Yeast Rev1 Protein Is a G Template-specific DNA Polymerase*

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Rev1 protein of Saccharomyces cerevisiae functions with DNA polymerase ζ in mutagenic trans-lesion synthesis. Because of the reported preferential incorporation of a C residue opposite an abasic site, Rev1 has been referred to as a deoxyribozyme. Here, we use steady-state kinetics to examine nucleotide incorporation by Rev1 opposite undamaged and damaged template residues. We show that Rev1 specifically inserts a C residue opposite template G, and it is ∼25-, 40-, and 400-fold less efficient at inserting a C residue opposite an abasic site, an O’-methylguanine, and an 8-oxoguanine lesion, respectively. Rev1 misincorporates G, A, and T residues opposite template G with a frequency of ∼10−3 to 10−4. Consistent with this finding, Rev1 replicates DNA containing a string of Gs in a template-specific manner, but it has a low processivity incorporating 1.6 nucleotides per DNA binding event on the average. From these observations, we infer that Rev1 is a G template-specific DNA polymerase.

The REV1- and RAD30-encoded proteins of Saccharomyces cerevisiae are members of the Y family of DNA polymerases (1). All of the proteins in this family share five highly conserved motifs from I to V, but they differ in their ability to promote replication through DNA lesions (2, 3). By contrast to the RAD30-encoded DNA polymerase η, which incorporates all four deoxynucleotides in a template-specific manner and has the ability to replicate through a variety of DNA lesions (4–7), the Rev1 protein has been reported to preferentially incorporate a C residue opposite an abasic site (8). Rev1 also could incorporate a C residue opposite template G and A but was only ∼20 and 10% as effective, respectively, in these reactions as the insertion opposite an abasic site (8). Because of the preferential ability of Rev1 to insert a C residue opposite an abasic site, a noninformational DNA lesion, this activity has been referred to as a deoxyribozyme.

The Rev1 and Rad30 proteins differ also in the manner in which they contribute to the replication of UV-damaged DNA. Whereas Rev1 functions with the REV3, REV7-encoded DNA polymerase ζ (9) in the mutagenic bypass of UV lesions, polymerase η (Polη)7 promotes the error-free bypass of UV-induced cyclobutane pyrimidine dimers. Polη replicates through a cis-syn-thymine-thymine (T-T) dimer with the same efficiency and accuracy as through undamaged Ts (10, 11), and genetic studies in yeast have also indicated a role for Polη in the error-free bypass of cyclobutane dimers formed at 5’-TC-3’ and 5’-CC-3’ sites (12). Polζ, which is a member of the Polζ family, promotes lesion bypass by extending from the nucleotide inserted by another DNA polymerase opposite the 3’-T of a T-T dimer or a (6-4)/T photoprotein (13, 14). Although the Rev1 protein is almost as indispensable for UV mutagenesis as is Polζ, its C-transferase activity is not needed for this function, because C insertion occurs only rarely opposite the UV lesions in yeast (15, 16) and inactivation of this biochemical activity has no impact on UV mutagenesis.2

The mutagenic bypass of abasic sites also requires Rev1 and Polζ (17), and it depends upon the sequential action of two DNA polymerases in which one inserts the nucleotide opposite the abasic site and Polζ subsequently extends from the inserted nucleotide (18). Although Rev1 is able to insert a C residue opposite an abasic site in an in vitro reaction, the inactivation of Rev1 C-transferase activity causes significant reduction in the incidence of mutations resulting from the bypass of abasic sites (18). This result is probably because of the fact that many different polymerases, including Polδ and Polγ, contribute to the insertion step (18, 19). The indispensability of the Rev1 protein, but not of its C-transferase activity, for the mutagenic bypass of UV lesions as well as that of abasic sites has suggested that the primary role of Rev1 in the mutagenic bypass of these lesions is structural. In this role, Rev1 could act as an intermediary, promoting the assembly of Polζ with Polδ stalled at a lesion site (18).

To provide for a better understanding of the biochemical activity of Rev1 protein, here we employ steady-state kinetic analyses to examine the efficiency of nucleotide incorporation by this protein on undamaged and damaged DNA templates.

MATERIALS AND METHODS

Proteins and DNA Substrates—S. cerevisiae Rev1 protein in fusion with glutathione S-transferase (8) was expressed in the yeast strain BY2 and purified on a glutathione-Sepharose 4B column followed by MiniS (Amersham Biosciences) chromatography as described previously (18). Oligonucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX). DNA substrates (S1-) were generated by annealing a 52-nucleotide (nt)-long oligonucleotide template 5’-TTC GTA TAA TGC CTA CAC TGC CTC AAG AAT TCG TAA-3’ to 52-nt 5’-AGC TAC CAT GCC TGC CTC AAG AAT TCG TAA-3’. The 5’-32P-labeled oligonucleotide primer N4456, 5’-GGT TTG CCA GTG ACC ACC ATG CTC CGG TAC TC-3’ (18) was generated by annealing a 52-nucleotide (nt)-long oligonucleotide template 5’-TTC GTA TAA TGC CTA CAC TGC CTC AAG AAT TCG TAA-3’ to the 32-nt 5’-P-labeled oligonucleotide primer N4456, 5’-GGT TTG CCA GTG ACC ACC ATG CTC CGG TAC TC-3’.

DNA substrates (S2-) were generated by annealing a 75-nt oligomer template 5’-AGC TAC CAT GCC TGC CTC AAG AAT TCG TAA-3’ containing a G (S1-G), an A (S1-A), a T (S1-T), a C (S1-C), or an abasic site (a tetrahydrofuran moiety) (S1-AP) at position 20 (X) to the 32-nt 5’-P-labeled oligonucleotide primer N4456, 5’-GGT TTG CCA GTG ACC ACC ATG CTC CGG TAC TC-3’.

The abbreviations used are: Pol, polymerase; nt, nucleotide; AP, abasic site; m6G, O’-methylguanine; 8-oxoG, 8-oxoguanine.

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2 L. Haracska, S. Prakash, and L. Prakash, unpublished observations.
template used for generating the substrate S2-G was annealed to the 5′-32P-labeled oligonucleotide primer N4577, 5′-GTT TTC CCA GTC AGC ATG CTG CGG TA-3′, yielding substrate S3, or this template was annealed to the 5′-32P-labeled oligonucleotide primer N4265, 5′-GTT TTC CCA GTC AGC ATG CTG CGG TA-3′, yielding substrate S4. S5 DNA substrate (Fig. 2) was generated by annealing a 43-nt oligonucleotide template, 5′-GGG GGG GGG GGG AGT ACC GGA GCA TCG TGA AAA C-3′, to the 5′-32P-labeled oligonucleotide primer N4456.

**Deoxynucleotide Incorporation Assays**—A standard primer extension reaction (10 μl) contained 40 mM Tris-HCl (pH 7.5), 8 mM MgCl2, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 10% glycerol. Assays were assembled on ice, incubated at 30 °C for 10 min, and stopped by the addition of loading buffer (40 μl) containing EDTA (20 mM), 95% formamide, 0.3% bromphenol blue, 0.3% cyanol blue. The reaction products were resolved on 10% polyacrylamide gels containing 8 M urea. The quantitation of the substrates and products was quantitated by PhosphorImager, and the data were fit by nonlinear regression using SigmaPlot 5.0 to the Michaelis-Menten equation describing a hyperbola, v = (Vmax × [dNTP]/Km + [dNTP]). Apparent Km and Vmax steady-state parameters were obtained from the fit and were used to calculate the efficiency of deoxynucleotide incorporation (kcat/Km).

**RESULTS**

**Nucleotide Incorporation by Rev1**—First we examined whether Rev1, besides its deoxycytidyltransferase activity, is able to incorporate other nucleotides opposite various undamaged and damaged template residues. DNA substrates containing a different template nucleotide at the primer-template junction were incubated with Rev1 in the presence of just one dNTP (Fig. 1A). Rev1 protein incorporated a C residue across from each of the four undamaged template nucleotides and also with various efficiencies opposite uracil, 8-oxoG, m6G, and abasic site. Whereas the C residue was incorporated most readily opposite template G (Fig. 1A, lanes 1–4). Because yeast Rev1 was shown to have only a deoxycytidyltransferase activity in the previous study (8), we examined the dependence of these other nucleotide incorporations by Rev1 on the sequence context and on the Tris buffer we used instead of the phosphate buffer employed in the previous study. The incorporation by Rev1 of each of four deoxynucleotides opposite various template G residues in different sequence contexts. DNA substrates (10 nM) were incubated with Rev1 (5 nM) in a phosphate buffer for 10 min at 30 °C.
a template G residue present in two other sequence contexts was examined in phosphate buffer (Fig. 1B). Here also, Rev1 incorporated all four nucleotides opposite template G; however, the level of incorporation of the G, A, and T nucleotides varied depending on the sequence context of the template (Fig. 1B, compare lanes 1–3 with lanes 7–9). Rev1 inserted a C residue opposite uracil, 8-oxoG, and m6G, whereas opposite an abasic site in addition to a C, Rev1 inserted some G and T as well (Fig. 1A).

**DNA Synthesis by Rev1 on a G-containing Template**—Next, we examined whether Rev1 could synthesize DNA on a template containing a string of Gs in a template-specific manner, and we found that Rev1 could in fact synthesize DNA almost to the end of this template, specifically inserting a C residue (Fig. 2A, lanes 5 and 6). This observation raised the possibility that instead of transferring only one nucleotide in a single DNA binding event, Rev1 was able to polymerize DNA, like DNA polymerases, in a G template-dependent manner and could incorporate more than one nucleotide per DNA binding event. We measured the processivity of Rev1 on a DNA substrate containing a string of 11 Gs toward the 5′ end of the template right after the primer:template junction (Fig. 2B). Processivity is a measure of how many deoxynucleotides a DNA polymerase incorporates in a single DNA binding event. To ensure that we were observing deoxynucleotide incorporation resulting from a single DNA binding event, we monitored DNA synthesis by Rev1 in the presence of excess of nonradiolabeled DNA substrate as a trap (Fig. 2B). The reactions were performed by first preincubating Rev1 with the radiolabeled DNA substrate without deoxynucleotide. DNA synthesis was initiated by the addition of increasing concentrations of all four deoxynucleotides (Fig. 2B, lanes 1–7) or a mixture of 100-fold excess of the same but nonradiolabeled DNA substrate and all four deoxynucleotides to the reaction (Fig. 2B, lanes 8–14). Under these conditions, any Rev1 molecule that dissociates from the labeled DNA substrate will be trapped by the excess of nonradiolabeled DNA. The effectiveness of the trap was verified by first preincubating Rev1 with the radiolabeled DNA substrate and the excess nonlabeled DNA followed by the addition of deoxynucleotides. The lack of DNA synthesis in this sample shows that the 100-fold excess of nonlabeled DNA is sufficient to trap all Rev1 molecules (Fig. 2B, lane 15). In the presence of saturating nucleotide concentration, Rev1 copied the template DNA, incorporating as many as nine nucleotides in a single DNA binding event (Fig. 2B, lane 14).

The percentage of active Rev1 molecules was calculated from the intensities of bands in Fig. 2B, lane 14 (see “Materials and Methods”), and the percentage of Rev1 molecules adding at least one deoxynucleotide was set as 100%. The percentage of active Rev1 molecules decreased after the addition of each subsequent nucleotide because of the dissociation of some fraction of Rev1 molecules from DNA (Fig. 2C). The processivity of Rev1, defined quantitatively as the probability (Pn) for each deoxynucleotide incorporation event n that Rev1 will move...
Steady-state kinetic parameters of nucleotide insertion reactions catalyzed by yeast Rev1 protein

<table>
<thead>
<tr>
<th>Site</th>
<th>dNTP added</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (min⁻¹ μM⁻¹)</th>
<th>$f_{inc}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insertion opposite G</td>
<td>dGTP</td>
<td>88 ± 9</td>
<td>0.21 ± 0.01</td>
<td>0.0024</td>
<td>2.0 × 10⁻³</td>
</tr>
<tr>
<td>5′--CTC</td>
<td>dATP</td>
<td>440 ± 130</td>
<td>0.034 ± 0.008</td>
<td>0.00008</td>
<td>6.5 × 10⁻⁵</td>
</tr>
<tr>
<td>--GAGGTC--</td>
<td>dTTP</td>
<td>110 ± 20</td>
<td>0.15 ± 0.01</td>
<td>0.0013</td>
<td>1.1 × 10⁻³</td>
</tr>
<tr>
<td>Insertion opposite A</td>
<td>dCTP</td>
<td>97 ± 8</td>
<td>0.25 ± 0.02</td>
<td>0.0025</td>
<td>2.0 × 10⁻³</td>
</tr>
<tr>
<td>5′--CTC</td>
<td>dCTP</td>
<td>48 ± 4</td>
<td>0.22 ± 0.01</td>
<td>0.0045</td>
<td>3.7 × 10⁻³</td>
</tr>
<tr>
<td>Insertion opposite T</td>
<td>dCTP</td>
<td>26 ± 3</td>
<td>0.24 ± 0.03</td>
<td>0.009</td>
<td>7.5 × 10⁻³</td>
</tr>
<tr>
<td>5′--CTC</td>
<td>dCTP</td>
<td>280 ± 60</td>
<td>0.17 ± 0.008</td>
<td>0.00060</td>
<td></td>
</tr>
<tr>
<td>Insertion opposite abasic site</td>
<td>dGTP</td>
<td>1200 ± 500</td>
<td>0.016 ± 0.009</td>
<td>0.0000103</td>
<td></td>
</tr>
<tr>
<td>5′--CAT</td>
<td>dATP</td>
<td>290 ± 70</td>
<td>0.12 ± 0.005</td>
<td>0.00040</td>
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<tr>
<td>Insertion opposite abasic site</td>
<td>dCTP</td>
<td>4.7 ± 0.2</td>
<td>0.24 ± 0.02</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>5′--CAT</td>
<td>dCTP</td>
<td>0.21 ± 0.03</td>
<td>0.18 ± 0.03</td>
<td>0.86</td>
<td>1.0</td>
</tr>
<tr>
<td>Insertion opposite abasic site</td>
<td>dCTP</td>
<td>5.2 ± 0.6</td>
<td>0.17 ± 0.02</td>
<td>0.032</td>
<td>3.7 × 10⁻²</td>
</tr>
<tr>
<td>5′--CAT</td>
<td>dCTP</td>
<td>7.2 ± 0.8</td>
<td>0.16 ± 0.02</td>
<td>0.022</td>
<td>2.6 × 10⁻²</td>
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<tr>
<td>Insertion opposite O-methylguanine</td>
<td>dCTP</td>
<td>18 ± 2</td>
<td>0.042 ± 0.013</td>
<td>0.0023</td>
<td>2.7 × 10⁻³</td>
</tr>
<tr>
<td>8-oxo</td>
<td>dCTP</td>
<td>100 ± 5</td>
<td>0.008 ± 0.002</td>
<td>0.0004</td>
<td></td>
</tr>
</tbody>
</table>

As indicated by the $k_{cat}/K_m$ values, Rev1 incorporates a C opposite template G ~130–500-fold more efficiently than opposite template A, T, or C. Opposite template G, Rev1 incorporates the correct C ~500-, 1000-, or 15000-fold more efficiently than it inserts the incorrect G, T, or A, respectively (Table I). This finding indicates that while inserting nucleotides opposite undamaged template bases, Rev1 uses the information of the template and discriminates between the correct and incorrect nucleotides.

Steady-state Kinetic Analysis of Nucleotide Incorporation by Rev1 Opposite Damaged Bases—Next, we compared the efficiencies of deoxyxynucleotide insertion opposite an undamaged G, an abasic site, an m6G, and an 8-oxoG (Fig. 3). As judged from the $k_{cat}/K_m$ values, Rev1 inserts a G, an A, a T, or a C ~4-, 6-, 3-, 24-fold less efficiently, respectively, opposite an AP site than opposite an undamaged G (Table I). In another sequence context, Rev1 incorporated a C residue opposite an AP site ~27-fold less efficiently than opposite an undamaged G template residue (Fig. 3 and Table I). Rev1 inserted a C residue opposite an m6G lesion, ~39-fold less efficiently, and opposite an 8-oxoG lesion ~370-fold less efficiently than opposite an undamaged G (Fig. 3 and Table I). Thus, Rev1 is sensitive to DNA lesions, and its activity is inhibited on lesion containing DNA substrates.

**DISCUSSION**

Here, we show that Rev1 is most efficient at inserting a C opposite template G, and Rev1 replicates a poly(dG) template DNA by specific insertion of Cx, and on this DNA, it incorporates 1.6 nucleotides per DNA binding event on the average. Rev1 misincorporates nucleotides opposite template G with a...
Yeast Rev1 Protein

Fig. 3. Steady-state kinetic analysis of deoxynucleotide incorporation by Rev1. A, dCTP incorporation opposite an undamaged G, an AP site, an m6G, and an 8-oxoG. Rev1 (1 nm) was incubated with the primer/template DNA (10 nm) and with the indicated concentrations of dCTP for 10 min at 30 °C. B, quantitation of dCTP incorporation reaction. The rate of incorporation is graphed as a function of dCTP concentration, and the data are fit to the Michaelis-Menten equation. The \( k_{\text{cat}} \) and \( K_m \) parameters obtained from the fit are listed in Table I.

In conclusion, our results indicate that Rev1 is a G template-specific DNA polymerase. Although Rev1 can also incorporate a C opposite template T, A, and C, it does so with a frequency of \( 10^{-2} \) to \( 10^{-3} \). In this regard also, Rev1 resembles Pol\( \eta \), which misincorporates a C opposite these templates with a similar frequency (20). However, in its specificity for primarily incorporating a C opposite template G, Rev1 is more similar to human Pol\( \eta \) (13, 23), another member of the Y polymerase family, than to Pol\( \eta \). Pol\( \eta \) is most efficient at inserting the correct nucleotide T opposite template A, and opposite this template, it misincorporates with a frequency of \( 10^{-4} \) to \( 10^{-6} \). Although Pol\( \eta \) incorporates the correct nucleotides C and G opposite templates G and C, respectively, it is much less efficient and accurate opposite these templates than opposite template A (13). Pol\( \eta \) is highly inefficient at inserting nucleotides opposite template T, and moreover, it incorporates a G opposite this template –10-fold better than an A (13). Thus, Pol\( \eta \) is most efficient and accurate on template A, and on the other three template nucleotides, it is much less efficient and accurate. However, in striking contrast to Pol\( \eta \), Rev1 is unable to insert the other three nucleotides with any reasonable efficiency, and its ability to primarily insert a C represents the most extreme deviation from normal polymerase behavior.

Rev1 and Pol\( \eta \) resemble each other in their ability to insert nucleotides opposite an abasic site. However, in contrast to the specific insertion of a C opposite this lesion by Rev1, Pol\( \eta \) primarily incorporates an A, and to a lesser extent, a G opposite this lesion site (13). The specificity of Rev1 for C insertion opposite an abasic site is enigmatic, because when a pyrimidine is positioned opposite the abasic site, both the pyrimidine and the abasic sugar are extrahelical and the helix collapses (24). By contrast, when an A is positioned opposite the AP site, the DNA retains the B-form, and both the unpaired A and the abasic residues remain intrahelical (24–26). Consequently, most DNA polymerases tend to insert an A opposite an abasic site (27). At low temperatures, a G opposite an abasic site is also predominantly intrahelical, and similar to Pol\( \eta \), it inserts a G or an A opposite an abasic site (19).

Pre-steady-state kinetic analyses of Pol\( \eta \) have indicated a two-step nucleotide binding mechanism in which the DNA bound polymerase first binds the nucleotide and then undergoes the rate-limiting induced fit conformational change prior to the chemical step of phosphodiester bond formation (28). The preference of Rev1 to insert a C opposite template G and also opposite DNA lesions might suggest that Rev1 undergoes the induced fit conformational change prior to the chemical step of phosphodiester bond formation far more readily when a C nucleotide is bound in the active site of the enzyme. In the presence of nucleotides other than C, such a conformational change may not occur or it becomes too slow. The over 20-fold preferential incorporation of a C opposite template G than opposite an abasic site and even a greater inhibition of C insertion opposite 8-oxoG and m6G lesions additionally suggest that the presence of G in the template is also important for such a putative conformational change to occur.

In conclusion, our results indicate that Rev1 is a G template-specific DNA polymerase. However, because of its inability to incorporate all four deoxynucleotides during the synthesis reaction, we consider it inappropriate to assign it a Greek letter nomenclature used for eukaryotic DNA polymerases.
REFERENCES


