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Pre-steady state kinetic studies show that an abasic site is a cognate lesion for the yeast Rev1 protein [†]

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Abstract

Rev1 is a eukaryotic DNA polymerase that rescues replication forks stalled at sites of DNA damage by inserting nucleotides opposite the damaged template bases. Yeast genetic studies suggest that Rev1 plays an important role in rescuing replication forks stalled at one of the most common forms of DNA damage, an abasic site; however, steady state kinetic studies suggest that an abasic site acts as a significant block to nucleotide incorporation by Rev1. Here we examined the pre-steady state kinetics of nucleotide incorporation by yeast Rev1 with damaged and non-damaged DNA substrates. We found that yeast Rev1 is capable of rapid nucleotide incorporation, but only a small fraction of the protein molecules possessed this robust activity. We characterized the nucleotide incorporation by the catalytically robust fraction of yeast Rev1 and found that it efficiently incorporated dCTP opposite a template abasic site under pre-steady state conditions. We conclude from these studies that the abasic site is a cognate lesion for Rev1.

Keywords

DNA replication; DNA repair; DNA polymerase; translesion synthesis; mutagenesis

1. Introduction

Structural distortions in DNA caused by damage interfere with DNA synthesis by classical polymerases. Consequently, replication forks stall upon encountering DNA lesions in the template strand. One pathway used to overcome replication fork stalling is translesion DNA synthesis (TLS), a pathway that employs non-classical DNA polymerases, which are capable of DNA synthesis on damaged DNA templates. Most of these non-classical polymerases are members of the Y family of DNA polymerases [1-9]. The yeast genome, for example, encodes two Y-family polymerases: DNA polymerase (pol) η and Rev1. The human genome encodes four Y-family polymerases: pol η , pol ι , pol κ , and Rev1. Studies of the structures and enzymatic mechanisms of these non-classical polymerases have begun to reveal the strategies used by these enzymes to accommodate the structural distortions in the DNA caused by lesions [2, 3].

In vitro, each of these non-classical DNA polymerases appears to be specific for one or more DNA lesions or groups of closely related DNA lesions. For example, pol ι is capable

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of synthesizing DNA on templates containing damaged purines with bulky adducts in both the minor groove and the major groove [10-15]. Similarly, pol κ is capable of synthesizing DNA on templates containing small N²-adducted guanines [16-21]. Studies such as these have led to the notion that each non-classical polymerase has one or more cognate lesions. While the definition of “cognate lesion” varies somewhat among researchers [5-7], one common feature is that non-classical polymerases incorporate nucleotides opposite their cognate lesions with very high catalytic efficiency (k_{cat}/K_m). The clearest example of this concept is pol η and the ultraviolet radiation-induced thymine dimer [22]. Steady state kinetic studies have shown that pol η incorporates an A opposite both damaged T residues with the same catalytic efficiency as it incorporates an A opposite a non-damaged T [23, 24]. Moreover, pre-steady state kinetic studies have shown that the incoming dATP binds to the pol η -DNA complex with the same affinity and is incorporated at the same rate regardless of whether the template is a thymine dimer or a non-damaged T [25].

Rev1, a member of the Y family of DNA polymerases, has an unusual specificity in that it prefers to incorporate C opposite all four non-damaged template bases, although most efficient incorporation occurs opposite a template G [26-29]. The X-ray crystal structure of yeast Rev1 in a ternary complex with dCTP and DNA shows that this enzyme uses a protein-template directed mechanism of nucleotide incorporation [30, 31]. The template G residue is flipped out of the DNA double helix and the incoming dCTP is positioned in the active site by hydrogen bonding with the side chain of an arginine residue. Using such a mechanism, Rev1 is able to incorporate nucleotides opposite certain N²-guanine adducts with high catalytic efficiency, indicating that these adducts are cognate lesions for Rev1. It has also been suggested that the abasic site may be a cognate lesion for Rev1, as genetic studies have shown that some abasic sites are bypassed *in vivo* by Rev1 [32-34]. However, steady state and pre-steady state kinetic studies have questioned this conclusion by showing that Rev1 incorporates nucleotides opposite abasic sites with a significantly lower catalytic efficiency than opposite non-damaged templates [27, 35, 36]. Similarly, *in vitro* primer extension assays have shown that Rev1 bypasses abasic sites with low efficiency [37].

Pre-steady state kinetics allows one to examine the mechanism of nucleotide incorporation by a DNA polymerase without complications arising from DNA binding or dissociation. Previously, pre-steady state kinetic studies of yeast Rev1 showed that this enzyme did not incorporate nucleotides with biphasic kinetics. This meant that the first turnover of nucleotide incorporation occurred slowly and was not faster than subsequent turnovers [35]. In addition, the kinetics showed that the efficiency of nucleotide incorporation opposite an abasic site was ~100-fold less than that opposite a non-damaged G. This implied that the abasic site was not a cognate lesion for Rev1. More recently, pre-steady state kinetic studies of human Rev1 showed that this enzyme did incorporate nucleotides with biphasic kinetics. This meant that the first turnover occurred rapidly and was faster than subsequent ones [38]. Although one might conclude from this that the mechanisms of nucleotide incorporation by yeast Rev1 and human Rev1 differ substantially, careful examination of the kinetics of human Rev1 revealed that the amplitude of the burst phase was only ~5% of the total protein concentration [38]. This indicated that only a small fraction of the total human Rev1 was capable of rapid nucleotide incorporation in the first turnover. This raised the possibility that a similar small fraction of yeast Rev1, which would have gone undetected under the conditions previously used to study this enzyme, might also be capable of rapid nucleotide incorporation. Moreover, this also raised the possibility that the abasic site might be a cognate lesion for Rev1.

To test these possibilities, we re-examined the pre-steady state kinetics of yeast Rev1. We found that, like human Rev1, a small fraction of yeast Rev1 (~7%) also incorporated nucleotides rapidly and displayed biphasic kinetics. Moreover, we examined the ability of

this fraction of Rev1 to incorporate nucleotides opposite an abasic site, and we found that dCTP bound to the Rev1-DNA complex containing a template abasic site with only a 9-fold weaker affinity than it bound to the analogous complex containing a non-damaged template G. Surprisingly, once bound, the C was incorporated at the same rate regardless of whether the template was an abasic site or a non-damaged G. These results, in combination with previously published *in vivo* results [32-34], provide compelling evidence that the abasic site is a cognate lesion for Rev1.

2. Materials and methods

2.1. Expression and purification of yeast Rev1

Rev1 was expressed in yeast strain BJ5464 carrying plasmid pRev1.41, which encodes Rev1 (amino acid residues 1-746) fused in frame with glutathione-S-transferase (GST). The fusion protein was purified as described previously [35], and the GST portion was removed by treatment with PreScission protease (GE Healthcare). Purified Rev1 was stored at -80°C in 20 μL aliquots.

2.2. DNA and nucleotide substrates

The synthetic oligonucleotides used as substrates in this study consisted of a 24-mer primer strand: 5'-GCC TCG CAG CCG TCC AAC CAA CTC annealed to a 45-mer template strand: 5'-GGA CGG CAT TGG ATC GAC CTX GAG TTG GTT GGA CGG CTG CGA GGC, where the X is a non-damaged guanine, an 8-oxoguanine lesion, or the abasic site analog tetrahydrofuran. The DNA was radiolabeled with ^{32}P and annealed as described previously [39]. Solutions of each dNTP were purchased from New England Biolabs and stored in 10 μL aliquots at -80°C .

2.3. Pre-steady state kinetics

Reactions were carried out in a buffer containing 25 mM TrisCl (pH 7.5), 5 mM MgCl_2 , 5 mM dithiothreitol, and 10% glycerol at 30°C using a rapid chemical quench flow apparatus (KinTek). Rev1 (200-800 nM final concentration) was pre-incubated with the ^{32}P -labeled DNA (2.5-200 nM) in one syringe of the quench flow apparatus, and the reaction was initiated by addition of the incoming nucleotide (1-1000 μM) from the other syringe. Reactions were stopped after various times (5 ms to 40 s) by mixing with 0.2 M EDTA. Each sample was then diluted 20:1 with formamide loading dye and resolved on a 15% polyacrylamide sequencing gel containing 8 M urea. The radiolabeled gel bands were quantified using a Phosphorimager (GE Healthcare). Reactions at each condition were repeated several times to ensure reproducibility.

2.4. Data Analysis

The concentrations of the extended primer products (P) were graphed as a function of time (t), and the data were fit by nonlinear regression to the burst equation (SigmaPlot 8.0) to determine the amplitude of the burst phase (A), the observed first-order rate constants for nucleotide incorporation during the burst phase (k_1), and the observed rate constant for the steady state phase (k_2) or the observed rate for the steady state phase (v_2). In most cases the data were fit using the simplified form of the burst equation (Eq.1), which assumes that $k_1 \gg k_2$; however, in some cases the data was fit to the full burst equation (Eq.2), depending on the relative values of k_1 and k_2 .

$$P = A \left(1 - e^{-k_1 t} \right) + v_2 t \quad \text{Eq. 1}$$

$$P=A[(k_1/(k_1+k_2))^2(1-e^{-(k_1+k_2)t})+((k_1k_2t)/(k_1+k_2))] \quad \text{Eq. 2}$$

To determine the dissociation constant for the incoming nucleotide ($K_{d\bullet\text{dNTP}}$) and the maximum first-order rate constant for nucleotide incorporation (k_{pol}), the observed rate constants for the burst phase (k_1) were graphed as a function of dNTP concentration ($[\text{dNTP}]$), and the data were fit by nonlinear regression to a hyperbolic equation (Eq. 3). To determine the dissociation constant for the DNA ($K_{d\bullet\text{DNA}}$) and the active site concentration of Rev1 ($[\text{Rev1}]$), the burst amplitudes (A) were graphed as a function of DNA concentration ($[\text{DNA}]$) and fit by nonlinear regression to the quadratic equation (Eq. 4).

$$k_1=k_{\text{pol}}[\text{dNTP}]/(K_{d\bullet\text{dNTP}}+[\text{dNTP}]) \quad \text{Eq. 3}$$

$$A=\left\{(K_{d\bullet\text{DNA}}+[\text{Rev1}]+[\text{DNA}])-\sqrt{(K_{d\bullet\text{DNA}}+[\text{Rev1}]+[\text{DNA}])^2-4([\text{Rev1}][\text{DNA}])}\right\}/2 \quad \text{Eq. 4}$$

3. Results

3.1. Yeast Rev1 displays biphasic kinetics

An earlier pre-steady state kinetic study with yeast Rev1 showed that this enzyme incorporated nucleotides slowly and did not display biphasic kinetics [35]. This study also suggested that the abasic site is not a cognate lesion of Rev1. A subsequent pre-steady state kinetic study with human Rev1 showed that this enzyme incorporated nucleotides faster and did display biphasic kinetics [38]. The amplitude of the burst phase, however, was very small (~5%), indicating that only a small percentage of human Rev1 was capable of rapid nucleotide incorporation. This raised the possibility that a small fraction of yeast Rev1 might also be capable of rapid nucleotide incorporation. This also raised the possibility that the abasic site might be a cognate lesion for Rev1. Here we test these possibilities by re-examining the pre-steady state kinetics of yeast Rev1.

To determine whether yeast Rev1 displays biphasic kinetics, we pre-incubated 400 nM Rev1 (final concentration) and 50 nM ^{32}P -labeled DNA (final concentration) with a non-damaged template G residue in one syringe of the rapid chemical quench flow instrument and mixed this solution with 500 μM dCTP from the other syringe. The reaction was quenched after various incubation times ranging from 0.005 to 5 s. The product concentration was graphed as a function of time (**Fig. 1**), and the best fit of the data to the burst equation yielded an observed rate constant (k_{obs}) equal to 17 s^{-1} and an amplitude of the burst phase equal to 26 nM. These data suggest that like the human enzyme, yeast Rev1 is capable of rapid nucleotide incorporation, but only a small fraction (~7%) of Rev1 molecules appear capable of robust incorporation.

3.2. Active site titration of Rev1 with non-damaged DNA

To determine the fraction of yeast Rev1 molecules capable of rapid nucleotide incorporation and to determine the DNA binding affinity of this enzyme, we carried out an active site titration of Rev1 using DNA substrates with a non-damaged G residue as the template. We pre-incubated 400 nM Rev1 with various concentrations of ^{32}P -labeled DNA (2.5 to 100 nM) and initiated the reaction by adding 500 μM dCTP (**Fig. 2A**). The amplitude of the burst phase was graphed as a function of DNA concentration, and the best fit of these data to the quadratic equation yielded an apparent dissociation constant for the DNA ($K_{d\bullet\text{DNA}}$)

equal to 12 nM and a concentration of active site Rev1 equal to 27 nM (**Fig. 2B**). This shows that ~7% of the Rev1 molecules are capable of rapid nucleotide incorporation. Additional kinetics experiments included as Supplementary Data suggest that the rest of the Rev1 molecules are not forming non-productive complexes with the DNA.

3.3. Kinetics of C incorporation opposite a template G

Next, we examined the pre-steady state kinetics of correct C incorporation opposite a template G by Rev1. Rev1 (400 nM) was pre-incubated with 50 nM ^{32}P -labeled DNA and then mixed with various concentrations of dCTP (2 to 500 μM). For each dCTP concentration, the amount of product was graphed as a function of time (**Fig. 3A**), and k_{obs} values were obtained from the best fits of these plots to the burst equation. The k_{obs} values were then graphed as a function of dCTP concentration (**Fig. 3B**), and the best fit of these of data to the hyperbolic equation yielded a maximum first-order rate constant for nucleotide incorporation (k_{pol}) equal to 18 s^{-1} and an apparent dissociation constant for dCTP ($K_{\text{d}\cdot\text{dCTP}}$) equal to 32 μM (**Table 1**).

3.4. Kinetics of G, A, and T incorporation opposite a template G

To compare the kinetics of correct C incorporation opposite a template G and the kinetics of incorrect G, A, and T incorporation, we determined the k_{pol} and apparent $K_{\text{d}\cdot\text{dNTP}}$ values for incorrect nucleotide incorporations as described above (**Table 1**). Rev1 incorporated the incorrect G most efficiently with an apparent $K_{\text{d}\cdot\text{dNTP}}$ that was 14-fold weaker and a k_{pol} that was 19-fold slower than in the case of C incorporation. Rev1 incorporated T less efficiently with an apparent $K_{\text{d}\cdot\text{dNTP}}$ that was 22-fold weaker and a k_{pol} that was 90-fold slower than in the case of C incorporation. Therefore, small to moderate differences in the $K_{\text{d}\cdot\text{dNTP}}$ and large differences in the k_{pol} contribute to the fidelity of Rev1 on a template G. Incidentally, incorporation of A opposite G by Rev1 was undetectable.

3.5. Kinetics of C incorporation opposite templates A, T, and C

To compare the kinetics of C incorporation opposite the optimal template G and the kinetics of incorporation opposite non-optimal templates A, T, and C, we determined the k_{pol} and apparent $K_{\text{d}\cdot\text{dNTP}}$ values with these templates as described above (**Table 2**). The $K_{\text{d}\cdot\text{dNTP}}$ values with the non-optimal A, T, and C templates were 11-fold, 4.1-fold, and 10-fold weaker than with the optimal G template, respectively. The k_{pol} values with the non-optimal A, T, and C templates were 49-fold, 320-fold, and 200-fold weaker than with the optimal G template, respectively. Therefore, small differences in the $K_{\text{d}\cdot\text{dCTP}}$ and large differences in the k_{pol} contribute to the specificity of Rev1 for a template G.

3.6. Kinetics of C incorporation opposite a template abasic site

To compare the kinetics of C incorporation opposite a template G and the kinetics of incorporation opposite a template abasic site – a potential cognate lesion – we determined the k_{pol} and apparent $K_{\text{d}\cdot\text{dCTP}}$ values with these templates as described above (**Fig. 4A** and **Table 2**). We found that the abasic site had only a 9.1-fold effect on the $K_{\text{d}\cdot\text{dCTP}}$, but it surprisingly had no effect on the k_{pol} . This suggested that the active site of Rev1 was adept at inserting C opposite an abasic site. Moreover, we carried out an active site titration of Rev1 with a DNA substrate containing a template abasic site to determine if the abasic site reduces the affinity of Rev1 for the DNA (**Fig. 4B** and **Table 3**). We found that the $K_{\text{d}\cdot\text{DNA}}$ for the abasic site-containing DNA substrate was equal to 10 nM and that ~8% of the Rev1 molecules were capable of robust incorporation. These data show that the mechanisms of nucleotide incorporation opposite a non-damaged template G and opposite a template abasic site are quite similar.

3.7. Kinetics of C incorporation opposite a template 8-oxoG lesion

To determine how C incorporation differs on a potential non-cognate lesion, we measured the k_{pol} and $K_{\text{d,dCTP}}$ for nucleotide incorporation opposite an 8-oxoG lesion (**Fig. 5A** and **Table 2**). We found that the 8-oxoG lesion had only a 6.3-fold effect on the $K_{\text{d,dCTP}}$, but it had a 16-fold effect on the k_{pol} . Small differences in the $K_{\text{d,dCTP}}$ and somewhat larger differences in the k_{pol} contribute to the bias of Rev1 against this lesion. Surprisingly, this lesion is not as poor of a substrate as was previously thought, and this raises the possibility that Rev1 might bypass 8-oxoG lesions in some physiological contexts. We also carried out an active site titration of Rev1 using a DNA substrate containing a template 8-oxoG lesion and found that the presence of the lesion does not significantly affect the $K_{\text{d,DNA}}$ (**Fig. 5B** and **Table 3**). Unexpectedly though, the presence of a template 8-oxoG lesion increased the active site concentration of Rev1 by roughly 4-fold. Therefore, this lesion affected a partial rescue of the sub-stoichiometric burst amplitude of Rev1.

4. Discussion

The Rev1 protein employs a remarkable protein-directed mechanism of nucleotide incorporation in which the specificity for the incoming nucleotide is achieved by the nucleotide binding to an arginine side chain in the protein's active site [30, 31, 40]. Pre-steady state kinetics has been used to examine the impact of this unusual mechanism of nucleotide selection. Previous studies of yeast Rev1 showed that the enzyme incorporated nucleotides slowly with a k_{pol} equal to $\sim 0.01 \text{ s}^{-1}$ [35]. This contrasted with similar studies of human Rev1 showing that the enzyme incorporated nucleotides considerably faster with a k_{pol} equal to $\sim 1 \text{ s}^{-1}$ [38] and $\sim 20 \text{ s}^{-1}$ [41]. While the kinetic parameters determined for human Rev1 are somewhat different in these two studies, it appeared that the yeast and human Rev1 proteins employed rather distinct kinetic mechanisms of nucleotide incorporation. The results reported in the present paper, however, show that a small fraction of the yeast Rev1 molecules ($\sim 7\%$) are capable of more rapid nucleotide incorporation with a k_{pol} equal to 18 s^{-1} . This suggests that while the principal findings of the earlier pre-steady state kinetics study of yeast Rev1 regarding nucleotide specificity are correct, this earlier study was not carried out under true pre-steady state conditions. Here we have shown that the kinetic mechanism of this highly active fraction of Rev1 more closely resembles the mechanism of human Rev1. The apparent $K_{\text{d,dCTP}}$ for yeast Rev1 ($32 \mu\text{M}$) determined here is nearly identical to the one determined in one of the studies of human Rev1 [38]. Moreover, the k_{pol} for yeast Rev1 is nearly identical to the one determined in the other study of human Rev1 [41]. In addition, the specificity for yeast Rev1 for incorporating a dCTP versus other incoming nucleotides and for incorporating opposite a template G versus opposite other non-damaged templates arises due to slight changes in the apparent $K_{\text{d,dCTP}}$ and larger changes in the k_{pol} . In these respects, the kinetic basis of the specificity of yeast Rev1 resembles that of human Rev1 [41]. Consequently, the mechanism of nucleotide incorporation by yeast and human Rev1 are more alike than had previously been thought.

The low burst amplitudes reported here show that only a small percentage of yeast Rev1 molecules are capable of rapid nucleotide incorporation opposite non-damaged templates and the abasic site. This is similar to what was observed previously for human Rev1 with certain damaged DNA templates where only about 5% of the enzyme was capable of rapid nucleotide incorporation [38]. Human Rev1 did not display a clear burst on the non-damaged template, but it does not appear that a significantly greater percentage of the enzyme was capable of rapid incorporation on this substrate either. These active site titrations and the kinetics experiments provided as Supplementary Data suggest that much of the Rev1 protein exists in an inactive conformational state. This does not seem to involve the formation of a non-productive complex, which is common among both classical and non-classical DNA polymerases in certain contexts [42-48]. What is particularly interesting

in the case of Rev1 is that the entry into this inactive state is reversible. This is because the sub-stoichiometric burst amplitude observed here can be partially rescued when the substrate used contained an 8-oxoG lesion. It is not clear why this specific lesion causes 4-fold more of the Rev1 molecules to enter the fully active state than does non-damaged templates or the abasic site, but it does show that the sub-stoichiometric burst amplitude is not due to permanently inactive protein.

Studies of the abilities of non-classical DNA polymerases to incorporate nucleotides opposite various types of DNA damage have led to the notion of cognate lesions. While the definition of cognate lesions differs somewhat among researchers [5-7], cognate lesions are generally considered to be those that a given non-classical polymerase has evolved to replicate with high efficiency in the physiological context. Thus, in order to demonstrate that a particular type of DNA damage is a cognate lesion for a non-classical polymerase, one must show that (i) the non-classical polymerase incorporates nucleotides opposite the lesion with high efficiency *in vitro* and (ii) the non-classical polymerase catalyzes the bypass of the lesion *in vivo*. In the case of Rev1 and abasic sites, evidence for the *in vivo* criterion comes from several prior studies. First, yeast cells with an abnormally high number of abasic sites generated by expressing an altered uracil-DNA glycosylase in the absence of the AP endonuclease Apn1 have a substantially increased number of mutations in reporter genes. The resulting mutation spectra suggest a major role for Rev1-catalyzed incorporation of C opposite abasic sites [32]. Second, *rev1Δ* yeast cells bypass abasic sites contained on plasmids with a substantially lower efficiency than do wild-type yeast cells. Depending on the sequence context around the lesion, C incorporation opposite the abasic site was observed in wild-type cells 60 to 90% of the time. No C incorporation was observed in the absence of Rev1 [33]. Third, another carefully designed plasmid-based assay in yeast showed a smaller role for Rev1 in the bypass of abasic sites. Yet even in this study, about 25% of the abasic sites were bypassed by the incorporation of a C that was dependent on the catalytic activity of Rev1 [34].

While evidence for the *in vivo* criterion is strong, evidence for the *in vitro* criterion is less clear. Often such evidence comes from steady state kinetics showing that the catalytic efficiency for incorporating nucleotides opposite the lesion is approximately as high as the catalytic efficiency for incorporation opposite non-damaged DNA. This is not the case with Rev1 as the catalytic efficiency of C incorporation opposite abasic sites was 20 to 30-fold lower than that opposite the preferred non-damaged G [27, 36]. While steady state kinetics is unlikely to yield false positives by arguing for a lesion to be classified as a cognate lesion when it shouldn't be, it can yield false negatives. This is because steady state kinetics takes into account the DNA association and DNA dissociation steps, which are not directly relevant to the physiological context. In the cellular environment, non-classical polymerases do not by themselves bind directly to naked primer-templates containing DNA damage. Instead their recruitment to stalled replication forks is facilitated by protein-protein interactions, most notably with mono-ubiquitylated PCNA [49-54]. What is relevant in the physiological setting is, once a non-classical polymerase is bound at a site of DNA damage, how well the polymerase incorporates nucleotides opposite the lesion. The best way to address this is by using pre-steady state kinetics where the polymerase and the DNA are pre-incubated to form a complex. One can then directly compare the apparent $K_{d,dNTP}$ and the k_{pol} values for damaged versus non-damaged DNA without any complications from the DNA association/dissociation steps. This approach is unlikely to yield either false positives or false negatives. Here we show that Rev1 binds the incoming dCTP with only a 9-fold weaker apparent $K_{d,dCTP}$ opposite an abasic site than it does opposite a non-damaged G and that Rev1 incorporates the bound nucleotide with the same k_{pol} in both cases. This is consistent with a recently determined X-ray crystal structure of Rev1 bound to DNA containing a template abasic site [40]. This structure was indistinguishable from the

structure of Rev1 bound to the optimal non-damaged G template. Overall, these pre-steady state kinetic and structural data demonstrates that, contrary to conclusions suggested previously by steady state kinetics, the abasic site is very likely a cognate lesion for Rev1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

dNTP	deoxynucleoside triphosphate
EDTA	ethylenediamine tetracetic acid
Pol	polymerase
8-oxoG	8-Oxoguanine

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Highlights

The mechanisms of nucleotide incorporation by yeast and human Rev1 are similar

Only a small fraction of Rev1 molecules are capable of rapid nucleotide incorporation

Rev1's specificity comes mainly from the rate of the nucleotide-incorporation step

Nucleotide incorporation by Rev1 opposite abasic sites occurs very rapidly

Kinetics and *in vivo* evidence show that abasic sites are cognate lesions for Rev1

Figure 1. Nucleotide incorporation by Rev1 displays biphasic kinetics

Rev1 (400 nM) was pre-incubated with DNA containing a template G (50 nM) and mixed with dCTP (500 μ M) for various lengths of time. The amount of nucleotide incorporation was plotted as a function of time, and the solid lines represent the best fit of the data to the burst equation. The amplitude was equal to 26 ± 2 nM, the k_{obs} was equal to 17 ± 4 s⁻¹, and the steady state rate was equal to 2.1 ± 0.8 nM/s.

Figure 2. Active site titration with non-damaged DNA

(A) Rev1 (400 nM) was incubated with various concentrations of DNA containing a template G (2.5 nM ($\diamond$), 5 nM ($\blacklozenge$), 10 nM ($\square$), 20 nM ($\blacksquare$), 35 nM ($\triangle$), 50 nM ($\blacktriangledown$), 75 nM ($\circ$), 100 nM ($\bullet$)) and then mixed with dCTP (500 μ M). The amount of nucleotide incorporation was plotted as a function of time, and the solid lines represent the best fits of the data to the burst equation. (B) The amplitudes were plotted as a function of DNA concentration, and the solid line represents the best fit of the data to the quadratic equation. The $K_{d\bullet\text{DNA}}$ was equal to 12 ± 1 nM and the concentration of active Rev1 was equal to 27 ± 1 nM, which is 6.8 % of the total Rev1 concentration.

Figure 3. Kinetics of C incorporation opposite a template G

(A) Rev1 (400 nM) was incubated with DNA containing a template G (50 nM) and then mixed with various concentrations of dCTP (2 μM (◇), 5 μM (◆), 10 μM (□), 20 μM (■), 50 μM (△), 100 μM (▼), 200 μM (○), 500 μM (●)). The amount of nucleotide incorporation was plotted as a function of time, and the solid lines represent the best fits of the data to the burst equation. (B) The k_{obs} values were plotted as a function of dCTP concentration, and the solid line represents the best fit to the hyperbolic equation. The k_{pol} equal to $18 \pm 1 \text{ s}^{-1}$ and $K_{d \cdot dCTP}$ equal to $32 \pm 7 \text{ μM}$.

Figure 4. Kinetics of C incorporation opposite a template abasic site

(A) Rev1 (400 nM) was incubated with DNA containing a template abasic site (50 nM) and then mixed with various concentrations of dCTP (25 to 1000 μM). The k_{obs} values were plotted as a function of dCTP concentration, and the solid line represents the best fit of the data to the hyperbolic equation. The k_{pol} was equal to $20 \pm 1 s^{-1}$ and the $K_{d \cdot dCTP}$ was equal to $290 \pm 30 \mu M$. (B) Rev1 (400 nM) was incubated with various concentrations of DNA containing a template abasic site (2.5 to 100 nM) and then mixed with dCTP (1000 μM). The amplitudes were plotted as a function of DNA concentration, and the solid line represents the best fit of the data to the quadratic equation. The $K_{d \cdot DNA}$ was equal to 10 ± 1

nM and the concentration of active Rev1 was equal to 33 ± 1 nM, which is 8.4 % of the total Rev1 concentration.

Figure 5. Kinetics of C incorporation opposite a template 8-oxoG

(A) Rev1 (400 nM) was incubated with DNA containing a template abasic site (50 nM) and then mixed with various concentrations of dCTP (25 to 500 μM). The k_{obs} values were plotted as a function of dCTP concentration, and the solid line represents the best fit of the data to the hyperbolic equation. The k_{pol} was equal to $1.1 \pm 0.1 \text{ s}^{-1}$ and the $K_{d \cdot dCTP}$ was equal to $200 \pm 30 \mu\text{M}$. (B) Rev1 (200 nM) was incubated with various concentrations of DNA containing a template abasic site (10 to 200 nM) and then mixed with dCTP (1000 μM). The amplitudes were plotted as a function of DNA concentration, and the solid line represents the best fit of the data to the quadratic equation. The $K_{d \cdot DNA}$ was equal to 25 ± 2

nM and the concentration of active Rev1 was equal to 63 ± 1 nM, which is 32 % of the total Rev1 concentration.

Table 1

Nucleotide incorporation opposite template G by Rev1

dNTP	k_{pol} (s^{-1})	K_d -dNTP (μM)	k_{pol}/K_d	k_{pol}/k_{pol} ^b	K_d/K_d ^c	f_{rel} ^d
dGTP	0.97 ± 0.04	460 ± 40	2.1×10^{-3}	19	14	270
dATP	ND ^a	ND	ND	ND	ND	ND
dTTP	0.20 ± 0.02	700 ± 100	2.9×10^{-4}	90	22	2,000
dCTP	18 ± 1	32 ± 7	0.56	1.0	1.0	1.0

^a Not detected

^b k_{pol} for dCTP divided by k_{pol} for the given dNTP

^c K_d for the given nucleotide divided by K_d for dCTP

^d relative efficiency of incorporating the given dNTP versus dCTP, which is k_{pol}/k_{pol} times K_d/K_d .

Table 2

C incorporation opposite non-damaged and damaged templates by Rev1.

Template	k_{pol} (s^{-1})	K_{d-dNTP} (μM)	k_{pol}/K_d	k_{pol}/k_{pol}^a	K_d/K_d^b	f_{rel}^c
G	18 ± 1	32 ± 7	0.56	1.0	1.0	1.0
A	0.37 ± 0.05	350 ± 60	1.1×10^{-3}	49	11	540
T	0.057 ± 0.014	130 ± 20	4.4×10^{-4}	320	4.1	1,300
C	0.090 ± 0.010	320 ± 60	2.8×10^{-4}	200	10	2,000
Abasic site	20 ± 1	290 ± 30	0.069	0.90	9.1	8.2
8-oxoG	1.1 ± 0.1	200 ± 30	5.5×10^{-3}	16	6.3	100

^a k_{pol} for dCTP divided by k_{pol} for the given dNTP

^b K_d for the given nucleotide divided by K_d for dCTP

^c relative efficiency of incorporating the given dNTP versus dCTP, which is k_{pol}/k_{pol} times K_d/K_d .

Table 3

Active site titrations of Rev1 with non-damaged and damaged DNA

Template	K _d -DNA (nM)	Active Rev1 (%) ^a
G	12 ± 1	6.8 ± 0.1
Abasic site	10 ± 1	8.4 ± 0.1
8-oxoG	25 ± 2	32 ± 1

^aDetermined by dividing [Rev1]_{active} by [Rev1]_{total}