 10 min; (C) RNase H2, DNA ligase I, and either no nuclease, FEN1, Exo1, or Dna2 for the times indicated. The products were treated with alkali and separated on an 1% alkaline agarose gel. In (A), the median product distribution was determined as in Figure 1B and Supplemental Information. See also Figure S3. The SE for two independent experiments is given.

DISCUSSION

Here we have reconstituted, for the first time, the excision repair of ribonucleotides in a highly purified system. Our results extend a previous study that used crude extracts to show incisions at single ribonucleotides in DNA by RNase H2 and FEN1. We demonstrate complete and efficient repair of ribonucleotides incorporated into DNA by the major leading and lagging strand replicases for the nuclear genome. The basic mechanism outlined in Figure 4 shows an employment of PCNA, Pol δ, FEN1, and DNA ligase that is analogous to that used during Okazaki

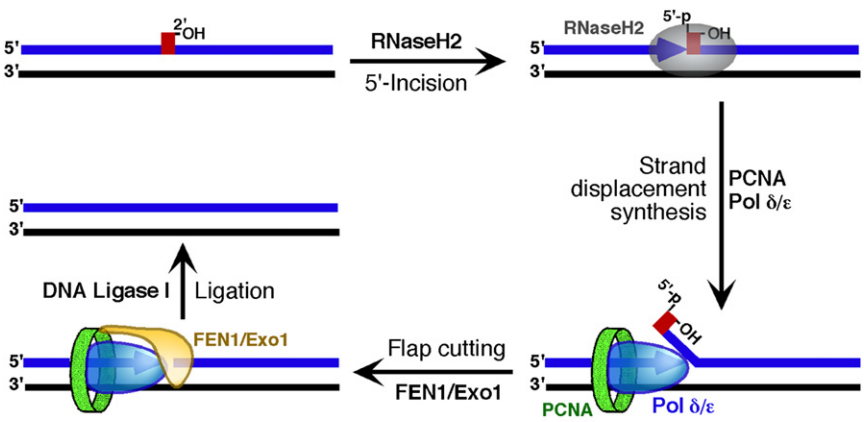

Figure 4. Model for Ribonucleotide Excision Repair

Redundant functions of FEN1 with Exo1 and Pol δ with Pol ε are indicated. See text for details.

fragment maturation or during PCNA-dependent base-excision repair (Matsumoto, 2001). Repair is absolutely dependent on RNase H2, and RNase H1 does not substitute for RNase H2. However, both enzymes contribute to genome stability (Lazzaro et al., 2012), indicating that RNase H1 has a role in processing RNA-DNA substrates other than a single embedded ribonucleotide, and that a defect in this role results in cellular stress. For example, R-loops formed during transcription, or a stretch of several consecutive ribonucleotides left on the DNA as a result of incorrect maturation of Okazaki fragments would require the activity of RNase H1 in a *rnh201Δ* mutant.

On the other hand, there is redundancy for DNA polymerase and nuclease activities in RER. The observed redundancy between FEN1 and Exo1 for RER would indicate that *RAD27* (FEN1) mutants should not be defective for RER, yet a defect in RER was observed in extracts from a *rad27Δ* strain (Rydberg and Game, 2002). Possibly, since Exo1 has a very low abundance compared to FEN1 (Ghaemmaghami et al., 2003) and is very sensitive to proteolysis (Fiorentini et al., 1997), extracts may have been largely deficient for Exo1, thereby emphasizing a dependence of FEN1. Our data suggest that after a first cleavage by RNase H2 in a PCNA-independent manner, PCNA is required for nick translation by Pol δ and FEN1, which is the coupling of strand displacement synthesis by the polymerase with flap cutting by the nuclease (Figure 4).

The incorporation of a relatively large percentage of ribonucleotides into DNA during DNA replication has only recently been appreciated (Nick McElhinny et al., 2010b). They can be considered "damage" because they are only weakly proofread during replication by Pol ε (Williams et al., 2012) and because, in the absence of RNase H2-dependent repair, they result in 2–5 base pair deletions that are independent of DNA mismatch repair (Clark et al., 2011). Ribonucleotides represent by far the largest pool of potential DNA damage in the cell. Based on our current data, indicating one ribonucleotide incorporation event per ~0.7 kb replicated by either Pol δ or Pol ε (Figure 1B), ~50,000 ribonucleotides would be incorporated during each yeast cell cycle. Are all of these repaired in an RNase H2-dependent pathway? If so, then alkali treatment of DNA isolated from a RNase H2 deletion strain should produce products of a similar size. One study showed a broad distribution of alkali-sensitive sites in the yeast chromosomal DNA isolated from an *rnh201Δ* strain that centered in the 2–20 kb range (Nick McElhinny et al., 2010a). Interestingly, a higher frequency of alkali-sensitive sites was detected in a double mutant of *rnh201Δ* with a Pol ε mutant that in vitro was shown to display an increased frequency of ribonucleotide incorporation. Importantly, chromosomal DNA isolated from a strain that is wild-type for RNase H2 is much less alkali-labile, indicating that RNase H2 is critical for the repair of ribonucleotides in DNA.

There are two potential reasons why the in vivo occurrence of alkali-sensitive sites in chromosomal DNA from a *rnh201Δ* strain, 1 per several kb, is less than the in vitro occurrence, 1 per ~0.7 kb. First, the local concentration of dNTPs at replication foci could be higher than that of the overall cellular concentration that we used in this study, e.g., through the localization of dNTP precursor enzymes at replication sites. Currently, this idea lacks conclusive experimental support, either in yeast or in human cells (Lee and Elledge, 2006; Pontarin et al., 2008). Second, alternative repair systems may exist for the repair of ribonucleotides. Topoisomerase I can incise ribonucleotide-containing DNA, via the covalent 3′-phosphotyrosyl linkage formed at the ribose moiety, into a 2′,3′-cyclic phosphate terminated chain (Kim et al., 2011; Sekiguchi and Shuman, 1997). In the cell, this intermediate can be aberrantly processed to yield 2–5 nt deletions within short tandem repeats, suggesting that topoisomerase I may initiate a secondary pathway that can ultimately result in ribonucleotide removal. The enzymes that act downstream of Topoisomerase 1 to ultimately repair ribonucleotides with consequent mutagenesis are as yet unknown. The complexity of processing ribonucleotides in DNA is further revealed by the fact that survival of yeast in the absence of both RNase H1 and H2 partly depends on two lesion tolerance pathways, Rad5-dependent template switching, and translesion synthesis by Pol ζ (Lazzaro et al., 2012). This is interesting, given our evidence here that RNase H1 does not initiate repair of ribonucleotides incorporated by Pols δ and ε in vitro. Collectively, the results suggest that the cytotoxic lesions in strains defective in both yeast RNases H could be (1) an R-loop formed during transcription (Huertas and Aguilera, 2003); (2) a stretch of several consecutive ribonucleotides placed into DNA during priming of Okazaki fragments or perhaps more rarely by a DNA polymerase; or (3) multiple interrupted ribonucleotides incorporated by a DNA polymerase within a short tract of DNA.

EXPERIMENTAL PROCEDURES

Proteins

Sources are listed in Supplemental Information.

RER Assay

The repair assay is generally carried out in two stages. In the first stage, single-stranded M13mp18 DNA is replicated at physiological cellular concentrations of rNTPs and dNTPs. After replication is complete, the DNA is either isolated or the second stage is initiated without prior isolation, by addition of factors required for RER. *Stage 1*: The standard 40 μl assay contained 30 mM Tris-HCl, pH 7.8, 13 mM magnesium-acetate, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 100 mM NaCl, 16 μM dATP, 14 μM dCTP, 12 μM dGTP, 30 μM dTTP, 3000 μM ATP, 500 μM CTP, 700 μM GTP, and 1700 μM UTP. [α-^{32}P] dATP was added as radioactive tracer. In "dNTPs only" assays, 100 μM ATP replaced all rNTPs, and the Mg concentration was lowered to 8 mM. Further additions were 60 fmol of singly primed single-stranded M13mp18 DNA (the 36-mer primer is complementary to nt 6330–6295), 15 pmol of replication protein A (RPA), 600 fmol PCNA as trimer (3 pmol for assays with Pol ε), and 120 fmol of replication factor C (RFC). Reactions were assembled on ice, preheated at 30°C for 1 min, and initiated by addition of 120 fmol of Pol δ or Pol ε. After 8 min (20 min for Pol ε), when replication was complete, either the RER enzymes (FEN1, DNA ligase 1, and RNase H2 as detailed below) were added, or the reaction was stopped with 0.3% SDS and 20 mM EDTA, heated at 55°C for 10 min, and purified by phenol/chloroform extraction and ethanol precipitation. The DNA was then dissolved in the same assay mixture (buffer/Mg/dNTPs/rNTPs/NaCl), and the following RER enzymes were added for *Stage 2*: 5 pmol RPA, 600 fmol PCNA, 120 fmol RFC, 120 fmol Pol δ, 120 fmol FEN1, and 600 fmol DNA ligase 1. After preheating to 30°C for 30 s, the reaction was initiated with 120 fmol of RNase H2. Aliquots were stopped with 0.3% SDS and 20 mM EDTA and incubated at 54°C for 10 min. 2.5 M NaOH was added to 0.3 M final, and after 2 hr incubation at 55°C in order to hydrolyze the DNA at ribonucleotide positions, the reaction was cooled, 2 M acetic acid was added to 0.4 M, ammonium acetate to 1 M, carrier DNA to 10 μg/ml, 2 μg of glycogen, and 2 volumes of ethanol. After cooling at −20°C for 20 min, the DNA was spun down for 20 min at 17,000 × g, and the pellet was washed with 70% ethanol, dried, and redissolved in TE with 0.1% SDS/50 mM NaCl. The samples were analyzed by electrophoresis on a 1% alkaline agarose gel at 1.3 V/cm for 16 hr. The gel was neutralized, dried, and analyzed by Phosphorimaging. Size distribution determinations were carried out as described in Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.06.035.

ACKNOWLEDGMENTS

The authors thank Carrie Stith and Else-Britt Lundström for protein purification and Bonnie Yoder for strain construction. This work was supported in part by Grant GM032431 from the National Institutes of Health to P.M.B.; the Swedish research council and Cancerfonden to E.J.; by Project Z01 ES065089 from the Division of Intramural Research of the National Institutes of Health, National Institute on Environmental Health Sciences, to T.A.K.; and the Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH to R.J.C. and T.A.K.

Received: May 7, 2012
Revised: June 14, 2012
Accepted: June 28, 2012
Published online: August 2, 2012

REFERENCES

Bubeck, D., Reijns, M.A., Graham, S.C., Astell, K.R., Jones, E.Y., and Jackson, A.P. (2011). PCNA directs type 2 RNase H activity on DNA replication and repair substrates. Nucleic Acids Res. *39*, 3652–3666.

Budd, M.E., Tong, A.H., Polaczek, P., Peng, X., Boone, C., and Campbell, J.L. (2005). A network of multi-tasking proteins at the DNA replication fork preserves genome stability. PLoS Genet. *1*, e61.

Burgers, P.M. (2009). Polymerase dynamics at the eukaryotic DNA replication fork. J. Biol. Chem. *284*, 4041–4045.

Cerritelli, S.M., and Crouch, R.J. (2009). Ribonuclease H: the enzymes in eukaryotes. FEBS J. *276*, 1494–1505.

Chon, H., Vassilev, A., DePamphilis, M.L., Zhao, Y., Zhang, J., Burgers, P.M., Crouch, R.J., and Cerritelli, S.M. (2009). Contributions of the two accessory subunits, RNASEH2B and RNASEH2C, to the activity and properties of the human RNase H2 complex. Nucleic Acids Res. *37*, 96–110.

Clark, A.B., Lujan, S.A., Kissling, G.E., and Kunkel, T.A. (2011). Mismatch repair-independent tandem repeat sequence instability resulting from ribonucleotide incorporation by DNA polymerase ε. DNA Repair (Amst.) *10*, 476–482.

Crow, Y.J., Leitch, A., Hayward, B.E., Garner, A., Parmar, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., et al. (2006). Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutières syndrome and mimic congenital viral brain infection. Nat. Genet. *38*, 910–916.

Eder, P.S., and Walder, J.A. (1991). Ribonuclease H from K562 human erythroleukemia cells. Purification, characterization, and substrate specificity. J. Biol. Chem. *266*, 6472–6479.

Fiorentini, P., Huang, K.N., Tishkoff, D.X., Kolodner, R.D., and Symington, L.S. (1997). Exonuclease I of Saccharomyces cerevisiae functions in mitotic recombination in vivo and in vitro. Mol. Cell. Biol. *17*, 2764–2773.

Garg, P., Stith, C.M., Sabouri, N., Johansson, E., and Burgers, P.M. (2004). Idling by DNA polymerase delta maintains a ligatable nick during lagging-strand DNA replication. Genes Dev. *18*, 2764–2773.

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. Nature *425*, 737–741.

Huertas, P., and Aguilera, A. (2003). Cotranscriptionally formed DNA:RNA hybrids mediate transcription elongation impairment and transcription-associated recombination. Mol. Cell *12*, 711–721.

Joyce, C.M. (1997). Choosing the right sugar: how polymerases select a nucleotide substrate. Proc. Natl. Acad. Sci. USA *94*, 1619–1622.

Kim, N., Huang, S.N., Williams, J.S., Li, Y.C., Clark, A.B., Cho, J.E., Kunkel, T.A., Pommier, Y., and Jinks-Robertson, S. (2011). Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science *332*, 1561–1564.

Lazzaro, F., Novarina, D., Amara, F., Watt, D.L., Stone, J.E., Costanzo, V., Burgers, P.M., Kunkel, T.A., Plevani, P., and Muzi-Falconi, M. (2012). RNase H and postreplication repair protect cells from ribonucleotides incorporated in DNA. Mol. Cell *45*, 99–110.

Lee, Y.D., and Elledge, S.J. (2006). Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1. Genes Dev. *20*, 334–344.

Li, X., Li, J., Harrington, J., Lieber, M.R., and Burgers, P.M. (1995). Lagging Strand DNA Synthesis at the Eukaryotic Replication Fork Involves Binding and Stimulation of FEN-1 by proliferating cell nuclear antigen. J. Biol. Chem. *270*, 22109–22112.

Matsumoto, Y. (2001). Molecular mechanism of PCNA-dependent base excision repair. Prog. Nucleic Acid Res. Mol. Biol. *68*, 129–138.

Montecucco, A., Rossi, R., Levin, D.S., Gary, R., Park, M.S., Motycka, T.A., Ciarrocchi, G., Villa, A., Biamonti, G., and Tomkinson, A.E. (1998). DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. EMBO J. *17*, 3786–3795.

Nick McElhinny, S.A., Kumar, D., Clark, A.B., Watt, D.L., Watts, B.E., Lundström, E.B., Johansson, E., Chabes, A., and Kunkel, T.A. (2010a). Genome instability due to ribonucleotide incorporation into DNA. Nat. Chem. Biol. *6*, 774–781.

Nick McElhinny, S.A., Watts, B.E., Kumar, D., Watt, D.L., Lundström, E.B., Burgers, P.M., Johansson, E., Chabes, A., and Kunkel, T.A. (2010b).

Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc. Natl. Acad. Sci. USA *107*, 4949–4954.

Pontarin, G., Fijolek, A., Pizzo, P., Ferraro, P., Rampazzo, C., Pozzan, T., Thelander, L., Reichard, P.A., and Bianchi, V. (2008). Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. Proc. Natl. Acad. Sci. USA *105*, 17801–17806.

Powell, R.D., Holland, P.J., Hollis, T., and Perrino, F.W. (2011). Aicardi-Goutieres syndrome gene and HIV-1 restriction factor SAMHD1 is a dGTP-regulated deoxynucleotide triphosphohydrolase. J. Biol. Chem. *286*, 43596–43600.

Reijns, M.A., Rabe, B., Rigby, R.E., Mill, P., Astell, K.R., Lettice, L.A., Boyle, S., Leitch, A., Keighren, M., Kilanowski, F., et al. (2012). Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell *149*, 1008–1022.

Rychlik, M.P., Chon, H., Cerritelli, S.M., Klimek, P., Crouch, R.J., and Nowotny, M. (2010). Crystal structures of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. Mol. Cell *40*, 658–670.

Rydberg, B., and Game, J. (2002). Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts. Proc. Natl. Acad. Sci. USA *99*, 16654–16659.

Sekiguchi, J., and Shuman, S. (1997). Site-specific ribonuclease activity of eukaryotic DNA topoisomerase I. Mol. Cell *1*, 89–97.

Stetson, D.B., Ko, J.S., Heidmann, T., and Medzhitov, R. (2008). Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell *134*, 587–598.

Stith, C.M., Sterling, J., Resnick, M.A., Gordenin, D.A., and Burgers, P.M. (2008). Flexibility of eukaryotic Okazaki fragment maturation through regulated strand displacement synthesis. J. Biol. Chem. *283*, 34129–34140.

Tadokoro, T., and Kanaya, S. (2009). Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. FEBS J. *276*, 1482–1493.

Tran, P.T., Erdeniz, N., Dudley, S., and Liskay, R.M. (2002). Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae. DNA Repair (Amst.) *1*, 895–912.

Vijayakumar, S., Chapados, B.R., Schmidt, K.H., Kolodner, R.D., Tainer, J.A., and Tomkinson, A.E. (2007). The C-terminal domain of yeast PCNA is required for physical and functional interactions with Cdc9 DNA ligase. Nucleic Acids Res. *35*, 1624–1637.

Watt, D.L., Johansson, E., Burgers, P.M., and Kunkel, T.A. (2011). Replication of ribonucleotide-containing DNA templates by yeast replicative polymerases. DNA Repair (Amst.) *10*, 897–902.

Williams, J.S., Clausen, A.R., Nick McElhinny, S.A., Watts, B.E., Johansson, E., and Kunkel, T.A. (2012). Proofreading of ribonucleotides inserted into DNA by yeast DNA polymerase ε. DNA Repair (Amst.), in press.