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Ribonucleotides as nucleotide excision repair substrates

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Abstract

The incorporation of ribonucleotides in DNA has attracted considerable notice in recent years, since the pool of ribonucleotides can exceed that of the deoxyribonucleotides by at least 10 to 20 fold, and single ribonucleotide incorporation by DNA polymerases appears to be a common event. Moreover ribonucleotides are potentially mutagenic and lead to genome instability. As a consequence, errantly incorporated ribonucleotides are rapidly repaired in a process dependent upon RNase H enzymes. On the other hand, global genomic nucleotide excision repair (NER) in prokaryotes and eukaryotes removes damage caused by covalent modifications that typically distort and destabilize DNA through the production of lesions derived from bulky chemical carcinogens, such as polycyclic aromatic hydrocarbon metabolites, or via crosslinking. However, a recent study challenges this lesion-recognition paradigm. The work of Vaisman *et al.* (A. Vaisman, J.P. McDonald, D. Huston, W. Kuban, L. Liu, B. Van Houten, and R. Woodgate, PLoS Genetics, in press, 2013) reveals that even a single ribonucleotide embedded in a deoxyribonucleotide duplex is recognized by the bacterial NER machinery *in vitro*. In their report, the authors show that spontaneous mutagenesis promoted by a steric-gate pol V mutant increases in *uvrA*, *uvrB*, or *uvrC* strains lacking *rnhB* (encoding RNase HII) and to a greater extent in an NER-deficient strain lacking both RNase HI and RNase HII. Using purified UvrA, UvrB, and UvrC proteins in *in vitro* assays they show that despite causing little distortion, a single ribonucleotide embedded in a DNA duplex is recognized and doubly-incised by the NER complex. We present the hypothesis to explain the recognition and/or verification of this small lesion, that the critical 2'-OH of the ribonucleotide--with its unique electrostatic and hydrogen

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We performed 55 ns MD simulations with AMBER 11 [65] utilizing the remodeled and completed crystal structure of UvrB [49] in a complex with DNA obtained by Jia *et al.* [61]; the benzo[a]pyrenyl lesion was replaced by a hydrogen atom, and the MD protocol described in [66] was used. The DNA sequence was 5'-ATCG (rC17) TACCGC-3' (Model 3 of [61]) with the UvrB residue 17 remodeled to a ribonucleotide, and a second 55 ns simulation with the ribonucleotide initially remodeled to have C3'-endo pucker was also performed. It has recently been shown that this sequence manifests NER of the single ribonucleotide similar to that reported in [34] (Roger Woodgate, Personal Communication). A normal all DNA control was also simulated for 55 ns. Model building was performed in the INSIGHT II 2005 program (Accelrys Software, Inc.). The structures shown here are the most representative structures from the MD ensemble obtained by clustering analyses with AMBER 11 [65].

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bonding properties--may act as a signal through interactions with amino acid residues of the prokaryotic NER complex that are not possible with DNA. Such a mechanism might also be relevant if it were demonstrated that the eukaryotic NER machinery likewise incises an embedded ribonucleotide in DNA.

Keywords

prokaryotic nucleotide excision repair; ribonucleotides; electrostatic and hydrogen bonding properties; lesion recognition and verification

Helix distorting and destabilizing DNA lesions are subject to repair by the nucleotide excision repair machinery in both prokaryotes and eukaryotes [1, 2]. However, what constitutes a helix-distorting lesion may not be obvious. For example, *cis-syn* cyclobutane pyrimidine dimers (CPD <TT>), the most prevalent lesions produced by ultraviolet (UV) light are inefficiently repaired by the NER machinery *in vitro*, while the 6-4 <TT> UV photoproduct, which occurs at about 25% lower frequency [3], is an excellent substrate for NER [4, 5]. How these photoproducts differ in their impact on duplex DNA structure might not be obvious from the structures of the lesions themselves: the CPD <TT> dimer is shaped like an open book with the two thymines at an angle of about 50° (PDB [6] ID: 4A0A [7]), while in the 6-4 <TT> lesion the pyrimidine/pyrimidone bases are near-perpendicular in a propeller-like orientation (PDB ID: 3EI1 [8]). However, NMR solution structures show that a 6-4 <TT> modified duplex decamer is more distorted: Watson-Crick hydrogen bonding is disrupted at the 3'-side of the 6-4 <TT> lesion but is present in the case of the CPD <TT> duplex which is much less bent [9].

Although the structural and mechanistic aspects of the prokaryotic and eukaryotic NER machineries are somewhat different, they share similar critical operational and functional features, particularly the recognition of the lesions and subsequent verification steps [1, 2]. In both cases, there are two NER pathways, transcription coupled repair (TC-NER) [10-12] and global genomic repair (GG-NER). In TC-NER, the lesion is first recognized by transcription stalling, but subsequent steps are similar to GG-NER [2, 13]. In GG-NER the damaged DNA is first recognized, in prokaryotes by UvrA [1] and in eukaryotes by XPC-RAD23B, with UV-DDB required upstream for UV-induced photoproducts [2, 8, 13]. Subsequently the damage is verified by UvrB in prokaryotes [1], and by TFIIH in cooperation with XPA and the helicase activity of XPD that is part of the multi-protein complex TFIIH in eukaryotes [2]. What follows in both cases is the excision of around ~13 and ~24-32 nucleotide-long oligonucleotide sequences containing the damage in the prokaryotic and eukaryotic systems, respectively, with subsequent gap-filling by one or more polymerases [1, 2].

The factors that determine whether a given lesion is recognized for subsequent excision has elicited much interest, particularly since different lesions are excised with remarkably different efficiencies [5, 13, 14]. Dual incision experiments with DNA substrates containing a variety of site-specifically inserted bulky lesions conducted with both prokaryotic and eukaryotic systems suggest that NER is promoted by local lesion-induced thermodynamic destabilization and enhanced dynamics [15-22]. These studies were focused mostly on DNA

lesions resulting from polycyclic aromatic derivatives, other bulky lesions, or intrastrand crosslinks that distort the local DNA structure [23-27].

The incorporation of ribonucleotides in DNA has attracted considerable notice in recent years since the pool of ribonucleotide can exceed that of the deoxyribonucleotides by 10 to 20 fold in prokaryotes [28] and even many thousand-fold in eukaryotes [29]. Hence single rNTP incorporation by DNA polymerases appears to be a common event, with human pol δ incorporating one rNTP per ~ 2000 dNTPs. rNTPs are mutagenic [30] and lead to genome instability [31], and hence DNA polymerases have defenses against incorporating ribonucleotides, known as steric gates [32] or fences [33].

The intriguing observation suggesting NER susceptibility for ribonucleotides came to light in the studies of Vaisman *et al.* [34], who used a steric gate mutant of DNA polymerase V to elucidate the pathways involved in ribonucleotide repair. In an earlier study, the *umuC_Y11A* steric gate mutant was shown to exhibit low-fidelity DNA synthesis similar to wild-type pol V *in vitro*. Importantly, however, it also has reduced ribonucleotide selectivity, such that the mutant enzyme incorporated ribonucleotides as efficiently as deoxyribonucleotides [34]. Furthermore, rather than promoting high levels of cellular mutagenesis, the *umuC_Y11A* mutant produced approximately one tenth of the level of mutagenesis promoted by wild-type pol V [35]. The low level of mutagenesis is due to efficient ribonucleotide repair that concomitantly repairs the misincorporated deoxyribonucleotides incorporated by low-fidelity pol V. The key player in ribonucleotide repair is RNase HII encoded by *rnhB*, since inactivation of the *rnhB*-dependent ribonucleotide repair pathway leads to an increase in *umuC_Y11A*-dependent mutagenesis [36]. However, even when RNase HII is inactivated, the level of *umuC_Y11A*-dependent mutagenesis is only ~ 30 -40% of that observed with wild-type pol V. Given that *umuC_Y11A* is just as error-prone as wild-type pol V [34], the authors hypothesized that alternate ribonucleotide repair pathways must operate to remove errantly incorporated ribonucleotides in the absence of *rnhB*. In search of these pathways, Vaisman *et al.* [34], generated a series of DNA repair-deficient strains in the *rnhB* background and assayed for an increase in *umuC_Y11A*-dependent mutagenesis (which is an indication of persistent misincorporated dNTPs, due to the inactivation of back-up ribonucleotide repair pathways). Inactivation of mismatch repair, or base excision repair had no effect on the level of relative *umuC_Y11A*-dependent mutagenesis. However, inactivation of nucleotide excision repair via mutations in *uvrA*, *uvrB* or *uvrC* resulted in an increase in mutagenesis [34]. Furthermore, by using the highly purified UvrABC complex with defined DNA substrates containing site-specific ribonucleotides, Vaisman *et al.* show that even a single ribonucleotide is recognized as “damage” *in vitro*. Similar to bulky DNA adducts, incision of the ribonucleotide occurs 8 bases 5' to the embedded ribonucleotide and 4-5 bases 3' to the ribonucleotide, indicating that a single ribonucleotide is a substrate of the prokaryotic nucleotide excision repair complex [34].

How could a single ribonucleotide embedded in DNA be recognized as a substrate by the NER complex? For bulky adducts, it is believed that UvrA as part of a UvrA₂UvrB₂ complex scans the DNA for helix distorting lesions using both three-dimensional jumping and ID-sliding, the latter being mediated by UvrB [37, 38]. According to present models, the

initial recognition step by UvrA is followed by the lesion verification by UvrB. Once the UvrB actively engages the damaged site, UvrA dissociates and nuclease UvrC is recruited [1] and catalyzes the dual incisions 3' and 5' to the damaged nucleotide.

How might such a mechanism be employed for ribonucleotide repair? A recent high resolution NMR solution structure [39] provided structural insights for consideration. The self-complementary Dickerson dodecamer DNA duplex [40], containing one embedded ribonucleotide symmetrically positioned on each strand (4th residue from the 5'-end) was investigated. This study revealed that each embedded rNTP adopts the C3'-endo sugar pucker that is characteristic of A-form double stranded RNAs [41]; however, the rest of the duplex is essentially normal B form, with only modest local disturbances in helical parameters at the site of the lesion and the adjacent base pairs. These disturbances do not seem quite in line with the more severe distortions in Watson-Crick pairing, groove dimensions, bending, unwinding, impaired stacking, and local dynamics that have been characteristic of NER-susceptible lesions in both prokaryotes [17, 22, 24, 42, 43] and eukaryotes [15-18, 20, 21, 26, 44, 45]. Such types of disturbances have also been observed in a crystal structure of UvrA containing a bulky fluorescein-modified thymine [1, 43], and were considered as recognition signals. In contrast, in the case of the eukaryotic XPC-HR23B which utilizes a β -hairpin, BHD3, to intrude between the damaged strand and its complement at the lesion site, according to a crystal structure of the yeast homologue [46], UvrA seems to sense DNA damage using positively charged residues and the C-terminal zinc finger [47, 48]. Damage verification by UvrB is mediated by a critical tyrosine 96 residue at the base of the β -hairpin in UvrB, as well as other nearby tyrosine residues [49-51].

While a ribonucleotide embedded in DNA does not induce the marked local distortions [39] that normally elicit damage recognition for NER, the unique electrostatic and hydrogen bonding properties of the ribonucleotide 2'-OH, in place of the hydrogen atom of the 2'-deoxyribonucleotide, might contribute to the NER recognition process. Notably the 2'-OH can act as a hydrogen bond donor and/or acceptor that plays a key role in RNA folding [52-54] and RNA-protein interactions [55]. One might speculate that a ribonucleotide in place of a deoxyribonucleotide could trigger recognition via hydrogen bonding/electrostatic interactions of the 2'-OH group with amino acid residues in UvrA and UvrB that are not possible in the case of DNA.

Using the experimental NMR solution structure of the DNA duplex containing the embedded central ribonucleotides [39], we computed the electrostatic potential of this duplex whose sequence is that of the classical Dickerson dodecamer [40], and also computed the potential for a normal all-DNA Dickerson dodecamer NMR structure (PDB ID: 1GIP) [56]. Figure 1 (A) shows the negative electrostatic patch near one of the ribonucleotides that is unique for the ribonucleotide case, as seen by comparison with a normal Dickerson dodecamer in Figure 1 (B); it provides hydrogen bonding as well as electrostatic properties that can alter the normal interactions of DNA with proteins of the UvrABC system. Such abnormal interactions with UvrA might act as the first recognition signal of a single ribonucleotide.

The Vaisman *et al.* [34] paper demonstrates both 5' and 3' incision surrounding a single ribonucleotide embedded in a DNA duplex, with a 12-13 base incision product, showing that the lesion has been verified. In prokaryotes, lesion verification is performed by UvrB [1]. During the process of damage verification it is believed that UvrB's helicase fold in the presence of ATP allows translocation of the damage from the damage detection site in UvrA to the β -hairpin of UvrB. A crystal structure of UvrB containing DNA (PDB ID: 2FDC [49]) has shown that a DNA strand threads behind the β -hairpin that has been implicated in the damage verification process by biochemical methods [51, 57, 58]. Exactly how the lesion is verified is still uncertain, but critical stacking interactions with tyrosine residues in UvrB are likely factors [59, 60]; it has been proposed that Tyr96 could play a key role in sensing DNA damage by utilizing stacking interactions with the damaged base positioned at the β -hairpin gate on the inner strand [1, 49]. Models that consider lesion placement in other locations near the β -hairpin have also been evaluated by molecular dynamics (MD) simulations [61].

Here, we speculate that the presence of the 2'-OH of the ribonucleotide might perturb local amino acid interactions in UvrB and thus provide signals for damage verification. We explored how a single ribonucleotide positioned on the inner strand at the β -hairpin gate would impact the UvrB structure, utilizing a UvrB model [61] based on the DNA-containing crystal structure of Truglio *et al.* [49], and first retaining the crystal C2'-endo sugar pucker for the modeled ribonucleotide. We reasoned that the pucker would change to C3'-endo during the MD if C2'-endo were unfavorable, but during the 55 ns MD simulation the sugar pucker of the single ribonucleotide in the UvrB complex remained C2'-endo. To explore this further, we performed a second UvrB simulation in which the ribonucleotide was remodeled to adopt the C3'-endo conformation. However, the pucker reverted quickly to C2'-endo and the two structures were similar at 55 ns. Since the ribonucleotide embedded in DNA is single stranded, it need not be C3'-endo, as is the case in A-form duplexes [41]. In non-double helical structures, ribonucleotides can readily adopt the C2'-endo sugar conformation found in B-DNA double helices [41, 62].

Our MD simulations provided insights into the impact of the ribonucleotide 2'-OH group on the hydrogen bonding patterns of amino acids in its immediate vicinity, and we compared them with a 55 ns simulation containing a normal 2'-deoxyribonucleotide. Figure 1C shows the best representative structure from the ribonucleotide-containing MD trajectory. Figures 1D and 1E show changes provoked by the 2'-OH group which might contribute to the verification. These changes involve hydrogen bonding/electrostatic interactions between the 2'-OH group and Tyr93 and Tyr96, concomitant with a disrupted hydrogen bond between Glu307 and Tyr96. Mutational studies have shown that substitutions of Tyr93 and Tyr96 with Ala strongly affect the function of UvrB and mutation of Glu307 has a modest impact [51]. These alterations may promote damage verification by UvrB [37], as is the case for the bulky fluorescein lesion [1, 60], where "... the engagement of UvrB with the lesion creates a trapped state for the helicase, rendering it incapable for further translocation..."[59]. Thus, we hypothesize that damage verification might include features that utilize electrostatic and hydrogen bonding properties of the damage. This hypothesis also helps clarify a previous equivocal observation made a decade ago by the Van Houten laboratory which showed that a one base gap and a nick in the DNA is efficiently processed by the UvrABC complex [63].

These gap and nick substrates would carry an extra negative charge [30], and thus might allow recognition in the same manner as the ribonucleotide moiety.

Although Vaisman *et al.* [34] used a steric gate mutant of pol V to increase the likelihood of ribonucleotide incorporation into the *E. coli* genome, it seems unlikely that the repair pathways uncovered using the mutant enzyme will be specific to pol V-dependent incorporation of ribonucleotides. Like many organisms, the pool of ribonucleotides in *E. coli* exceeds that of deoxyribonucleotides, by 10 to 20 fold in this case [28]. Despite possessing steric gate exclusion mechanisms, it has recently been reported that wild-type *E. coli* DNA polymerase III incorporates ~2000 ribonucleotides during genome duplication [35]. This roughly equates to a ribonucleotide incorporated once every ~2300 base-pairs and unless rapidly repaired, the ribonucleotides cause replisome pausing that may be ultimately deleterious to the cell [35]. Therefore, global ribonucleotide repair pathways, that include NER, to efficiently replace erroneously incorporated NTPs with dNTPs appear plausible. Clearly further work is needed to confirm this hypothesis.

The next question is whether NER in eukaryotes is able to process and excise ribonucleotides. Support for this notion comes from the fact that differential expression of NER genes in RNase H2-deficient yeast has been observed [36] and that NER can process ribonucleotides-containing oligonucleotides introduced into yeast [30]. If eukaryotic NER is demonstrated, it will be of great interest to determine if the prokaryotic and eukaryotic systems share recognition and verification features. Additionally, it would be interesting to learn how a mispaired ribonucleotide, incorporated by pol V [64] and also produced by various other processes [30]--that is an even better UvrABC NER substrate than one with a normal base partner [34]--is recognized and verified, since it may have a more distorting structural effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

rNTP	ribonucleotide
CPD <TT>	<i>cis-syn</i> cyclobutane pyrimidine dimers
6-4 <TT>	6,4 pyrimidine-pyrimidone
dNTP	deoxyribonucleotide
RER	ribonucleotide excision repair
MD	molecular dynamics
NER	nucleotide excision repair
GG-NER	global genomic repair
TC-NER	transcription coupled repair

Highlights

- !! Prokaryotic nucleotide excision repair incises ribonucleotides embedded in DNA.
- !! Ribonucleotides embedded in DNA cause little distortion in contrast to bulky lesions.
- !! Ribonucleotides have unique electrostatic and hydrogen bonding properties due to the presence of the 2'-OH.
- !! These electrostatic and hydrogen bonding properties might provide lesion recognition and verification signals.

Figure 1.

(A and B) Electrostatic surfaces computed on the PDB2PQR Server (http://nbc-222.ucsd.edu/pdb2pqr_L8/). (A) represents the electrostatic surface of the NMR solution structure of the Dickerson dodecamer containing an embedded ribonucleotide [39] and (B) represents an NMR solution structure of the same normal dodecamer structure [56]. The negative (red) electrostatic patch due to the 2'-OH group depends little on the sugar pucker which is C3'-endo in (A). (C, D and E): Molecular dynamics simulations suggest how a ribonucleotide on the inner strand at the gate of the β-hairpin in UvrB could alter the interactions of nearby amino acid residues. (C) is a view of the full UvrB structure together with the area of interest at the β-hairpin gate. These are also featured in the movie given in Supplementary Data. The ribonucleotide (D) as compared to the deoxyribonucleotide (E), provides opportunities for hydrogen bonding/electrostatic interactions with Tyr93 and Tyr96

that the 2'-deoxyribonucleotide lacks. These interactions disrupt the normal hydrogen bond between Tyr96 and Glu307. The black dashed lines indicate the relevant hydrogen bonding/electrostatic interactions. Electrostatic interactions are outside of hydrogen bonding distance but within the range of the Coulomb's law attractive potential. All graphics were prepared with PyMOL Molecular Graphics System Version 1.3. (Schrodinger, LLC). See Footnote for details.