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Biochemical properties of the human REV1 protein

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Abstract It has been proposed that the REV1 protein plays an important role in the induced-mutagenesis pathway. We show that purified REV1 protein inserts dCMP opposite template G, A, T and C, and dGMP and dTMP opposite template G in the presence of magnesium, while in the presence of manganese the specificity for dCMP was found to be relaxed and the REV1 protein acquired the ability to insert dCMP, dGMP, dAMP and dTMP opposite templates G, A, T, and C. Kinetic analysis provided evidence for high affinity for dCTP with template G, suggesting that the REV1 protein is specialized for dCTP and template G. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mutagenesis; Postreplication repair; Translesion DNA synthesis; Y-family DNA polymerase

1. Introduction

The damage-induced mutagenesis pathway is a tightly regulated cellular function. Genetic studies in *Saccharomyces cerevisiae* have contributed to the discovery of genes required for the mutagenesis, including *RAD6*, *RAD18*, *REV1*, *REV3*, and *REV7* [1–5]. Mutations in the *RAD6* and *RAD18* genes disrupt the tight regulation so that strong sensitivity to a wide range of DNA-damaging agents is exhibited [6]. Rad6, one of 13 ubiquitin-conjugating enzymes in *S. cerevisiae*, forms heterodimers with Rad18, the complex possessing single-stranded DNA-binding and ATPase activities [7–9]. It has been postulated to recognize single-stranded gaps and activate the damage-induced mutagenesis pathway. The *rev* mutants were initially isolated by reduced mutations after UV treatment [1]. *REV3* encodes the catalytic subunit of DNA polymerase ζ, which also contains Rev7, capable of translesion DNA synthesis [10]. This DNA polymerase is specifically required for damage-induced mutagenesis in yeast.

Rev1 belongs to the Y family of DNA polymerases [11]. It contains a BRCT domain at its N-terminus and possesses dCMP transferase activity in a template-directed reaction [12]. However, the main role of the *REV1* gene in the mutagenesis pathway is not due to action as a deoxyribonucleotidyl transferase. Although the Rev1 protein does not allow bypassing T-T (6-4) photoproducts in vitro, the gene is required for translesion DNA synthesis in vivo [13]. A mutagenesis-deficient mutant has a mutation in the BRCT domain but the encoded protein retains normal dCMP transferase activity [13], and methyl methanesulfonate-induced mutagenesis has been shown to be normal in a site-directed mutant lacking dCMP transferase activity [14]. Therefore, the main function of the Rev1 protein may be to incorporate a large complex with DNA polymerase ζ, by protein–protein interactions [14].

The damage-induced mutagenesis pathway is evolutionarily conserved from yeast to humans. The genes involved in yeast have mammalian homologues, including *HR6A*, *HR6B*, *RAD18*, *REV1*, *REV3* and *REV7* [15–24]. The human REV1 protein contains a BRCT domain at the N-terminus like the yeast Rev1 protein [19,20,23] and a domain required for binding to the human REV7 protein at the C-terminus [24]. Since these domains are not required for dCMP transferase activity [23], their conservation may further point to an importance for incorporation of large complexes with DNA polymerase ζ.

In the present study, we developed a method to purify the REV1 protein without any tags for rapid purification, and investigated the enzyme properties using the intact protein. The intact REV1 protein may be suitable for investigations of protein–protein interactions in the mutagenesis pathway.

2. Materials and methods

2.1. Construction of a REV1 expression plasmid

To remove the portion encoding a histidine tag from the h6-*REV1S* expression plasmids, pBAD-REV1S and pBAD-REV1SA [23], each was digested with *Nde*I and *Eco*RV, and the respective *Nde*I–*Eco*RV fragments were inserted into the corresponding site of the pET20b(+) vector (Invitrogen). *Xba*I fragments of each of the resulting plasmids (pET-I-REV1S and pET-I-REV1SA), containing the Shine–Dalgarno sequence and the start codon of the REV1S cDNA, were replaced with the corresponding fragment of pBAD-REV1S [23]. The resulting plasmids pBAD-I-REV1S and pBAD-I-REV1SA produce wild type and mutant type (D569A/E570A) proteins, respectively. They contain the *araC* gene and the p_{BAD} promoter of the arabinose operon, and gene expression is consequently induced by L-(+)-arabinose (Fig. 1).

2.2. Overproduction and purification of human REV1 protein

The bacterial strain, BL21(DE3) [25], harboring pBAD-I-REV1S was inoculated into 40 ml of Luria broth supplemented with ampicillin (100 μg/ml). Cells were grown at 15°C for 24 h with vigorous shaking. This starter culture was transferred into 2 l of SB medium (32 g NZ-amine/20 g yeast extract/5 g NaCl/0.5 ml 10 M NaOH, all per liter) [26] supplemented with ampicillin (250 μg/ml), and cells were further grown at the same temperature. The doubling time of the cells was 5.5 h under these conditions. When the A_{600} value reached 0.6, 20 g of L-(+)-arabinose was added and incubation was continued for 11 h. Then cells were harvested by centrifugation at 4°C. The resultant cell paste (12 g) was resuspended in 24 ml buffer I (50 mM HEPES–NaOH, pH 7.5/1 M NaCl/0.1 mM EDTA/10 mM β-mercaptoethanol) at 4°C, frozen in liquid nitrogen and stored at −80°C. The frozen cells were thawed in ice water with shaking, and lysed after addition of phenylmethylsulfonyl fluoride to 1 mM by introduction of 4 ml of buffer I containing 100 mM spermidine and 4 mg/ml lysozyme. The cells were incubated on ice for 30 min, heated in a 37°C water bath for

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2 min with mild mixing, and then further incubated on ice for 30 min. The cell lysate was clarified by centrifugation at 35 000×*g* for 30 min at 4°C and 25 ml of clarified supernatant was obtained (fraction I; 25 ml, 250 mg protein).

The following column chromatography was carried out at 4°C using an FPLC system (Pharmacia). Fraction I was mixed with 240 μl of buffer A (50 mM HEPES–NaOH, pH 7.5/1 M NaCl/10% glycerol/10 mM β-mercaptoethanol) containing 1 M imidazole and applied at 0.2 ml/min to a 1-ml HiTrap chelating column (Pharmacia), which had been treated with 2 ml of 0.5 M $NiSO_4$ and equilibrated with buffer A containing 10 mM imidazole. The column was washed with 15 ml of equilibration buffer at 0.1 ml/min and then the REV1 protein was eluted with 10 ml of a linear gradient of 10–100 mM imidazole in buffer A and collected in 500-μl fractions. A sample (5 μl) of each fraction was analyzed by SDS–PAGE and peak fractions containing the REV1 protein, eluted in a main peak at the beginning of the gradient, were pooled (fraction II; 2.5 ml, 6.3 mg protein) and dialyzed against buffer B (50 mM HEPES–NaOH, pH 7.5/10% glycerol/10 mM β-mercaptoethanol) containing 400 mM NaCl.

Fraction II was then loaded at 0.1 ml/min onto a 1-ml HiTrap heparin column (Pharmacia) equilibrated with the dialysis buffer. The column was washed with 10 ml of the equilibration buffer and the REV1 protein was eluted with 10 ml of a linear gradient of 400–700 mM NaCl in buffer B and collected in 500-μl fractions. A sample (5 μl) of each fraction was analyzed by SDS–PAGE and the peak fraction containing REV1 protein, which was eluted in a main peak at about 550 mM NaCl, was collected (fraction III; 500 μl, 150 μg protein).

Fraction III was then applied at 0.1 ml/min to a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with buffer B containing 500 mM NaCl, and 500-μl fractions were collected. The peak fractions of REV1 protein were pooled, frozen in liquid nitrogen and stored at −80°C (fraction IV; 1 ml, 135 μg protein). Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin (BSA; Bio-Rad) as the standard.

The mutant form of the REV1 protein, D569A/E570A, was overproduced in BL21(DE3) harboring pBAD-I-REV1SA plasmid and purified as for the wild type protein.

2.3. Transferase assays

Oligonucleotide templates 5′-CTCGTCAGCATCTTCAXCATACAGTCAGTG-3′ (X = G; 30G, A; 30A, T; 30T, C; 30C, tetrahydrofuran; 30F) and the primer 5′-CACTGACTGTATG-3′ (P13) were purchased [23]. The latter was labeled using polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Amersham Pharmacia Biotech), and annealed to the templates. The standard reaction mixture (25 μl) contained 50 mM Tris–HCl buffer, pH 8.0, 25 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 5 mM dithiothreitol, 0.1 mM dNTP, 100 nM primer template, indicated amounts of divalent cations and 1 μl of protein sample, diluted with buffer B containing 500 mM NaCl and 0.1 mg/ml BSA as indicated. After incubation at 30°C for the indicated time, reactions were terminated with 10 μl of stop solution (30 mM EDTA/94% formamide/0.05% bromophenol blue/0.05% xylene cyanol) and products were resolved on 20% polyacrylamide gels containing 8 M urea and autoradiographed at −80°C. The amount of DNA present in each band was quantified using a Bio-Imaging Analyzer BAS2000 (Fuji Photo Film).

3. Results

3.1. Purification of the intact human REV1 protein from overproducing Escherichia coli cells

Human *REV1* expression from pBAD-I-REV1S, under control of the p_{BAD} promoter, was induced by addition of arabinose (Fig. 1). When the cells were grown at 37°C, many bands of degradation products of the REV1 protein were detected by Western blotting (data not shown). The degraded fractions were much reduced when the cells were grown at 15°C and a clear 150-kDa band was observed (Fig. 2, lane 3). We used those cells as the starting material for purification of human REV1 protein.

As REV1 protein is readily precipitated at low salt concentrations [19], a crude lysate was prepared in the presence of 1 M NaCl and a large amount of induced REV1 protein was recovered in the soluble fraction (Fig. 2, lane 4). We found the intact REV1 protein to have an affinity for the nickel-chelating column. This finding facilitated the purification. The REV1 protein, but not the majority of *E. coli* proteins, bound to the column in the presence of 10 mM imidazole and eluted on increasing the imidazole gradient. In this chromatography step, REV1 represented the major band in peak fractions (Fig. 2, lane 5). After dialysis against buffer containing 400 mM NaCl, no precipitate was observed. Then the sample was

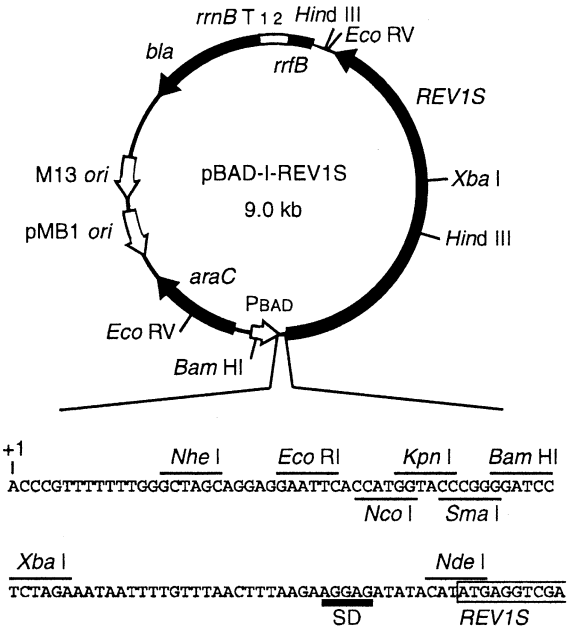

Fig. 1. Structure of the *REV1* expression plasmid. The human *REV1* gene is under the control of the p_{BAD} promoter regulated by the *araC* gene, whose transcription is induced by arabinose. +1 represents the position of the 5′-terminus of the transcript [30]. The induced protein is a short form of the REV1 protein, REV1S [23].

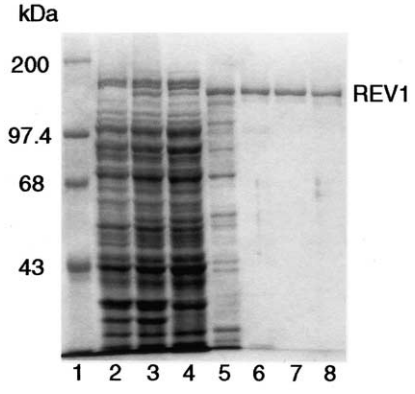

Fig. 2. Results of SDS–PAGE showing overproduction and stages of purification of wild type (lanes 2–7) and D569A/E570A (lane 8) REV1 from *E. coli*. Lane 1, molecular weight markers (Invitrogen); lanes 2 and 3, total *E. coli* proteins before and after induction with arabinose, respectively; lane 4, crude *E. coli* lysate (fraction I, 25 μg); lane 5, pooled fractions eluted from the nickel-chelating column (fraction II, 3 μg); lane 6, the peak fraction eluted from the heparin column (fraction III, 0.6 μg); lanes 7 and 8, pooled fractions eluted from the gel filtration column (fraction IV, 0.5 μg).

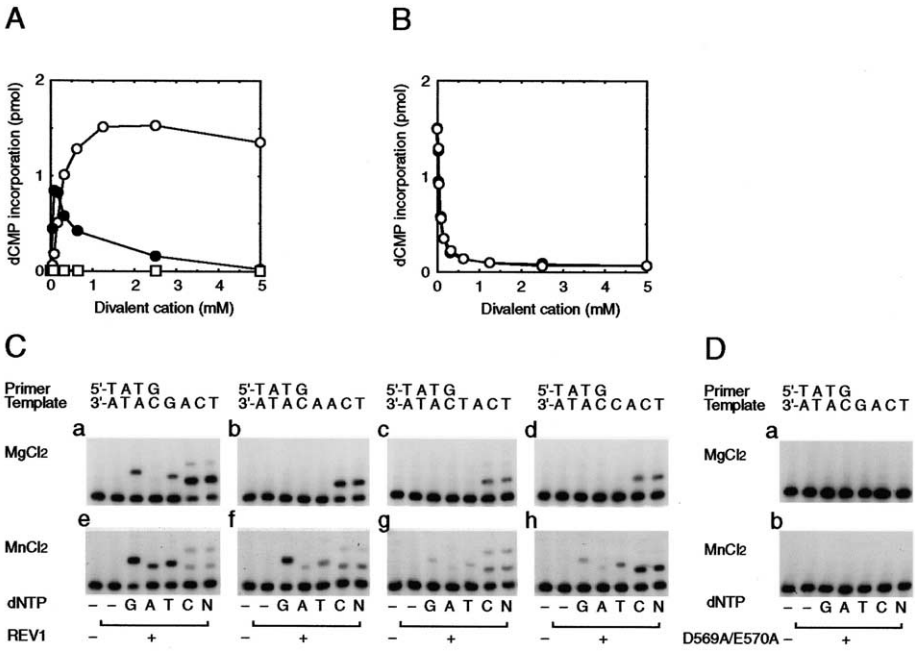

Fig. 3. The effects of divalent cations. A: Titration of divalent cations. The 5′-^{32}P-labeled primer P13 was annealed with the template 30G (P30G). Aliquots of 10 ng REV1 protein were incubated with P30G and dCTP under standard reaction conditions with the indicated concentrations of magnesium chloride (open circles), manganese chloride (closed circles) or calcium chloride (open squares) at 30°C for 10 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea, the band intensities of substrates and products were determined using a Bio-Imaging Analyzer BAS2000, and the amounts (in pmol) of products in 25 μl of reaction solution were calculated. B: Inhibitory effects of manganese chloride and calcium chloride in the presence of 5 mM magnesium chloride. Aliquots of 10 ng REV1 protein were incubated with P30G and dCTP under standard reaction conditions with 5 mM magnesium chloride and the indicated concentrations of calcium chloride (open circles) or manganese chloride (closed circles) at 30°C for 10 min. C: Substrate specificity of the transferase reactions. The 5′-^{32}P-labeled primer, P13, was annealed with each of the templates, 30G (panels a and e), 30A (panels b and f), 30T (panels c and g), 30C (panels d and h). The nucleotide sequences adjacent to the primer terminus are shown at the top of each panel. Aliquots of 10 ng REV1 protein and the indicated primer template were incubated with no dNTP (−), a single dNTP (G, A, T, C), or all four dNTPs (N) under standard reaction conditions with 2 mM magnesium chloride (panels a–d) or 1 mM manganese chloride (panels e–h) at 30°C for 10 min. The reaction products were resolved in 20% polyacrylamide gels containing 8 M urea and visualized by autoradiography. D: Transferase reactions with a mutant form of the REV1 protein, D569A/E570A. Aliquots of 100 ng D569A/E570A protein and P13G were incubated with no dNTP (−), a single dNTP (G, A, T, C), or all four dNTPs (N) under standard reaction conditions with 2 mM magnesium chloride (panel a) or 1 mM manganese chloride (panel b) at 30°C for 10 min.

loaded onto a heparin column. In the presence of 400 mM NaCl, almost all *E. coli* proteins were found in the flow-through fraction, but REV1 protein was bound and eluted at about 550 mM NaCl (Fig. 2, lane 6). For further purification, the peak fraction from heparin chromatography was applied to a Superdex 200 column (Pharmacia). The REV1 protein eluted with an apparent molecular mass of 380 kDa and a Stokes radius of 59 Å. Analysis by SDS–PAGE revealed a full-length REV1 protein of 150 kDa and minor contaminating degraded forms (Fig. 2, lane 7), detected specifically when the REV1 protein was induced in *E. coli* cells (data not shown). The mutant REV1 protein, D569A/E570A, was purified by exactly the same procedures (Fig. 2, lane 8). In these purified protein fractions, neither DNA polymerase nor deoxyribonuclease activities were detected (Fig. 3C,D, data not shown).

3.2. Optimum conditions for the transferase reaction

To test the reaction conditions for dCMP transferase activity of the REV1 protein, glycerol was first omitted from the reaction buffer because it had a slight inhibitory effect and increased the rate of dCMP insertion opposite the template adenine. Next, buffer system conditions and pH were investigated using sodium phosphate, potassium phosphate, HEPES–NaOH, and Tris–HCl buffers. Maximal activity was observed between pH 7.8 and 8.5 and Tris–HCl, but not HEPES–NaOH, exerted an enhancing effect. Then we tested the effects of increasing concentrations of sodium sulfate, sodium chloride, sodium acetate, potassium chloride, potassium acetate, ammonium chloride, ammonium acetate and ammonium sulfate. We found these were stimulatory at low concentrations. We selected 25 mM ammonium sulfate because it produced good stimulation and decreased the rate of dCMP insertion opposite adenine.

3.3. Effects of divalent cations

Divalent cations are an absolute requirement for transferase reactions. When the effects of magnesium chloride, manganese chloride and calcium chloride were tested (Fig. 3), maximal activity was observed in the reaction with 2 mM magnesium chloride (Fig. 3A). Manganese chloride also supported the transferase reaction at low concentrations, the titration curve demonstrating a sharp peak at 0.1 mM, with strong inhibition on increasing the concentration (Fig. 3A). This suggested the affinity of manganese for the REV1 protein was higher than that of magnesium, but the efficiency for catalysis lower. This idea was supported by an experiment in which the effects of manganese chloride were analyzed in the presence of 5 mM magnesium chloride (Fig. 3B). The results showed manganese to exert inhibitory effects on the transferase reaction with

magnesium (Fig. 3B). Calcium chloride could not support the catalysis at concentrations between 0.02 mM and 20 mM (Fig. 3A, data not shown) and inhibitory effects were shown in the presence of 5 mM magnesium chloride (Fig. 3B). The inhibition curve of calcium was identical to that of manganese (Fig. 3B), suggesting similar affinities of the two cations for the REV1 protein.

Then substrate specificity of the transferase reaction was analyzed in the presence of different divalent cations. In the presence of magnesium, REV1 protein inserts dCMP opposite template G, A, T and C, and dGMP and dTMP opposite template G (Fig. 3C, panels a–d) [27]. The REV1 protein has a weak ability to extend a mismatch terminus (Fig. 3C, panel c). In the presence of manganese, REV1 transferred dGMP, dAMP, dTMP and dCMP opposite all the templates examined (Fig. 3C, panels e–h). However, in the presence of all four dNTPs, the dCMP transferase reaction predominated. Of the transferase reactions for dGMP, dAMP and dTMP, template G proved to be the most efficient substrate (Fig. 3C, panels e–h). Templates A, T and C were less efficient, even though the respective dTMP, dAMP and dGMP transfer reactions made Watson–Crick base pairs (Fig. 3C, panels e–h). It seems likely that REV1 is specialized for transfer of dNMP moieties to template G.

To determine whether activity is intrinsic to the REV1 protein, we examined a mutant, D569A/E570A, which lacks transferase activity [23,27]. Even with 100 ng protein, 10 times the wild type protein amount, no activity was observed on incubation with magnesium or manganese (Fig. 3D).

3.4. Steady-state kinetics of the transferase reactions

Kinetic parameters were determined by steady-state gel kinetic assays under defined optimum conditions (Table 1). The assays were all carried out with 5 min incubation, because the time course of the reactions was linear until 10 min (data not shown). In the dCMP transferase reaction, the template nucleotides slightly affected k_{cat} values, which were 3.0, 3.1, 1.6 and 1.8 min^{-1} opposite templates G, A, T and C respectively. The template nucleotides strongly affected the K_m value for dCTP (Table 1). The affinity of dCTP for template G was 43, 330 and 310 times higher than for templates A, T and C, respectively.

The REV1 protein possesses an activity capable of inserting a dCMP opposite AP site [12,19]. The velocity of the reaction with template AP site was 1.5 times faster than the reaction with template G, while the affinity of dCTP for template AP site was 14 times lower than for template G.

Table 1
Kinetic analysis of dCMP incorporation with various DNA templates

Template	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (min^{-1} μM^{-1})
G	3.0	0.54	5.5
A	3.1	23	0.14
T	1.6	180	0.009
C	1.8	170	0.011
AP	4.9	7.6	0.65

Kinetic assays were performed for 5 min in 25 μl reaction solutions with 2 mM magnesium chloride using 71 fmol (10 ng) REV1 and 2.5 pmol of the primer templates shown in Fig. 3C. The AP template contained a tetrahydrofuran as an AP site analogue. dCTP concentrations ranged from 1 to 2500 μM. K_m and k_{cat} were evaluated from the plot of the initial velocity versus the dCTP concentration using a hyperbolic curve-fitting program. Data from two to four independent experiments were plotted together and the correlation coefficients (R^2) were more than 0.93.

4. Discussion

In the present study, a method was established to purify human REV1 protein without any tags, and conditions for the dCMP transferase reaction were optimized. These were then applied to determine kinetic parameters. The results revealed high efficiency for the dCMP transferase reaction with template G, in line with the earlier report of specific transfer of dCMP opposite template G by mouse Rev1 [27]. However, the mammalian REV1 protein seems to have a lower fidelity than the yeast protein [28]. Previously, we showed that the reaction efficiency was strongly affected by the surrounding nucleotide sequence [27]. It is also possible that the difference is due to the assay conditions.

Substrate specificity was strongly affected by a variety of divalent cations, magnesium chloride limiting the transferase reaction to dCMP, except with the G template, while manganese chloride disrupted the specificity. Thus with manganese, REV1 protein transferred all four dNTPs opposite templates G, A, T and C. On the other hand, calcium chloride did not support any transferase activity. In this context it is of interest that Ling et al. [29] described a calcium ion to be chelated in an active site of crystal DNA polymerase IV of *Sulfolobus solfataricus* P2, a Y-family DNA polymerase including REV1 protein. We have shown 0.5 mM calcium chloride to completely inhibit the transferase reaction with 5 mM magnesium chloride, suggesting that calcium binds to the REV1 protein, consistent with the crystallographic finding.

Human REV1 protein contains a BRCT domain at its N-terminus [19,20,23] and a domain required for binding to human REV7 at its C-terminus [24]. The BRCT domain may be necessary for binding unidentified factor(s) to regulate enzyme activity [13]. Haracska et al. [14] recently proposed an essential structural role of the REV1 protein in mutagenic bypass in assembling DNA polymerase ζ with DNA polymerase δ. Protein tags might interfere with protein–protein interactions and the intact form of REV1 protein is most appropriate for investigations of these interactions, a next step in elucidating the molecular mechanisms of mutagenesis.

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