Partial Functional Deficiency of E160D Flap Endonuclease-1 Mutant in Vitro and in Vivo Is Due to Defective Cleavage of DNA Substrates

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To assess the roles of the active site residues Glu160 and Asp181 of human FEN-1 nuclease in binding and catalysis of the flap DNA substrate and in vitro biological processes of DNA damage and repair, five different amino acids were replaced at each site through site-directed mutagenesis of the FEN-1 gene. The mutants were then expressed in Escherichia coli and purified using a His-tag. Even though the mutants bind to the flap DNA to different degrees, most of the mutants lost flap nuclease activity with the exception of an E160D mutant. This mutant retained wild type-like binding ability, specificity, and partial catalytic activity. Detailed steady state and pre-steady state kinetic analysis revealed that the functional deficiency of this mutant was due to retardation of the endonucleolytic cleavage. When the mutant enzyme E160D was expressed in yeast, it partially complements the biological functions of the homologous yeast gene, RAD27, and reverses the hyper-temperature lethality and hypersensitivity to methyl methanesulfonate, in a manner corresponding to the in vitro activity.

Accumulations of mutations are a fundamental mechanism of carcinogenesis. Many DNA mutations occur independent of local sequence. The DNA repair systems must be able to recognize infidelity in the genome. One key characteristic which distinguishes normal DNA strands from one that contains a mutation is abnormal structures such as a mismatched nucleotide, T-T dimer, or abasic sites deviating from the customary Watson-Crick base pairing. In order to repair lesions, DNA repair enzymes must be structure-specific. The DNA repair enzyme, FEN-11 (flap endonuclease-I or five’ exonuclease-1) is a structure-specific nuclease involved in DNA replication and repair (1, 2). FEN-1, in the presence of Mg2+ or Mn2+, recognizes and cleaves a DNA flap structure composed of double-stranded DNA and a loose single-stranded 5’-flap oligonucleotide. The enzyme has been relatively well characterized biochemically in several laboratories (3–10). In replication, it is essential to remove the last ribonucleotide after RNAse H action or to remove displaced RNA primers dispensed by DNA polymerase during lagging strand synthesis (2, 11–13). FEN-1 acts as an exo- or endonuclease in a cooperative manner with a DNA polymerase (14), helicase (15, 16), proliferating cell nuclear antigen (17, 18), and maybe replication protein A (19) in order to remove multiple RNA primers before the Okazaki fragments are ligated. FEN-1 has also recently been shown to be involved in base excision repair pathways, both in a DNA polymerase β-dependent pathway and in a proliferating cell nuclear antigen-dependent pathway (20, 21). In the polymerase β-dependent pathway, FEN-1 is functional without proliferating cell nuclear antigen and replication factor C but requires DNA synthesis, which leads to a flap structure formation.

Null mutants of FEN-1 homologs (RAD27 and rad2) in Saccharomyces cerevisiae and Schizosaccharomyces pombe respectively, showed marked sensitivity to alkylating agents, modest sensitivity to ultraviolet radiation, and chromosomal instability (22–25). In addition, these mutants display a moderate growth defect at 30 °C, but accumulate in S phase at 37 °C, apparently owing to a block in DNA replication (23). In addition, a rad27 (fen-1) null mutant is a strong mutator as unexcised flap strand in Okazaki fragments lead to double-stranded DNA breaks, which are subsequently processed via a Rad51 and Rad52-dependent double-strand break repair mechanism, and to a lesser extent, by a mutagenic repair pathway (26). In addition, RAD27/FEN-1 has an active role in preventing trinucleotide repeat expansion and contraction as deletion mutants in S. cerevisiae lead to length-dependent destabilization of CTG tracts and a marked increase in expansion frequency (27, 28). Thus FEN-1 mutants in humans may lead to genetic disease such as myotonic dystrophy, Huntington’s disease, several ataxias, and fragile X syndrome (29).

The evolutionary conservation of the flap structure-specific nucleases extends from viral, eubacterial, archaeabacterial, yeast, plant to mammalian cells. The structural and functional homologs have been summarized recently (30, 31). By comparing the two conserved regions of 6 eukaryotic FEN-1/XPG family members, 5 archaeabacterial homologs, 6 5’-nuclease domains of prokaryotic DNA polymerases, and 4 bacteriophage exonucleases, the amino acid residues that we identified to be essential for DNA substrate binding and cleavage are strictly conserved in this extended family. In this report, we focus on two amino acid residues at the 160 and 181 positions, which are highly conserved throughout these 21 structure-specific nucleases (Fig. 1A). The naturally occurring amino acid at position 160 is glutamic acid, and at position 181 the residue is aspartic acid in the human FEN-1. These two positions are proposed to coordinate to a divalent metal center, primarily Mg2+, and to be involved in DNA substrate cleavage. Previous results indicate that replacement of Asp181 with an alanine results in

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1 The abbreviations used are: FEN, flap endonuclease; MMS, methyl methanesulfonate.
complete retention of binding ability, but loss of cleavage ability, while the E160A replacement leads to a partial defectiveness of binding and complete inactivation of cleavage. We have replaced the residues at the 160 position with Ala, Gln, Asn, His, and Ser and at the 181 position with Ala, Asn, Glu, His, and Ser using site-directed mutagenesis. We characterized the mutants using various nuclease assays to answer the following questions. Can the hydroxyl group in the original residues be replaced or partially replaced by a hydroxyl group from any other amino acid residue? What is the effect of the carbon chain length, Asp versus Glu?

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemical reagents used were ultrapure or > t. J. Baker analytical reagent grade. Solutions were prepared with double distilled water. Tubes and glassware for metal sensitive experiments were first washed with 10 mM EDTA and then rinsed extensively with metal-free water before use. Plasmid DNA was purified with a Qiagen kit (Qiagen, Santa Clarita, CA). Restriction enzymes and bovine serum albumin were obtained from New England Biolabs (Beverly, MA), while chromatography materials for chelating chromatography and fast protein liquid chromatography were obtained from Pharmacia Biotech (Piscataway, NJ). Oligonucleotides were synthesized on an Applied Biosystems, Inc. DNA synthesizer. E. coli ( strain in this study were cultured with the Chameleon site-directed mutagenesis kit from Stratagene as described previously (32). The follow-

**Site-directed Mutant Construction, Overexpression, and Purification**—Plasmid pBS-FEN, with the Not

**Oligonucleotides were synthesized on an Applied Biosystems, Inc. DNA synthesizer in The City of Hope National Cancer Center core facility.

**Flow Cytometric Kinetics**—Techniques for immobilization of the fluorescent DNA substrates onto the flow beads have been described in Nolan et al. (9). Flow cytometric measurements of microsphere fluorescence were made on a Becton-Dickenson FACScalibur (San Jose, CA). Samples were illuminated at 488 nm (15 milliwatts) and forward angle light scatter and 90 light scatter (side scatter). The mean fluorescence channel numbers were recorded. Kinetic experiments were started by adding substrate beads to a baseline. The sample tube was removed from the tube holder, enzyme was added at 10 s, the tube vortexed, and the sample reintroduced into the instrument. The time between mixing and data acquisition was typically 10–20 s. The mean fluorescence channel number as a function of time was calculated using the IDLYK flow cytometry data analysis program created at Los Alamos National Laboratory, and the data were presented as normalized fluorescence intensity.

**Construction of rad27 Strain**—S. cerevisiae wild type strain W1021-7C (MATa, ade2–1, can1–100, his3–11, 17, leu2-3, ura3-1) was transformed with a DNA fragment with LEU2 flanked by two 53-base pair regions upstream and downstream of RAD27 gene using the LiAc-

**Phenotypic Observation of the Yeast Mutant Strains**—The phenotypic observation of the yeast mutant strains, respectively.

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RESULTS

Two Conserved Residues in the I Domain Have Been Converted to Five Different Amino Acids—Two conserved domains have been identified in the FEN-1 family of 21 cloned enzymes (30). Proper folding of these two domains results in an active nuclease center which is specifically involved in recognition, binding, and catalysis of a flap nucleic acid substrate. Amino acid residue Asp181 is absolutely identical among these enzymes and Glu160 is conserved but may be replaced by an aspartate in the prokaryotic homologues (Fig. 1a). Previous results indicate that replacement of Asp181 with an alanine results in complete retention of binding ability, but loss of cleavage ability while the E160A replacement leads to partial defectiveness of the binding ability and a complete inactivation of cleavage ability. To determine the precise role(s) of residues 160 and 181, we have replaced them with five different amino acids with or without a hydroxyl group. For glutamate at position 160, we have changed it to alanine, glutamine, aspartate, histidine, and serine while aspartate at position 181 has been changed to alanine, asparagine, glutamate, histidine, and serine (Fig. 1b).

Nuclease Activity Screening Showed That E160D Is a Partially Active Mutant—Nine constructed mutants were overexpressed in E. coli and purified as described previously (32, 33). D181S was not purified due to its insolubility. Therefore, it was not included in further experiments. Flap endonuclease activities were assayed using a flow cytometry-based nuclease assay system that we have established (9). The relative activities were calculated from a single point of the initial cleavage velocity. The results showed that one mutant, E160D, has partial deficiency in cleavage.

FIG. 1. A, conserved active site amino acid residues and their replacements. Active site amino acid residues Glu160 and Asp181 (boxed) are strictly conserved in the flap endonuclease family. Shown is the sequence alignment of nucleas I domains of 11 FEN-1 family members from eukaryotic and archaebacterial cells: 1) human; 2) murine; 3) Xenopus laevis; 4) Arabidopsis thaliana; 5) S. cerevisiae; 6) S. pombe; 7) M. jannaschii; 8) P. furiosus; 9) Archaeglobius fulgidus; 10) Pyrococcus horikoshii; and 11) Methanobacterium thermoautotrophicum. FEN-1s were aligned with the sequence alignment program CKWHENCE written by C. M. Bruns at The Scripps Research Institute. B, the five different residues replaced at positions 160 and 181.

FIG. 2. Relative activity of mutant enzymes screened by flow cytometry. The reactions were carried out in a 500-μl reaction volume containing 10 mM MgCl2, 0.1 mg/ml bovine serum albumin, 50 mM Tris (pH 8.0), 10 μl of fluorescence labeled flap beads containing approximately 50 pm flap substrate and 50 nm recombinant enzymes (5 μl). The initial cleavage rates of enzymes were determined from the linear portions of the flow cytometric profiles. The relative activities were normalized to that of the wild type.
outstanding residual nuclease activity (about 3%) while the activities of all other mutants were comparably lower (Fig. 2). Moreover, when the activities of the mutant E160D and wild type enzyme were assayed in a steady state kinetic manner by incubating the enzymes with a relatively large amount of flap substrate at 30 °C for 30 min, approximately 30% of the wild type enzyme activity was detected in the mutant E160D (Fig. 3). Other mutants did not show detectable activity when they were assayed in the same manner.

Most Catalytically Inactive Mutants Retain Binding Ability to Flap DNA Substrate—Inactivity of the mutants could be due to a defect in the binding, cleavage, or both steps of the nuclease catalysis process. All mutant enzymes, which did not show detectable flap endonuclease activity in the gel assay, have been considered as inactive mutants. These mutants were subjected to a competition assay, where the mutant proteins were preincubated with DNA substrate without co-factor Mg^{2+} and the reactions were initiated by adding Mg^{2+} and wild type enzyme. The flap endonuclease activities were measured at different concentrations of mutant proteins. All of the mutants could inhibit the wild type enzyme activity to a certain degree (Fig. 4). The order of binding abilities of these six mutants are D181A > D181H/D181N > E160Q > E160A/E160S. Mutants D181E and E160D, which have more than 1% activity, were not included in the competition experiments. The binding ability of wild type and E160D was tested using a gel retardation assay.

Mutant E160D Has Similar Binding Ability and Specificity as the Wild Type Enzyme—The activity of E160D mutant is significantly reduced. Is this reduction due to defective binding, cleavage or both? To answer this question, we tested the substrate binding ability and preference of the E160D mutant by employing seven different substrates (Fig. 5A), and comparing the results with wild type FEN-1. After incubating FEN-1 enzymes with the substrates in the absence of Mg^{2+}, binding was visualized on a nondenaturing polyacrylamide gel. Both wild type and E160D enzymes had similar binding specificities with approximately the same affinity (Fig. 5B). The enzymes bound to flap, pseudo-Y, 3' overhang, and nicked double-stranded DNA structures, while they bound weakly to 5' overhangs and showed no apparent affinity toward either single-stranded or duplex DNA. Such qualitative results show the abilities of E160D to bind various substrates, yet exact quantitative substrate affinities will help further elucidate the enzymatic mechanism.

Kinetics of Wild Type and E160D FEN-1 Nuclease—The activities of the purified wild type and E160D mutant FEN-1 were tested by conventional steady state kinetic analysis using radiolabeled substrate and gel electrophoresis and by pre-steady state kinetic analysis using immobilized fluorescently labeled substrate and flow cytometry. We measured cleavage activity on both the flap DNA substrate and pseudo-Y substrate which lacks the upstream primer.
The gel-based assay involves the incubation of a constant amount of enzyme with increasing amounts of substrate. These data were analyzed using the Michaelis-Menten formula to derive the apparent enzyme-substrate binding constant, $K_{\text{m}}$, and an apparent catalytic rate constant, $V_{\text{max}}$. The analysis (Table I) indicated a slower reaction velocity for E160D reacted with flap DNA substrate than wild-type reacted with flap DNA substrate.

TABLE I

|        | $V_{\text{max}}$ | $K_{\text{m}}$ |
|--------|------------------|----------------|
| Flap   | Pseudo-Y         | Flap           | Pseudo-Y       |
| Wild type | 2.18 ± 2.61     | 0.38 ± 0.03    | 0.58 ± 0.05    | 9.14 ± 0.66 |
| E160D  | 1.38 ± 1.83     | 0.13 ± 0.02    | 0.41 ± 0.07    | 10.3 ± 1.48 |

The flow cytometric cleavage assay is a continuous kinetic assay in which low concentration of substrate (approximately 50 pm) was incubated with increasing concentrations of enzyme. The kinetics of DNA substrate cleavage in this assay is very sensitive to the concentration of enzyme (Fig. 6). At low enzyme concentrations, binding of enzyme to substrate is the rate-limiting step of the reaction. At high enzyme concentrations, the binding step is fast and the reaction rate approaches a maximum value associated with the single turnover of enzyme coupled with cleavage of substrate as measured in the $Mg^{2+}$-jump experiment (Fig. 7). By this interpretation, the concentration dependence of the overall reaction rate may be interpreted as a measure of the enzyme-substrate binding, with an apparent binding constant being estimated from the enzyme concentration which gives the half-maximal reaction rate.

For both the wild type cleavage of the pseudo-Y substrate and of the E160D cleavage of the flap DNA substrate, the maximal reaction rate and the enzyme concentration that gives the half-maximal reaction rate are very similar, and both are lower than those for the cleavage of the flap DNA substrate with wild type FEN. The maximal observed rate constant, measured at saturating enzyme concentrations as well as by $Mg^{2+}$-jump, was approximately 40-fold lower than that for wild type FEN-1 acting on the flap DNA substrate. This result is consistent with the gel-based steady state kinetic analysis described above.

Reversal of the Hyper-temperature Lethality—The in vitro biochemical characterization revealed that the E160D mutant is partially defective in the cleavage step. The null mutant of the RAD27 gene, which encodes the hFEN-1 homolog in yeast, displayed several phenotypes distinct from wild type, which include the cell growth arrest at 37 °C and hypersensitivity to the DNA alkylating agent methyl methanesulfonate. To test the functional compensations of hFEN-1 to the rad27 deletion mutant and to relate the in vitro biochemical activity of this enzyme to the in vivo biological functions, we expressed three human FEN-1 enzymes: wild type, E160D, E160A, in a yeast wild type strain, FSC-15B, and rad27 null mutant strain, IC2-1, using a yeast multicopy expression vector, pDB20 with an ADH1 promoter.

To test reversal of temperature sensitivity, we grew the yeast strains harboring the expression constructs of the above three FEN-1 proteins in SD-URA medium. All of the strains transformed with the vector alone, wild type FEN-1 gene, E160D FEN-1, and E160A FEN-1 can grow at 30 °C in both wild type and RAD27 minus background. When the duplicated plate is incubated at 37 °C, however, the RAD27 minus strains can only survive when they are transformed with a wild type FEN-1 expression plasmid and to a lesser extent with the E160D FEN-1 gene expressed, but there was no growth with E160A gene expressed (Fig. 8).

Reversal of MMS Hypersensitivity—In order to test the complementation of MMS hypersensitivity with a human gene...
and to correlate the in vitro and in vivo functions of this enzyme, we measured the MMS survival rates of the complete set of the above yeast strains. Fig. 9 shows the survival frequency of these six different strains at MMS concentrations ranging from 0.01 to 0.1%. The inset figure shows that at the concentration of 0.01%, the E160D mutant protein could partially support survival of the yeast deletion mutant. E160D supported 26% survival compared with the wild type strain grown on the medium without MMS. In the control, the deletion strain transformed with the wild type hFEN-1 gene could support approximately 70% of the survival at the same concentration of MMS.

DISCUSSION

While biochemical and genetic analysis has provided convincing evidence for the uniqueness and importance of FEN-1 (for recent reviews, see Refs. 1 and 2), knowledge of the intramolecular mechanisms of how the FEN-1 enzyme recog-

Fig. 6. Enzyme concentration dependent cleavage kinetics assayed by flow cytometry. Cleavage kinetics as a function of enzyme concentration: A, wild type enzyme + flap substrate; B, mutant E160D + flap substrate; C, wild type enzyme + pseudo-Y substrate; D, mutant E160D + pseudo-Y substrate. E, the observed rate constant ($k_{obs}$) calculated from the half-time versus wild type and E160D mutant enzyme concentrations (see technical details in the text and Ref. 9). ●, wild type FEN-1/flap substrate; ○, E160D FEN-1/flap substrate; ▼, wild type FEN-1/pseudo-Y substrate.
nizes, binds, and cleaves its substrate and of the organization of its Mg$^{2+}$ or Mn$^{2+}$-oriented active center is limited. To assess what roles the active site residues Glu$^{160}$ and Asp$^{181}$ of human FEN-1 nuclease might play in binding and catalysis of the flap DNA substrate and in vivo biological processes of DNA damage and repair, five different amino acids were replaced at each site through site-directed mutagenesis of the FEN-1 gene.

Structure-function Relationship of the conserved Glu$^{160}$ and Asp$^{181}$ in the Mg$^{2+}$-Oriented Active Center—FEN-1, in the presence of Mg$^{2+}$ or Mn$^{2+}$, recognizes and cleaves a DNA flap structure, composed of double-stranded DNA and a loose single-stranded 5'-flap oligonucleotide. The DNA structure-specific nature of this enzyme depends on the unique structural organization of the protein. Crystallographic structures of viral and eubacterial functional homologs, T4 RNase H, T5 exonuclease, and Taq DNA polymerase 5’-nuclease (39–41) revealed that the N-terminal and intermediate conserved regions fold together to form an “arch” structure with the nonconserved region between them. This three-dimensional structure leaves a hole in the molecule, which is large enough for a single-stranded DNA molecule, but not for a double-stranded one, to thread through (40, 42). The aromatic and bulky amino acid residues in the inner side of the arch directly interact with the DNA substrate. Site-directed mutagenesis analysis provided evidence that the conserved amino acid residues located in the inner cleft of the enzyme molecule provide ligands for two metal ions (Mg$^{2+}$ or Mn$^{2+}$) (32, 33, 43–45). One or two metal ion mechanisms utilizing carboxylates for coordination have been proposed for EcoRI and EcoRV restriction endonucleases, E. coli DNA polymerase I 3’-exonuclease, certain bacterial transposases and retroviral integrases to explain how the diphosphate bonds in a DNA polymer are hydrolyzed (46–51). More recently published crystal structures of Methanococcus jannaschi and Pyrococcus furiosus FEN-1 nuclease provided us more accurate and detailed structural information on the structure-specific nature and active center of the enzyme (52, 53).

Fig. 7. Cleavage kinetics initiated by addition of Mg$^{2+}$. Substrate and an excess amount of enzymes were preincubated for 15 min in 10 mM Tris (pH 8.0) plus 1 mM EDTA. Then the reaction was initiated by the addition of 10 mM MgCl$_2$. The cleavage reactions in the complexes of wild type FEN-1/flap substrate, E160DFEN-1/flap substrate and wild type FEN-1/pseudo-Y substrate were recorded.

From the previous data (32, 33), we know that two of the acidic amino acid residues in the active center, Glu$^{160}$ and Asp$^{181}$, are particularly interesting for further structural/functional analysis due to the following reasons: 1) they may coordinate the same metal ion based on the T4 RNase H structure (39); 2) both of the the amino acid residues are positioned such that they could activate a water molecule for nucleophilic attack on the substrate or serve as a ligand for the metal ion. However, their three-dimensional origins are different due to the physical locations, therefore restrictions on their geometry, angle, and carbon chain length are expected to be different; 3) Asp$^{181}$ is absolutely evolutionarily conserved among 22 functional homologs while Glu$^{160}$ is conserved, but replaced by an aspartate in the eubacterial and viral organisms; 4) the initial replacement of Asp$^{181}$ and Glu$^{160}$ with an alanine showed both mutants lost their cleavage activity; however, D181A completely retained its binding ability while E160A partially lost its binding capacity. If these two sites are scanned by the same set of amino acid residues, different functional effects are expected, which may reflect mechanistic details.

Five amino acids were chosen in this study: alanine is an excellent substitutive amino acid because it has no active side chain to participate in any reaction but fills physical space. Conversion of an aspartate to an asparagine or a glutamate to a glutamine (amidation) removes the active hydroxyl group, thus preventing hydrogen bond formation with metal ions, water, or other amino acids and eliminating the catalysis function but retaining a similar physical topology as the original amino acid. Glutamate has the same carboxyl group as aspartate but it is one -CH$_2$- longer. The length of the side chain could affect the orientation of the Mg$^{2+}$ center and consequently affect the enzyme’s activity. This is the reason for converting glutamate to aspartate. Serine has a hydroxyl group in its side chain which has the potential to form hydrogen bond(s) with a metal, but the length of the side chain is much shorter. The histidine residues have been previously implicated in metal binding or in the activation of the water molecules. It would be of interest to see if any of these residues can replace the function of aspartate or glutamate.

Among 10 total mutants made at both sites, only E160D had...
Implications of the E160D Mutant—As the first partially active mutant created for the entire family of structure-specific nucleases, it is particularly interesting to use E160D in answering fundamental mechanistic questions since it retained an almost identical conformation as wild type protein. To support this statement, we have done four experiments which are not shown under “Results” due to space limitations: 1) both wild type and E160D migrate very similarly on a nondenaturing gel; 2) both of the proteins have a very similar CD spectra indicating they have very similar exposed secondary structures; 3) both have the same metal ion preferences (Fig. 6, B and C). However, when the Glu160 and upstream primer are both missing, the cleavage rate is dramatically reduced, approaching zero (Fig. 6D). Therefore, we propose that Glu160 and the upstream primer are involved in two independent catalytic steps. Possibly, the upstream primer interacts with the enzyme to stimulate a proper orientation of the protein for catalysis. The Glu160, in context of correct orientation, helps to induce a molecular rearrangement at the active site that allows catalysis to occur. The slow reaction rate of the E160D FEN-1 is indeed due to the retardation of cleavage.

In Vivo and in Vitro Relationship—The human and yeast enzymes share about 85% similarity at the protein sequence level. We have predicted that the human protein can support the required function in a yeast cell. This is supported by data presented by Murray et al. (22), where the UV sensitivity of the yeast S. pombe rad2 deletion mutant can be complemented by the expression of the hFEN-1 gene in a plasmid. In this report, biochemically well characterized enzymes (wild type, E160D, and E160A) have been expressed in S. cerevisiae cells to see if each can support the biological functions of the yeast homologous enzyme Rad27 in order to correlate the in vitro and in vivo functional analysis data. Our results indicated that the wild type human enzyme can support the survival of the yeast at 37 °C while the deletion mutants accumulate in the S phase apparently due to a block in DNA replication. In addition, expression of wild type FEN-1 enzyme in a yeast cell complemented the MMS resistance ability, while mutant E160D had reduced recovery ability.

In summary, by changing amino acid residues at two highly conserved sites in human FEN-1, residues Glu160 and Asp181, a partially active mutant, E160D, was created, which retained similar three-dimensional properties of wild type. Kinetic analysis revealed that residue 160 is critical in DNA substrate cleavage. Replacement of this residue with an aspartate causes 10–40-fold reduction in cleavage efficiency. The adjacent strand is important for cleavage as well. Changing Glu160 to Asp and eliminating the adjacent DNA strand had an additive effect on the cleavage velocities and therefore, we propose a

Outstanding residual flap endonuclease activity (Figs. 2 and 3). The other six purified mutants retained their binding ability to the flap substrate, which suggests that the proteins had reasonably well folded conformations. It is interesting to note that Glu160 can be replaced by an aspartate while Asp181 cannot be replaced by a glutamate. The result indicated that our predictions are reasonable. The restrictions on the geometry, angle, and carbon chain length of Glu160 are more flexible than at Asp181 due to their different three-dimensional locations. The evolution of these two sites would also seem to predict such a result. The aspartate at 181 is absolutely conserved while the glutamate at 160 can be an aspartate in different evolutionarily related organisms.

**Fig. 9. Reversal of MMS hypersensitivity.** The same set of transformants used in Fig. 8 were plated on glucose based SD-URA plates (supplemented with His, Ada, Trp, and Leu) containing various amounts of MMS (0, 0.01, 0.05, and 0.1%). Colonies were counted and a survival percent was calculated using the 0% MMS plates as the standard of 100% growth. The figure shows that the survival frequencies of these six different strains at different MMS concentrations ranging from 0.01 to 0.1%. *Inset* shows the survival rates at the concentration of 0.01%.
two-step mechanism for the cleavage of the flap substrate. In vivo complementation of wild type and E160D FEN-1s correlates well with our in vitro data and support our two-step cleavage hypothesis.

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