Free radicals are produced in cells by ionizing radiation, a variety of chemical agents, and normal oxidative metabolism. The spectrum of free radical-induced damage to DNA is broad and includes a wide variety of modifications to the purine and pyrimidine bases, sites of base loss and single-strand breaks (for reviews, see references 11, 39, and 66). The 5,6 double bond of pyrimidines is particularly vulnerable to free radical attack (66), and for DNA thymine, the products include 5,6-dihydroxy-5,6-dihydrothymine (produced under anaerobic conditions), 6-hydroxy-5,6-dihydrothymine, and 5,6-dihydroxy-5,6-dihydrothymine (thymine glycol [Tg]), 5-hydroxy-5,6-dihydrothymine, 6-hydroxy-5,6-dihydrothymine, and 5,6-dihydrothymine (produced under anaerobic conditions). 5-Hydroxymethyluracil (24) and a number of ring contraction and fragmentation products such as 5-hydroxy-5-methylhydantoin, methylarctonyl urea, and urea are also formed. A major product of free radical attack on cytosine is 5,6-dihydroxy-5,6-dihydrocytosine (cytosine glycol) (67), which is unstable and dehydrates to form 5-hydroxycytosine (5-OHC) or deaminates to form uracil glycol (Ug), which can also dehydrate to form 5-hydroxyuracil (5-OHU). Contraction and fragmentation products can also be found after free radical attack on DNA cytosine. Of the above-mentioned structures that retain intact ring conformation and base-pairing capabilities, only Tg has been shown to be a potentially lethal lesion. Tg is a potent block to DNA synthesis in vitro (17, 28, 31, 56), using a variety of DNA polymerases, and when present in biologically active single-stranded phage DNA molecules is a lethal lesion with an inactivation efficiency of 1 (1, 29). Tg is also lethal in duplex DNA, where it takes about 10 to 12 Tg lesions to kill (41).

Other intact pyrimidine products such as Ug, dihydrothymine, 5-OHC, and 5-OHU do not block synthesis in vitro (32, 52, 53) and, where tested, in vivo (22, 23). However, the pyrimidine contraction and fragmentation products, of which only urea has been well studied (41, 42), are presumed to be potentially lethal, but these are less prevalent.

Many of the oxidized cytosines are premutagenic lesions. 5-OHC mispairs in vitro (53) and has been shown to be mutagenic in *Escherichia coli* (23). Ug and 5-OHU pair correctly with A (52, 53); however, since these products are derived from C, they are presumably potent premutagenic lesions. In contrast, dihydrothymine is not a mutagenic lesion (22) and Tg is rarely mutagenic (29) except when present in a sequence that is readily bypassed, and in this case it pairs with G (6). Urea is a premutagenic lesion in *E. coli* under SOS conditions which favor lesion bypass (42).

Oxidative damage produced by normal metabolism appears to be responsible for a substantial fraction of endogenous DNA damage, and cells have evolved an efficient and accurate repair mechanism, base excision repair, to remove these lesions from DNA. Base excision repair is highly conserved from bacteria to humans (for reviews, see references 20 and 68). In *E. coli*, there are three known DNA glycosylases that recognize oxidized purines and pyrimidines. FAPY-DNA glycosylase (Fpg) recognizes and removes formamidopyrimidine products of adenine and guanine (16) as well as 8-oxoguanine (45, 63) and 5-hydroxypyrimidines (27) from DNA. Pyrimidine damages are recognized and removed by endonuclease III (endo III) (21, 27, 54, 62; for reviews, see references 20 and 68) and endonuclease VIII (endo VIII) (43), which share a common range of substrates. In addition to their glycosylase activities, all three of these enzymes possess a lyase activity which cleaves the DNA backbone, leaving a blocked 3’ terminus in the resulting nick (references 20 and 68 and references therein). The
blocked 3' terminus is removed by the phosphodiesterase activities of either exonuclease IV (endo IV) or exonuclease III (exo III), leaving a single-base gap that is filled in by DNA polymerase I and sealed by DNA ligase (references 20 and 68 and references therein).

The genes for endo III, nth (18), and Fpg, fpg (10), have been cloned and sequenced, and mutants have been constructed. The product of fpg is the same as that of mutM (45), originally isolated as a mutator for G→T transversions. fpg mutants were reported to be insensitive to any DNA-damaging agents that produce free radicals (9), although recent evidence suggests that fpg mutants may be sensitive to hydrogen peroxide (71). nth mutants exhibit a weak mutator phenotype (69) but are not sensitive to any DNA-damaging agents that produce the substrates for the enzyme (18). However, when duplex DNA is transfected into wild-type and nth mutant hosts, the survival of the phage is decreased about 2.5-fold in the mutant cells (37, 41), indicating that the enzyme functions in vivo as predicted by its substrate specificity. It was not clear why nth mutants exhibited wild-type sensitivity to DNA-damaging agents while at the same time they were less effective at supporting replication of damaged viral transfacing DNA. It seemed likely that either the DNA-damaging agents (X rays and H2O2) may not have produced enough lethal lesions for expression of a phenotype or an additional enzyme was present in the cell that did not act on exogenous supercoiled DNA but functioned to protect cellular DNA. It was the lack of a cellular phenotype for nth mutants that originally led to the examination of cell extracts for an additional pyrimidine-specific enzyme and the characterization of the purification after each chromatographic step, the protein fractions containing endo VIII were collected per tube. The matrix of the affinity column consisted of redAP and showed that mutants deficient in both endo III and endo VIII were less effective at supporting replication of damaged viral DNA containing Tg. The enzyme activity was assayed by using double-stranded BlueScript psk DNA containing Tg (Tg-psk) in the alkaline fluorometric assay (38). Specifically, 100 ng of double-stranded Tg-psk was incubated with 1 μl of eluate at 37°C for 5 min and immediately transferred to ice to stop the reaction; 2 ml of buffer B (20 mM potassium phosphate [pH 1.8], 0.5 EDTA, 500 μg of ethidium bromide per liter) was added. Fluorescence of the reaction mixture was measured with a Ratio-2 System filter fluorometer (Farrand Optical Inc., Valhalla, N.Y.) both before and after boiling the sample. The number of nicks per DNA molecule was calculated from the two readings as previously described (36, 38). One unit was defined as the amount of enzyme needed to nick 1 pmol of DNA in 1 min.

Cloning and sequencing of the nei gene. Amino acid sequencing analysis of the purified protein was performed by the Microchemistry Facility of Harvard University and the Protein Chemistry Laboratory of the University of Texas Medical Branch at Galveston. The N-terminal amino acid sequence, PEGEPARRAALN LEAAIKGKPLT7(S)FAPFPQ(LK)Y, and two internal amino acid sequences, [D]LNAAQLDALAHALLEIP(R) (int1) and GQVDENKHHGALFR (int2) (brackets indicate probable and parentheses indicate indeterminate, probable), were obtained. Three oligodeoxynucleotides, whose degenerate sequences either code for the N-terminal amino acid sequence (excluding the uncertain sequence of the last 12 amino acids) or are complementary to the coding sequence for each of the two internal amino acid sequences, were synthesized and used as primers (designated primer-N, primer-nt, primer-int1, and primer-int2, respectively) for PCR. E. coli genomic DNA, prepared as previously described (4), was used as the PCR template. The PCR products were set up with 1 μg of E. coli genomic DNA, 2 μM each primer (primer-N/primer-nt or primer-N/primer-int1 or primer-N/primer-int2), and 200 μM each of four deoxynucleotides in 50 μl of PCR buffer supplemented with 1.5 mM Mg2+ and 2.5 U of Taq polymerase (Life Technologies) and run with denaturation for 5 min at 95°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and extension for 10 min at 72°C. The reaction mixtures were then electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg of ethidium bromide per liter) for 4 h and then loaded onto a hydroxylapatite FPLC column. Proteins were eluted with a linear gradient of 0 to 500 mM potassium phosphate in buffer B at a flow rate of 0.5 ml/min and collected at 1 ml per tube. Affinity chromatography was not used as the final step of purification. The purity of the pooled peak fractions was determined by the alkali fluorometric assay (38). Specifically, 100 ng of double-stranded Tg-psk was incubated with 1 μl of eluate at 37°C for 5 min and immediately transferred to ice to stop the reaction; 2 ml of buffer B (20 mM potassium phosphate [pH 7.4], 100 mM KCl, 14 mM 2-mercaptoethanol) was added. The enzyme activity was assayed by using double-stranded BlueScript psk DNA containing Tg (Tg-psk) in the alkaline fluorometric assay (38). Specifically, 100 ng of double-stranded Tg-psk was incubated with 1 μl of eluate at 37°C for 5 min and immediately transferred to ice to stop the reaction; 2 ml of buffer B (20 mM potassium phosphate [pH 1.8], 0.5 EDTA, 500 μg of ethidium bromide per liter) was added. Fluorescence of the reaction mixture was measured with a Ratio-2 System filter fluorometer (Farrand Optical Inc., Valhalla, N.Y.) both before and after boiling the sample. The number of nicks per DNA molecule was calculated from the two readings as previously described (36, 38). One unit was defined as the amount of enzyme needed to nick 1 pmol of DNA in 1 min.

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TABLE 1. Purification scheme for endo VIII*  

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>4.629</td>
<td>735</td>
<td>0.16</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>S-Sepharose</td>
<td>269</td>
<td>871</td>
<td>3.23</td>
<td>119</td>
<td>20.2</td>
</tr>
<tr>
<td>Mono S</td>
<td>14</td>
<td>704</td>
<td>51</td>
<td>95.8</td>
<td>317</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>1.22</td>
<td>109</td>
<td>94</td>
<td>1.0</td>
<td>1,562</td>
</tr>
<tr>
<td>Heparin-lysatope</td>
<td>0.72</td>
<td>159</td>
<td>221</td>
<td>21.6</td>
<td>1,379</td>
</tr>
<tr>
<td>DNA-cellulose</td>
<td>0.024</td>
<td>112</td>
<td>4,710</td>
<td>15.2</td>
<td>29,438</td>
</tr>
</tbody>
</table>

* For this purification, 400 g of cell paste of BW434 (nth mutant) was used.

† One unit was defined as the amount of enzyme needed to nick 1 pmol of T4-gS DNA at 37°C in 1 min.

‡ calf thymus DNA was depurinated by incubation in 10 mM sodium citrate (pH 4) at 80°C for 1 h. The AP sites were reduced with 100 mM NaBH4 (pH 13).

After neutralization, the redAP-DNA was coupled to cellulose.


RESULTS

Purification of endo VIII to apparent homogeneity. Since E. coli Fpg stably binds to an oligodeoxyadenosine nucleotide containing a redAP site (13), and since endo VIII, like Fpg, is a DNA N-glycosylase with an associated AP lyase activity that does not cleave DNA containing redAP sites (43), we examined endo VIII binding to redAP-DNA by using an affinity column made of redAP-DNA-cellulose. Endo VIII eluted at 0.9 M NaCl (data not shown), indicating that endo VIII bound tightly to the affinity column. Table 1 shows a representative purification scheme for endo VIII. Significantly, S-Sepharose and redAP-DNA-cellulose affinity chromatography resulted in about a 20-fold purification with yields of about 100 and 70%, respectively. The final yield was 15%, and purification was about 30,000-fold. The enzyme activity in the peak fractions appeared to be higher than that of the loading sample after the S-Sepharose and hydroxyapatite columns. The underestimation of the loading activity possibly resulted from reversible inactivation due to endo VIII due to inhibitory proteins or to the high concentration or the type of salt in the crude extracts as well as in the phenyl-Superose eluate. Silver staining of the electrophoresed peaks after each chromatographic step (Fig. 1) showed that an apparently homogeneous preparation of
endo VIII was obtained and that endo VIII indeed had a molecular mass of 29 to 30 kDa as estimated previously (43). The apparent homogeneity of endo VIII was later supported by the data from the amino acid sequence analysis of the endo VIII preparation.

Cloning and the sequencing of the nei gene. Since apparently homogeneous endo VIII protein had been obtained, the cloning of the nei gene coding for endo VIII was accomplished by reverse genetics. Amino acid sequence analysis of the purified protein gave two internal and the N-terminal amino acid sequences. The N-terminal sequence and 26 to 28% identity and 43 to 46% similarity of the 76-amino-acid sequence, [D]LNAAQLDALAHALLEIP(R) (int1), and PEGPEIRRAADNLEAAIKGK PLT?V(S)FAFFQ(LKP)Y, respectively. To obtain a probe for screening the E. coli Kohara library, we carried out two PCRs using degenerate primers whose sequences were determined from the amino acid sequences. A unique DNA product was obtained from each PCR (data not shown). Using a 32P-labeled 650-bp fragment as a probe, in situ hybridization showed that two adjacent clones of the Kohara library, 1H5 and 1H6 (representing H5 and H6 in plate 1 of the library region included all three sequences obtained from the amino acid sequence analysis of the purified endo VIII protein). In addition, in the 5′-upstream region of nei, there were two sequences, positions 26 to 31 and 50 to 55 corresponding to E. coli promoter consensus sequences, −35 and −10 hexamers, respectively (51), and a Shine-Dalgarno-like sequence at positions 66 to 70 (61). Taken together, the data show that the nucleotide sequence of 792 bp from position 178 to 969 is the nei structural gene coding for endo VIII and that this sequence is identical to the open reading frame in this region submitted by Mori (46a).

Features of the nei-encoded protein, endo VIII. The predicted sequence of 263 amino acids from the coding region included all three sequences obtained from the amino acid sequence analysis of the purified endo VIII protein (underlined in Fig. 2). The total number (263 versus 264) and the composition of amino acid residues of the predicted protein were very close to those obtained from amino acid composition analysis of the purified endo VIII protein. In addition, 23 to 35 and 10 hexamers, respectively (51), and a Shine-Dalgarno-like sequence at positions 66 to 70 (61). Taken together, the data show that the nucleotide sequence of 792 bp from position 178 to 969 is the nei structural gene coding for endo VIII and that this sequence is identical to the open reading frame in this region submitted by Mori (46a).

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Overexpression of the \( \textit{nei} \) gene. DNA of the \( \textit{nei} \) gene was cloned into an overexpression vector, and a fusion protein with an intein and a chitin binding domain was overproduced. Figure 4 shows a silver-stained SDS gel (lanes 1 to 3) and a nitrocellulose filter immunochemically stained with Fab Nei-31 (lanes 4 to 6) on which were crude cell extracts containing the overexpression vector with the intein-endoprotease VIII fusion protein (lanes 2 and 5) and overexpressed endoprotease VIII protein eluted from a chitin column (lanes 3 and 6). In lanes 2 and 5, Fab Nei-31 staining of the fusion protein (ca. 70 kDa) as well as apparent protein breakdown products can be seen, while a single band was observed (lanes 3 and 6) when purified endoprotease VIII was cleaved from the chitin column. It should be noted that only one band was observed in crude \( \textit{E. coli} \) extracts (see Fig. 6B), indicating that Fab Nei-31 does not cross-react with other \( \textit{E. coli} \) proteins. Significant amounts of endoprotease VIII activity (data not shown) were found in extracts from cells containing the plasmid construct. These data further confirm that the cloned \( \textit{nei} \) gene is the structural gene coding for endoprotease VIII.

Construction and identification of \( \textit{nei} \) single and \( \textit{nei} \) double null mutants. In vitro studies showed that endoprotease VIII shared substrate specificity with endoprotease III (33, 43), suggesting
that both enzymes contribute to the repair of oxidative pyrimidine damage in the cell. To determine the biological role of endo VIII and its relationship to endo III, mutants lacking endo VIII activity and lacking both endo III and endo VIII were constructed. By deleting an internal region of ~50% (397 bp) from the middle of the nei structural gene (792 bp), which includes the codon for the first cysteine of the zinc finger motif, and replacing it with a chloramphenicol resistance gene (Cm) and its promoter (775 bp), a construct of deleted nei, Δnei::Cm (which included the flanking ~260 bp upstream and ~400 bp downstream of nei), was made and presumed to be incapable of encoding a functional endo VIII. A chromosomal mutant was made by linear transformation, and various mutant constructs were made by P1 transduction. The PCR products obtained from wild-type, SW2-8 (nei), and SW2-38 (nth nei) genomic DNA confirmed the gene replacement. As can be seen in Fig. 5, for the two sets of primers used, the PCR products from the nei and nei nth mutants, which contained the Cm insert in the nei gene, were larger than those obtained from the wild type and in agreement with their expected sizes. These data also show that the mutants did not contain intact copies of the genes which might result from tandem duplications in the recipient.

Analysis of crude extracts for endo VIII and endo III activity on Tg-containing DNA (Fig. 6A) showed that both wild-type (BW35 and KL16) (lane 2) and nei mutant (SW2-8) (lane 4) cells contain significant amounts of activity. Wild-type cells contain both endo VIII and endo III activities, while nei mutants contain endo III only. Little activity was observed in endo III and endo VIII, due to their lyase activity, may produce lethal breaks from X-ray- or hydrogen peroxide-induced damage. These data support the idea that there are additional lethal damaging agents that produce lethal substrates recognized by the enzyme. One explanation for this observation is that cellular endo VIII provides a backup activity for endo III. To examine this possibility, we measured the X-ray sensitivity of isogenic wild-type and nth, nei, and nth nei mutant strains. In Fig. 7A, it can be seen that the nth nei double mutant was 1.4-fold more sensitive than the wild type, as determined by comparing the mean lethal doses. As previously observed (18), nth single mutants lacking endo III were not X-ray sensitive (data not shown). Interestingly, single nei mutants consistently showed a slightly increased X-ray sensitivity compared to their isogenic wild-type strain (Fig. 7B). The sensitivity of the nth nei double mutant, however, was not nearly as great as the sensitivity of an nth nfo mutant which is totally devoid of base excision repair. In agreement with published results of Cunningham et al. (19), the nth nfo mutant was about fourfold more sensitive than its isogenic wild-type strain (data not shown). nth double mutants were also about three- to fourfold more sensitive to hydrogen peroxide than wild-type cells but less sensitive than nth mutants (data not shown).

These data support the idea that there are additional lethal lesions produced by X rays or hydrogen peroxide and processed by base excision repair, such as sites of base loss, strand breaks, and lethal purine lesions, that are not recognized by either endo III or endo VIII. Alternatively, in the absence of exo III and endo IV, endo III and endo VIII, due to their lyase activity, may produce lethal breaks from X-ray- or hydrogen peroxide-induced damage.

**Mutator phenotypes of nei, nth, and nth nei strains.** Many of the free radical-modified DNA bases are not blocks to DNA polymerases but miscode and are thus potentially mutagenic. It has previously been established, using several reversion assays, that cells lacking endo III exhibit a mild mutator effect (18, 69), and so it was of interest to examine the role, if any, that endo...
PHOTON IRRADIATION AND MUTATION FREQUENCY

VIII might play in this phenotype. Using a rifampin forward mutation assay, the ratio of revertants to total cells plated was used to calculate the mutation frequency. Table 2 corroborates existing data showing that nth mutants exhibit a mild mutator phenotype (threecfold). nei single mutants showed the same mutation frequency as the wild type; however, the nth nei double mutant exhibited a strong mutator phenotype, about 20-fold. Thus, in the absence of endo III and endo VIII, a significant number of premutagenic pyrimidine lesions are un-repaired.

**DISCUSSION**

In an effort to clone the gene coding for endo VIII and to further characterize the enzyme biochemically and structurally, endo VIII was purified to apparent physical homogeneity. The development of the redAP-DNA-cellulose affinity column was a critical step in the successful purification of endo VIII because cells contain a very small amount of the protein (~95% endo III, compared with 5% endo VIII [43]).

The nei structural gene with its flanking regions was cloned and sequenced. The overall predicted protein from the nei gene showed significant homology with five bacterial Fpg proteins, especially in N-terminal and C-terminal regions. The proteins also have similar sizes (263 and 269 amino acids), similar molecular masses (29.7 and 30.2 kDa), similar hydrophobicities (~0.32 and ~0.26 hydrophathy), and similar hydrophobicity patterns (data not shown), especially for the carboxy half (~160 amino acids) of the proteins. (The second number in each comparison is for *E. coli* Fpg.) Endo VIII contains a zinc finger motif (Cys-X2-Cys-X16-Cys-X2-Cys) at the C terminus, as does Fpg. *E. coli* Fpg proteins with mutations in the zinc finger consensus sequences do not bind Zn2+ as strongly; in addition, these mutant proteins fail to specifically bind to DNA containing a redAP site and exhibit a significantly lower efficiency for all catalytic activities of Fpg (47, 64). Thus, the zinc finger is essential for binding of Fpg to DNA and, probably as a consequence, is essential for Fpg functions. The zinc finger motif of endo VIII is likely to play a similar role. In addition to sequence homology, endo VIII also shares several catalytic activities with Fpg, including cleavage of the DNA backbone by β,δ-elimination as well as a 5′ deoxyribose phosphatase (33). Thus, nei and fpg, the genes coding for endo VIII and Fpg, may have originated from a common ancestral gene.

In the upstream region of the nei structural gene, there are several −35- and −10-like hexamers. Considering the interregion spacing as well as homology to each consensus sequence, TTGGTG and TAATAA, it is possible that positions 26 to 31 and 50 to 55 (Fig. 2) serve as the −35 and −10 hexamers of the nei promoter, but this puts the start site of the message fairly close to the putative Shine-Dalgarno sequence. The distance between these two hexamers is 18 nucleotides, which matches the consensus distance (17 ± 1 nucleotides) (26). The sequence of AAGGA (positions 166 to 170 in Fig. 2) overlaps with five of nine nucleotides of the Shine-Dalgarno consensus sequence (UAAGGAGGU) (25), and the spacing from it to the start codon (AUG) is seven nucleotides, which is equal to the average and optimal spacing between this particular partial Shine-Dalgarno sequence and the start codon of an open reading frame for prokaryotes (15). Therefore, AAGGA (positions 66 to 70) is proposed as a potential ribosome binding site for translation of endo VIII.

The nei gene also contains an unusually large number of rare codons, 26%, compared to 25 *E. coli* nonregulatory genes which utilize 12% rare codons (35). This use of rare codons by nei is high but comparable to that for the products of fpg, uvrC, ada, alkA, and tagA (25, 21, 26, 34, and 21%, respectively). In contrast, only 12% rare codons are used for endo III. The implication for a significantly increased use of rare codons for some proteins has been controversial (35, 60), but it has been
suggested that the use of rare codons may result in lower translation because of smaller cellular amounts of the corresponding tRNA. Although this idea is consistent with the difference between the amounts of endo III and endo VIII in the cell, it should be pointed out that when nei was expressed behind a strong promoter, significant overexpression of the protein resulted (Fig. 4).

The nei gene turned out to be upstream of abrB, the gene coding for a putative regulator of a DNA repair gene, aidB, but with an opposite orientation. In fact, the two genes overlap by 3 bp on their 3’ ends (65a). The significance of this, if any, is not clear. The nei gene is downstream from two open reading frames, also in the opposite orientation. Thus, it is unlikely that nei is part of an operon.

Deletion-substitution mutants of nei were constructed by linear transformation, and deletion of nei was confirmed by genomic PCR, activity analysis in extracts, and Western blot analysis (Fig. 5 and 6). Hypersensitivity to ionizing radiation (Fig. 7A) and hydrogen peroxide was exhibited by nth nei double mutants; thus, these agents produce some lethal pyrimidine lesions subject to repair by endo III and endo VIII. Tg and urea are produced by both ionizing radiation and hydrogen peroxide and are lethal in duplex DNA (41), and so they are likely to be two of the potentially lethal lesions repaired by endo III and endo VIII. Other likely candidates are cytosine- and thymine-derived hydantoinos, methylthioaroyl urea, and other contraction, fragmentation, and ring-open products. As previously shown (18), wild-type X-ray sensitivity was exhibited by nth mutants although a very slight X-ray sensitivity was observed with nei mutants (Fig. 7B). Although these data should not be overinterpreted, it might be that endo VIII can repair some potential lesions not repaired by endo III; alternatively, endo III may produce some lethal lesions from otherwise nonlethal lesions.

Double mutants defective in both endo III and endo VIII showed a significant defect in the repair of spontaneous premutagenic lesions (~20-fold) (Table 2) comparable to the mutator effect observed in fpg mutants (~15-fold) (12, 44). It is of interest that this mutator phenotype would not have been detected in a screen for spontaneous mutators because both nei and nth would have had to have had defective. nei mutants showed no mutator defect, while nth mutants showed a much smaller mutator effect than the double mutant. These data suggest that endo VIII cannot repair all of the premutagenic lesions handled by endo III or that in the absence of endo III, endo VIII fixes otherwise repairable premutagenic lesions. The fact that nth and nei are not completely epistatic to one another suggests that they are compartmentalized and/or act in independent pathways; however, in certain situations, such as repair of X-ray- or hydrogen peroxide-induced lethal lesions, endo III and endo VIII can substitute for one another, while in others, such as repair of potentially mutagenic lesions, they do not. In the latter case, enzyme abundance may play a role. It is clear from studies with uniquely introduced lesions that there are more oxidized pyrimidine base lesions that are premutagenic than are potentially lethal. Thus, it might be that there is not enough endo VIII present in the cell to compensate in the repair of premutagenic pyrimidine lesions in the absence of otherwise abundant endo III.

There is ample precedence in E. coli for both compartmentalization of repair enzyme function as well as effects of enzyme abundance on observed phenotypes. For example, endo IV and exe III are the major E. coli AP endonuclease activities. Both enzymes share catalytic activities, and in some cases they can completely substitute for one another while in other cases they cannot (19). Exo III constitutes about 90% of the cellular hydrolytic AP activity, while endo IV is responsible for only 10% of this activity. Similarly to endo III and endo VIII, exe III and endo IV substitute for one another in the repair of X-ray-induced lethal lesions since single mutants exhibit wild-type sensitivity whereas double mutants are X-ray sensitive (19). However, endo IV, the low-abundance activity, cannot substitute in the repair of hydrogen peroxide-induced lethal lesions since single xth mutants lacking exe III are extremely sensitive to hydrogen peroxide (19). Lack of abundance of endo IV has also been attributed to its inability to overcome the lethal phenotype of dut xth mutants (70). Endo IV also appears to act in a separate repair pathway since it is induced by redox cyclers (14) and appears to be better able to repair bleomycin-induced lesions than exe III (nfo mutants are sensitive to bleomycin, while exe mutants are not) (19).

Good candidates for premutagenic lesions recognized by endo III and endo VIII are oxidized cytosines such as 5-OHC, 5-OHU, and Ug. Ug and 5-OHC are the most abundant oxidized pyrimidine lesions (67), 5-OHC mispairs with A in vitro (53) and has been shown to be mutagenic (23). Ug and 5-OHU pair with A (52, 53) and since they are derived from C are expected to be potent premutagenic lesions. All three of these oxidized cytosines would lead to C→T transitions. The thymine ring saturation products all pair correctly with A (22, 29); Tg is at best a poor premutagenic lesion (6). Since the spontaneous premutagenic lesions repaired by endo III and endo VIII are presumed to be the result of oxidative metabolism, the cytosine lesions are the most likely candidates for the spontaneous mutator effect observed in cells lacking these activities. This prediction is currently under investigation.

ACKNOWLEDGMENTS

The first four authors contributed equally to this work.

We are grateful to Michael Volkert for communicating his results with abrB prior to publication and for helpful discussion and to Yoke Wah Kow and Min Yao for their help and advice. Bernard Weiss generously supplied BW35 (KL16), the parent host for constructing the nei mutants. His advice on this strain enabled us to obtain reproducible X-ray survival data. We are also grateful to Yuji Kohara for supplying his E. coli ordered library.

This work was supported by National Institutes of Health grant R37 CA33657 awarded by the National Cancer Institute.

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