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Endonuclease IV (*nfo*) Mutant of *Escherichia coli*

RICHARD P. CUNNINGHAM,[1] SUSAN M. SAPORITO,[1] SILVIA G. SPITZER,[2] AND BERNARD WEISS[2]*

Department of Biological Sciences, State University of New York, Albany, New York 12222,[1] and Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205[2]

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A cloned gene, designated *nfo*, caused overproduction of an EDTA-resistant endonuclease specific for apurinic-apyrimidinic sites in DNA. The sedimentation coefficient of the enzyme was similar to that of endonuclease IV. An insertion mutation was constructed in vitro and transferred from a plasmid to the *Escherichia coli* chromosome. *nfo* mutants had an increased sensitivity to the alkylating agents methyl methanesulfonate and mitomycin C and to the oxidants *tert*-butyl hydroperoxide and bleomycin. The *nfo* mutation enhanced the killing of *xth* (exonuclease III) mutants by methyl methanesulfonate, H_2O_2, *tert*-butyl hydroperoxide, and gamma rays, and it enhanced their mutability by methyl methanesulfonate. It also increased the temperature sensitivity of an *xth dut* (dUTPase) mutant that is defective in the repair of uracil-containing DNA. These results are consistent with earlier findings that endonuclease IV and exonuclease III both cleave DNA 5′ to an apurinic-apyrimidinic site and that exonuclease III is more active. However, *nfo* mutants were more sensitive to *tert*-butyl hydroperoxide and to bleomycin than were *xth* mutants, suggesting that endonuclease IV might recognize some lesions that exonuclease III does not. The mutants displayed no marked increase in sensitivity to 254-nm UV radiation, and the addition of an *nth* (endonuclease III) mutation to *nfo* or *nfo xth* mutants did not significantly increase their sensitivity to any of the agents tested.

Endonuclease IV (23) of *Escherichia coli* is an apurinic-apyrimidinic (AP) DNA endonuclease, i.e., a DNase specific for apurinic and apyrimidinic sites in DNA. It catalyzes the cleavage of a phosphodiester bond 5′ to the AP site and is an example of the most prevalent form of AP endonuclease found throughout nature. Within *E. coli*, however, the enzyme is a minor one; it accounts for no more than 10% of the AP endonucleolytic activity measured in crude extracts (24). The major AP endonucleolytic activity of *E. coli* is that associated with exonuclease III (39, 42). Like endonuclease IV, it also cleaves 5′ to the AP site, but it is responsible for about 85% of the AP endonuclease activity in the cell (39, 42). A third enzyme, endonuclease III, is about equal in activity to endonuclease IV, but it cleaves 3′ to the AP site and has an associated glycosylase activity that releases ring-damaged pyrimidines from DNA (2, 19).

AP sites appear as both the direct and indirect result of DNA damage in vivo. Pathways of base-excision repair involve glycosylases that remove unusual or damaged bases, leaving AP sites (12). AP endonucleases are therefore important repair enzymes that are required in the subsequent removal of the resulting base-free sugar residues. It was surprising, therefore, that mutants deficient in exonuclease III or endonuclease III had so few biological abnormalities. Exonuclease III mutants, for example, had only a slight increase in sensitivity to alkylating agents (26, 42), although such agents readily generate AP sites (12). The explanation had to lie in the existence of back-up enzymes, and endonuclease IV was the prime candidate. In addition, the presence of multiple AP endonucleases in the same organism suggested that they might function in different pathways. It was the object of this study, therefore, to obtain a mutation affecting endonuclease IV and to examine the effects of this mutation, both alone and in combination with those affecting other AP endonucleases.

* Corresponding author.

MATERIALS AND METHODS

Strain construction. The bacterial strains and plasmids used in this study are listed in Table 1. All strains are derivatives of *E. coli* K-12. Congenic sets of mutants were constructed by transduction with bacteriophage P1. *nfo-1*::*kan* and *nth-1*::*kan* were transferred by selection for kanamycin resistance. To introduce them into the same strain, an *nfo-1*::*kan* Δ(*manA-nth*)*84* derivative was first prepared. The *manA-nth* deletion was transferred by selection for a nearby kasugamycin resistance marker, *ksgB1* (40). The deletion was then replaced by *nth-1*::*kan* by using transductional selection for mannose utilization. *nfo*, *xth*, and *nth* genotypes were confirmed by enzymatic assay. Transductional methods were as previously described (40). *xth* point mutations were transduced into Hfr KL16 strains that bore Δ(*xth-pncA*)*90* (34). *dut-1* alleles were introduced by selecting for transductional replacement of a previously transferred *pyrE-zia-207*::Tn*10* segment (34). Plasmid pWB21 was constructed by replacing a *Hin*dIII-*Bam*HI segment of pKC7, which distinguishes pKC7 from pBR322 (29), with the corresponding 3.9-kilobase (kb) *nfo*$^+$ restriction fragment from pLC38-27.

Media. Tryptone-yeast (TY) medium, minimal medium, and supplements were as previously described (41). Kanamycin was used at 25 μg/ml, and ampicillin was used at 100 μg/ml. Plain soft agar contained 5 g of NaCl and 6 g of agar per liter.

DNA substrates. ^{3}H-labeled partially depyrimidinated T4 phage DNA (T4 · AP) was prepared by treating uracil-containing T4 DNA with uracil-DNA glycosylase (6). Treatment with methoxyamine (CH_3ONH_2) was performed at a DNA nucleotide concentration of 0.4 mM in 0.14 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)–KOH (pH 7.2)–10 mM CH_3ONH_2 (33). After incubation for 30 min at 37°C, the DNA was precipitated with ethanol (25) and dissolved in 10 mM Tris buffer (pH 8.0)–1 mM trisodium EDTA. The substrates were tested by measuring the release of acid-soluble material (measured as for

TABLE 1. *E. coli* strains and plasmids used

Strain[a] or plasmid	Relevant genotype[b]	Source[c] or reference
AB1157 strains		
BW372	*nth-1*::*kan*	6
BW434	*nth-1*::*kan* Δ(*xth-pncA*) *90*	P1(BW372) × BW9109 (6)
BW534	*nfo-1*::*kan nth-1*::*kan*	2-Step transductant of RPC500[d]
BW535	*nfo-1*::*kan nth-1*::*kan* Δ(*xth-pncA*)*90*	2-Step transductant of RPC501[d]
BW9109	Δ(*xth-pncA*)*90*	41
RPC500	*nfo-1*::*kan*	This study
RPC501	*nfo-1*::*kan* Δ(*xth-pncA*) *90*	P1(RPC500) × BW9109
Hfr KL16 strains		
BW285	*dut-1*	This study[d]
BW287	*dut-1 xthA3*(Ts)	This study[d]
BW295	*xth-1*	This study[d]
BW296	*xth-9*	This study[d]
BW540	*nfo-1*::*kan*	P1(RPC500) × Hfr KL16
BW541	*nfo-1*::*kan xth-1*	P1(RPC500) × BW295
BW542	*nfo-1*::*kan xth-9*	P1(RPC500) × BW296
BW548	*dut-1 nfo-1*::*kan*	P1(RPC500) × BW285
BW549	*dut-1 xthA3*(Ts) *nfo-1*::*kan*	P1(RPC500) × BW287
Plasmids		
pUC4K	Km^r	36
pKC7	Ap^r Km^r derivative of pBR322	29
pLC38-27	ColE1 *nfo*$^+$	4
pLC28-48	ColE1 *nth*$^+$	4, 6
pWB21	pBR322 *nfo*$^+$ Ap^r	This study[d]

[a] All strains are derivatives of *E. coli* K-12 λ$^-$.
[b] Nomenclature is that of Bachmann (1), with the following exceptions: *nth*, gene affecting endonuclease III (6, 40); *nfo*, gene affecting endonuclease IV; *nth-1*::*kan*, insertion mutation produced by cloned segment of Tn*5* (6); *nfo-1*::*kan*, insertion mutation produced by cloned segment of pUC4K (see text). Abbreviations: Ap^r, ampicillin resistance; Km^r, kanamycin resistance. For complete genotypes of AB1157 and Hfr KL16, see reference 40.
[c] P1 transductional crosses are described as follows: P1(donor) × recipient.
[d] See Materials and Methods.

the AP endonuclease assay) upon incubation in 0.15 M NaOH for 20 min at 37°C; 40% of the DNA was alkali labile before methoxyamine treatment, but only 0.2% was alkali labile afterward. Sodium borohydride treatment of T4 · AP DNA was performed as described by Gossard and Verly (15), and the preparation was dialyzed against 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM trisodium EDTA. Four percent of the reduced DNA was alkali labile.

The ^{3}H-labeled plasmid DNA, used in the supercoil nicking assays, was that of the pBR322 dimer. Strain BW475 was grown with aeration at 37°C in M9 medium (27) containing 0.4% glucose, 0.5% Casamino Acids (Difco Laboratories), 1 mM $MgSO_4$, and thiamine at 1 μg/ml. At a cell density of 2 × 10^8/ml, chloramphenicol was added to a final concentration of 0.17 mg/ml, followed 1 h later by deoxyadenosine (0.2 mg/ml) and [^{3}H]thymine (10 μCi/ml). After 4 h of further incubation, plasmid DNA was purified by the rapid alkaline method (30), and the supercoils were isolated by electrophoresis in low-melting-point agarose (25). DNA concentration was estimated from the intensity of fluorescent staining with ethidium bromide (7). The yield from a 20-ml culture was 300 nmol of DNA nucleotide containing about 3,000 cpm/nmol.

AP endonuclease assays. Sonicates of log-phase cells were prepared and diluted as previously described (6). Plasmid-bearing strains were grown in the presence of appropriate selective agents, i.e., colicin E1 (4) or ampicillin (100 μg/ml), to prevent curing. The acid-solubility assay for endonuclease IV activity was performed in a reaction mixture (200 μl) containing 0.4 nmol of ^{3}H-labeled T4 · AP DNA (10^4 cpm/nmol), 50 mM HEPES–KOH (pH 8.2), 1 mM trisodium EDTA, 1 mM dithiothreitol, bovine serum albumin at 0.1 mg/ml, and 0.01 to 0.04 U of enzyme. After 30 min of incubation at 37°C, the tube was chilled, and 0.1 ml of salmon sperm DNA (2.5 mg/ml) and 0.3 ml of cold 10% trichloroacetic acid were added. After centrifugation at 12,000 × *g* for 5 min, radioactivity was measured in 0.5 ml of the supernatant. One unit of enzyme released 1 nmol of nucleotide.

Assays for the DNase activity of endonuclease III were performed as previously described (6). Exonuclease III assays were performed as for endonuclease IV, except that 5 mM $MgCl_2$ replaced trisodium EDTA in the reaction mixture.

The substrate for the supercoil nicking assays was prepared immediately before use by incubating ^{3}H-labeled pBR322 dimer DNA at a concentration of 90 μM in 0.1 M NaCl–20 mM sodium acetate buffer (pH 4.8) for 10 min at 70°C (5). The reaction mixture (20 μl) contained 1 nmol (3,000 cpm) of DNA nucleotide and enough enzyme to produce 0.5 to 2.0 nicks per molecule. Other components were the same as for the acid solubility assay. Because of the small volumes, the enzyme was diluted in reaction buffer (minus DNA) and 10 μl were added to an equal volume of the mixture containing the twofold-concentrated substrate. After incubation at 37°C for 10 min, the tubes were chilled, and 4 μl was added of a solution containing 40% glycerol, 0.6% sodium dodecyl sulfate, 60 mM trisodium EDTA, and bromphenol blue (2.5 mg/ml). The intact and nicked supercoils were separated by electrophoresis in low-melting-point agarose (25), located by ethidium bromide staining, and excised in agarose plugs. The plugs were melted in 0.5 ml of water in a microwave oven, and 5 ml of a liquid scintillation fluid was added before cooling. The average number of nicks was determined in each sample by Poisson analysis (21). One unit of enzyme, as defined by this assay, produces an average of one single strand break per DNA molecule.

Sedimentation analysis. Cells of strain BW434(pWB21) were harvested by centrifugation while in the midlogarithmic phase of growth in TY broth containing ampicillin (100 μg/ml) and frozen in a dry ice-ethanol bath. The cells were thawed in 5 volumes of 50 mM Tris hydrochloride buffer (pH 8.0)–50 mM KCl–0.1 mM dithiothreitol and disrupted by sonication at 0°C. Subsequent operations were at 0 to 4°C, and centrifugations were for 10 min at 10,000 × *g*. After the cell debris was removed by centrifugation, a 10% (vol/vol) solution of Polymin P (polyethyleneimine solution; BDH Poole, England), adjusted to pH 7.4 with HCl, was added to a final concentration of 0.35%. The mixture was stirred for 30 min and centrifuged. Two volumes of a saturated $(NH_4)_2SO_4$ solution were added to the supernatant. The mixture was stirred for 30 min and centrifuged. The precipitate was dissolved in 50 mM Tris hydrochloride buffer (pH 8.0)–50 mM KCl–1 mM dithiothreitol, dialyzed against the same buffer, and frozen in a dry ice-ethanol bath. The final preparation had a protein concentration of 5.4 mg/ml and a

specific activity of 295 U/mg (by the acid-solubility assay). A mixture of 20 μl of this fraction and 100 μl of a bovine carbonic anhydrase A solution (0.5 mg/ml) was layered on one sucrose gradient, and 100 μl of ovalbumin solution (2 mg/ml) was layered on another. Sedimentation analysis was performed as previously described (6). Endonuclease IV and carbonic anhydrase (8) were detected by an enzymatic assay, and ovalbumin was detected by a protein assay.

Gradient plates. Agar media containing linear concentration gradients of lethal agents were prepared by a modification of the method of Bryson and Szybalski (3) in which two agar wedges are overlaid. To 25 ml of molten TY agar at 50 to 55°C were added the agent to be tested plus 25 μl of an autoclaved 10% suspension of Dow Corning Antifoam B (to prevent meniscus formation in the plastic dishes). The mixture was poured into a 9-cm square polystyrene petri dish, which was tilted so that the tapered edge of the agar coincided with a bottom edge of the plate. After the agar had hardened, the plate was leveled and overlaid with another 25 ml of molten TY agar (without additions). The plates were inverted, uncovered, and dried in a 37°C incubator for 1 h. Twenty microliters of a fresh stationary-phase bacterial culture was diluted in 2 ml of molten plain soft agar (at 45°C), which was transferred to a microscope slide on a temperature block. The 3-in. (ca. 7.5-cm) edge of a fresh microscope slide was dipped into the cell suspension and touched to the surface of the agar plate parallel to the gradient. Incubation was for 20 to 24 h at 37°C. The plate was stained with 1.5 ml of an acridine orange solution (0.2 mg/ml in ethanol), which was permitted to soak in and evaporate. It was then photographed in blue light with a set-up used to detect flavin fluorescence (32), but a UV light box could also be used.

Survival curves. Sensitivity of H_2O_2 was measured with logarithmically growing cultures (9). Other tests were performed on fresh stationary cultures that were prepared by diluting saturated overnight cultures into 10 to 20 volumes of fresh TY broth containing 0.1% glucose and growing with aeration at 37°C for 5 h. Tenfold serial dilutions were prepared in 50 mM potassium phosphate buffer (pH 7.4). Chemical agents (other than H_2O_2) were added to 2 ml of molten plain agar (at 46°C), followed immediately by 0.1 to 0.2 ml of each cell dilution, and the mixture was poured onto the surface of a TY agar plate. Colonies were counted after the maximum number appeared on each plate (1 to 2 days at 37°C). For UV exposure, 5 ml of a 10^{-4} dilution of each culture was exposed in an open petri dish to a 15-W germicidal lamp at a dose of 1 W/m^2 in 20-s increments. Samples were further diluted and plated in a soft agar overlay as above. The UV and mitomycin C experiments were done in dim light. Sensitivity to gamma rays was tested with oxygenated cell suspensions as previously described (6).

Other biological tests. Spontaneous mutation of strain AB1157 derivatives to His^+ or Arg^+ was scored by growth on limiting media (37). Twenty independent cultures were used for each test, and the median values were recorded (11). Mutation to azauracil resistance was determined by plating samples of washed cultures on minimal medium supplemented with 0.2% vitamin-free Casamino Acids and with 6-azauracil at 40 μg/ml. Spontaneous mutation to valine resistance was tested by plating the washed cells on minimal medium containing 2% glucose in 2 ml of a plain soft agar overlay containing 2 mg each of L-valine and of the required amino acids. Colonies were counted after 3 to 5 days of incubation at 37°C. Induced reversion to His^+ and Arg^+ was performed as described previously (37) with 0.2 μl of methyl methanesulfonate (MMS) per plate. For induced mutation to valine resistance, the top agar (2 ml) was supplemented with 1 mg of valine and 24 μg of isoleucine, and the cells were washed before plating. Uracil sensitivity was measured as previously described (18).

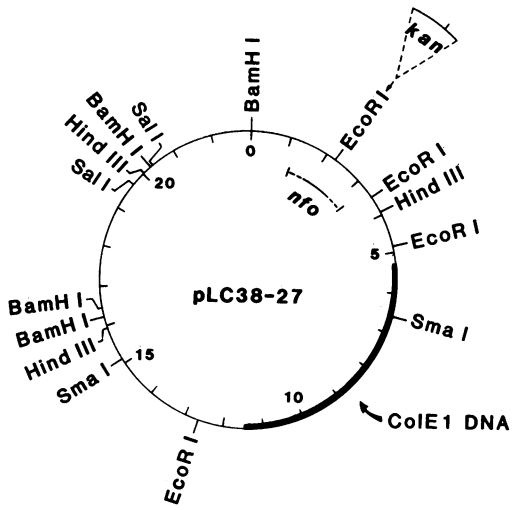

FIG. 1. Restriction site map of plasmid pLC38-27 (ColE1 *nfo*$^+$, 22.8 kb). Also shown is the *nfo-1::kan* insertion (labeled *kan*), a 1.2-kb kanamycin resistance cassette from plasmid pUC4K, which was ligated into the *Eco*RI site of pLC38-27. Its orientation is denoted by the placement of its *Hin*dIII site (short line); its other restriction sites (36) are not depicted.

Other materials and methods. *tert*-Butyl hydroperoxide (*t*-BuO_2H), mitomycin C, and bleomycin were obtained from Sigma Chemical Co., and MMS was obtained from Aldrich Chemical Co. Methods for restriction enzyme digestions, DNA ligase reactions, bacterial transformation, plasmid isolation, and gel electrophoresis of DNA were as described by Maniatis et al. (25). Protein was determined with the bicinchoninate reagent (31) with bovine serum albumin as a standard. Radioactivity was measured in 5 ml of a Triton X-100-containing liquid scintillation medium.

RESULTS

Isolation of *nfo* plasmids and mutants. The strategy was to isolate a plasmid-bearing the gene, to create an insertion mutation containing a drug resistance marker, and then to transfer the mutation to the chromosome. As previously described (6), we screened strains of the Clarke and Carbon strain library (4), which contain ColE1 plasmids bearing cloned chromosomal fragments. We assayed them for EDTA-resistant AP endonuclease activity, a property shared by only two known enzymes of *E. coli*, namely, endonucleases III and IV. The enzymes should be overproduced in those clones bearing the genes for these enzymes on the multicopy recombinant plasmids. Two of the plasmids contained similar regions of cloned DNA. They were found to specify endonuclease III (thymine glycol-DNA glycosylase) and were the subject of a previous communication (6). The third plasmid, pLC38-27, had a pattern of restriction sites (Fig. 1) unlike those described for the others (6). It was likely, therefore, to specify endonuclease IV.

The *nfo* was localized to a 3.9-kb *Bam*HI-*Hin*dIII segment of pLC38-27 when it was found that plasmids containing the subcloned segment (e.g., pWB21), overproduced the en-

TABLE 2. EDTA-resistant endonuclease activities in crude extracts: supercoil nicking assays

Strain	Relevant genotype	Enzyme sp act (U/μg)	
		Acid-treated DNA	Untreated DNA
BW434	*nfo*$^+$ *nth xth*	4.6	–[a]
BW535	As BW434 but *nfo-1*	2.1	0.3
BW554	BW434(pBR322 *nfo*$^+$)[b]	37	0.3

[a] –, Not assayed.
[b] pBR322-*nfo*$^+$ is pWB21.

zyme. The segment has two *Eco*RI sites. Insertions were produced at these sites with the expectation that one might be within the *nfo* gene. pLC38-27 was partially digested with *Eco*RI endonuclease and ligated with a 1.2-kb *Eco*RI segment of pUC4K (36), which specifies kanamycin resistance. The insertion at 2.2 kb on the map (Fig. 1), which we have designated *nfo-1::kan*, abolished endonuclease IV overproduction, whereas that at 3.5 kb had no effect. The *nfo-1::kan* mutation was then transferred to the chromosome by the same method that had been used for an endonuclease III (*nth*) mutation (6). Briefly, a *polA*(Ts) strain was transformed with the recombinant plasmid, and the cells were then grown in the presence of kanamycin at 42°C to select for chromosomal integration of the plasmid. Phage P1 was then used to transduce the kanamycin resistance marker (*nfo-1::kan*) to the chromosome of other strains, independent of the vector DNA, which was detected by colicin immunity.

Enzyme levels. The screening for *nfo* mutants was performed with an assay based on the release of acid-soluble material from partially depyrimidinated DNA in the presence of EDTA. This assay, however, is not specific for AP endonucleases; it would also detect other endonucleases as well as exonucleases. To confirm that the *nfo* gene affects an endonuclease activity, we measured the cleavage of circular DNA. To reduce possible interference from other enzymes, the assays were performed in the presence of EDTA and in strains bearing mutations in the genes for endonuclease III (*nth*) and exonuclease III (*xth*). BW554, which has the intact *nfo* gene on a multicopy plasmid, overproduced an endonuclease specific for acid-treated (i.e., partially depurinated) DNA (Table 2). However, BW535, an *nfo* mutant, had a reduced level of activity.

Because the acid-solubility assay was more convenient, it was used to measure AP endonuclease activity in crude extracts of wild-type, mutant, and plasmid-bearing strains. Our first substrate was uracil-containing phage T4 DNA that had been treated with uracil-DNA glycosylase to introduce AP sites (6). This substrate could be used to detect overproduction of AP endonuclease in strains bearing *nfo* or *nth* plasmids as well as to detect enzyme deficiencies in *nth* and *xth* mutants (Table 3). As expected, the *xth* mutation did not significantly affect the measured activity because exonuclease III is inhibited by EDTA (39).

In an effort to enhance the specificity of the assay, the substrate was treated with methoxyamine and with sodium borohydride. Both reagents react with the C-1 carbonyl groups at AP sites; methoxyamine forms a relatively stable adduct (33), whereas sodium borohydride reduces the carbonyl group to an alcohol. It was hoped that the treatments would block endonuclease III activity (19) as well as cleavage at AP sites that might occur via β-elimination reactions mediated by basic proteins in the crude extracts (22). The results with extracts prepared from the plasmid-bearing strains (Table 3) indicated that these treatments largely inhibited endonuclease III but had little effect on endonuclease IV. With the other strains, however, the specificity of the assay was not significantly enhanced by the substrate modifications. So much cell extract had to be used in these latter assays that perhaps the desired effect created by modifying the AP sites may have been offset by other interfering enzymes that attack at AP sites, at cleaved AP sites (exonucleases), or at other lesions introduced by the treatments. Nevertheless, in an *nth* background, the addition of the *nfo-1::kan* mutation produced an average loss of 71% of EDTA-resistant AP endonuclease activity (Table 3). Moreover, as described below, most of the residual activity is probably not due to endonuclease IV.

Sedimentation rate. Sedimentation analysis was used to confirm the identity of the endonuclease specified by the *nfo* gene. Enzymatic activity was assayed with the $NaBH_4$-treated substrate. An extract was prepared from strain BW554, which contains an *nfo* plasmid and which is deficient in exonuclease III and endonuclease III. As described under Materials and Methods, the cells were disrupted by sonication, and the extracts were treated with polyethyleneimine to remove DNA that otherwise might bind to the enzyme during sedimentation. The protein was then concentrated by $(NH_4)_2SO_4$ precipitation. The overall yield was 89%, with a 2.1-fold increase in specific activity (to 295 U/mg). However, a similar fractionation of strain BW535 (*nfo xth nth*) led to a decrease in specific activity by a factor of 4 (to 0.37 U/mg). Therefore most of the residual endonuclease activity in the triple mutant was not due to the *nfo* gene enzyme. These results (295 versus 0.37 U/mg) also indicate that the *nfo* enzyme probably constituted over 99% of the measured activity in the BW554 $(NH_4)_2SO_4$ fraction. Samples of this fraction were sedimented in sucrose density gradients. The AP endonuclease activity appeared in a single peak (64% recovery). Its sedimentation coefficient was 3.3S, as measured against bovine carbonic anhydrase (3.06S), and 3.2S with respect to ovalbumin (3.66S). The values are similar to that obtained by Ljungquist (23) for purified endonuclease IV (3.4S).

Mutation rates. Cells exposed to MMS acquire AP sites via spontaneous and enzymatic hydrolysis of the resulting alkylated bases. The agent was therefore tested for its ability

TABLE 3. EDTA-resistant AP endonuclease activities: acid-solubility assays[a]

Strain	Relevant genotype	Enzyme sp act (U/mg)		
		T4 · AP DNA	CH_3ONH_2-treated DNA	$NaBH_4$-treated DNA
AB1157	Wild type	9.4	11	6.3
RPC500	*nfo*	3.4	2.5	1.4
BW372	*nth*	5.1	5.4	3.5
BW534	*nfo nth*	1.3	1.9	1.1
BW535	*nfo nth xth*	1.8	1.6	1.6
BW434	*nth xth*	6.6	–[b]	6.1
BW452	BW434(ColE1 *nfo*$^+$)[c]	78	66	68
BW554	BW434(pBR322 *nfo*$^+$)	110	160	140
BW411	AB1157(ColE1 *nth*$^+$)	30	13	14

[a] The assays, performed on unpurified sonicates of the designated strains, measured the release of acid-soluble material from partially depyrimidinated [^{3}H]DNA that was either untreated (T4 · AP DNA), treated with CH_3ONH_2, or treated with $NaBH_4$.
[b] –, Not assayed.
[c] Plasmids: ColE1-*nfo*$^+$, pLC38-27; ColE1-*nth*$^+$, pLC28-48; pBR322-*nfo*$^+$, pWB21.

TABLE 4. MMS-induced mutagenesis

Strain[a]	Relevant genotype	Induced mutants (no./plate)[b]		
		His^+	Arg^+	Val^r
AB1157	Wild type	62	92	22
RPC500	*nfo*	109	125	43
BW9109	*xth*	79	83	9
RPC501	*nfo xth*	268	198	128
BW535	*nfo xth nth*	395	202	131

[a] The congenic strains were derived from AB1157 (*hisG4 argE3*, valine sensitive).

[b] The number of induced mutants equals the number of colonies growing on selective media in the presence of MMS minus the number growing in its absence (37). The MMS dose (0.2 μl/plate) was sublethal (Fig. 2A).

to induce mutations in cells lacking one or more of the AP endonucleases (Table 4). Strains bearing combined mutations in *nfo* and *xth* displayed a modest (four- to sixfold) increase over the wild type in the frequency of MMS-induced reversions to His^+ and mutations to Val^r (Table 4). In addition, spontaneous mutation frequencies were measured for 6-azuracil resistance as well as those traits listed in Table 4. *nfo* and *xth* single and double mutants had less than a twofold increase in mutation frequency over the wild-type strain. The *nth nfo xth* triple mutant had a fivefold increase in *argE3* reversion attributable to the previously reported mutator effect of the *nth* allele (6).

DNA uracil repair. *dut* mutants, which are deficient in dUTPase, incorporate large amounts of uracil into their DNA (10, 35). The uracil is removed by uracil-DNA glycosylase, thereby generating apyrimidinic sites. In contrast to other treatments we used in this study (irradiation, alkylation, or oxidation), the *dut* mutation enabled us to generate AP sites without introducing many other ill-defined lesions. Whereas *dut-1 nth* double mutants are viable (B. Weiss, unpublished results), *dut-1*(Ts) *xth* strains are temperature sensitive; their survival at 42°C was less than 10^{-4} (34). This result suggested an essential role for the exonuclease III in the repair of AP sites and prompted us to similarly examine endonuclease IV. Whereas the *nfo* mutation had little effect on the survival of a *dut-1* mutant (Table 5), it significantly reduced the survival of a *dut xth* double mutant. The results were compatible with a discernible but nevertheless minor role of endonuclease IV in the repair of AP sites.

dut mutants have a reduced plating efficiency in the presence of high concentrations of uracil (18), which may be due to excessive breakage of DNA resulting from the excision repair of misincorporated uracil. This uracil sensitivity might provide a more sensitive test for the possible role of endonuclease IV in this repair pathway. Therefore, strains BW285 (*dut*) and BW548 (*dut nfo*) were tested for their ability to grow in the presence of 0.5, 1.0, and 2.0 mM uracil. Their rates of survival were similar. At a 1 mM level, for example, their plating efficiencies were 1.4×10^{-4} and 2.4×10^{-4}, respectively. Therefore, either the endonuclease IV level is not limiting in this repair pathway, or else unrepaired AP sites are as lethal as excessive single strand breaks.

TABLE 5. Effect of *nfo* mutations on the temperature sensitivity of *dut*(Ts) and *xth*(Ts) mutants

Strain	Relevant genotype	Survival (42°C/30°C)[a]
Hfr KL16	Wild type	1.00
BW285	*dut-1*	0.92
BW548	*dut-1 nfo*	0.83
BW287	*dut-1 xthA3*	1.1×10^{-4}
BW549	*dut-1 xthA3 nfo*	6.6×10^{-6}

[a] Relative plating efficiency at 42°C versus 30°C.

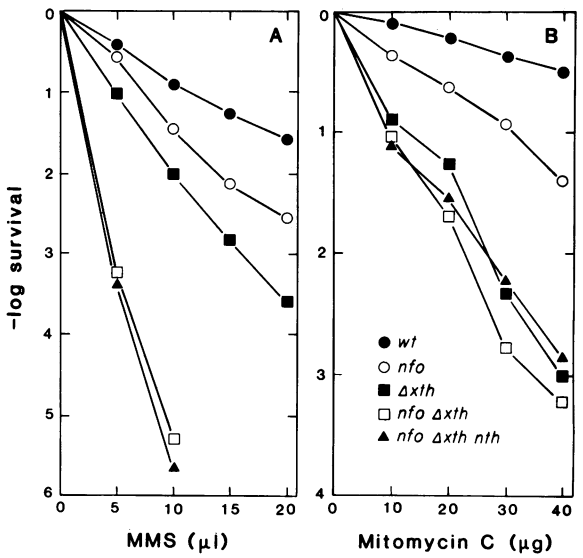

FIG. 2. Sensitivity of mutants to MMS (A) and to mitomycin C (B). The strains used were AB1157 (wild type [wt]) and its congenic derivatives RPC500, RPC501, BW534, BW535, and BW9109.

Sensitivity to MMS and mitomycin C. The *nfo* mutation was tested for its effect on resistance to MMS and to mitomycin C (Fig. 2). MMS alkylates mainly purine nitrogens in DNA and generates AP sites via spontaneous and enzymatic hydrolysis of glycosylic bonds (12). In addition it produces O^6-methylguanine, a minor but lethal lesion that is repaired without depurination via a methyltransferase (12). Mitomycin C is a bifunctional alkylating agent that can cross-link the DNA strands and that produces bulky adducts that are repaired in part by the *uvr* gene products (12). The *nfo* mutant demonstrated a slightly increased sensitivity to these agents (Fig. 2), which was not as great as that of the *xth* mutant. The most striking result, however, was that the *nfo xth* double mutant was quite sensitive to MMS, more than either of the single mutants. An *nth* mutation produced no significant additional effect.

Radiation sensitivity. The *nfo xth* double mutant was also unusually sensitive to the lethal effects of gamma rays (Fig. 3A), whereas the single mutants were not. With respect to UV sensitivity (Fig. 3B), the mutations had only a slight effect of questionable significance, but again the most sensitive strains were those possessing mutations in both *nfo* and *xth*.

Sensitivity to oxidizing agents. *xth* mutants are known to be unusually sensitive to H_2O_2 (9) (Fig. 4A). Although the *nfo* mutation alone did not result in a level of sensitivity different from that of the wild type (Fig. 4A), the mutation markedly increased the sensitivity of an *xth* strain. With the agent *t*-BuO_2H (Fig. 4B), the lowest survival was again shown by the *nfo xth* double mutant, but the *nfo* mutation alone specified an unusual degree of sensitivity, even greater than that of the *xth* mutant.

Bleomycin sensitivity. The antibiotic bleomycin causes release of bases from DNA and the breakage of DNA strands (13, 28). Like the peroxides and ionizing irradiation, its effects are believed to be mediated by oxygen radical formation, and the limit products produced by the action of

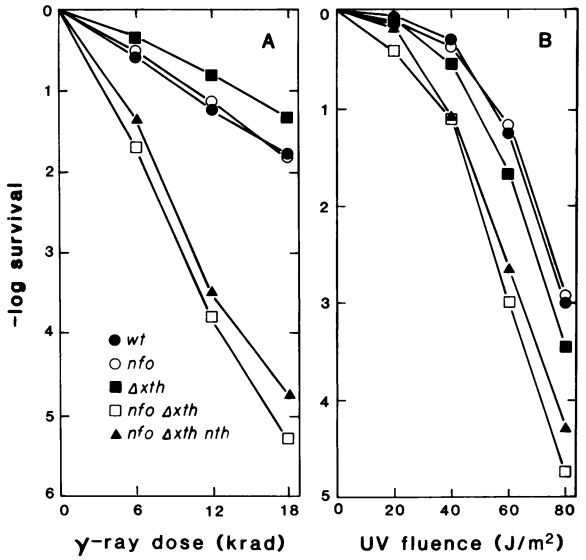

FIG. 3. Sensitivity of mutants to gamma rays (A) and short wave UV light (B). The strains used were those listed in the legend to Fig. 2.

bleomycin on DNA are similar to those produced by ionizing radiation (17). Bleomycin killed the *nfo* mutant at lower doses than it did the wild type (Fig. 5), and the effect was potentiated by the presence of an *xth* mutation.

Analysis of other strains. The survival curves (Fig. 2 through 5) do not include data on all of the eight possible permutations of the three mutations and their wild-type alleles. The *nth* and *nth xth* strains, at least, were previously tested for sensitivity to H_2O_2 and to gamma rays (6). A more thorough analysis of allele combinations was necessary. It was accomplished with a simpler screening technique involving gradient plates. Cell suspensions were applied in uniform lines on an agar surface containing a linear gradient of the substance to be tested. After incubation, the point in

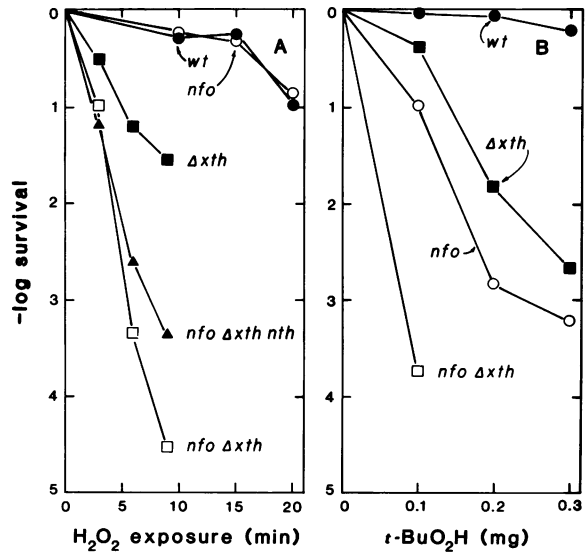

FIG. 4. Sensitivity of mutants to 10 mM H_2O_2 (A) and to t-BuO_2H (B). The strains used were those listed in the legend to Fig. 2.

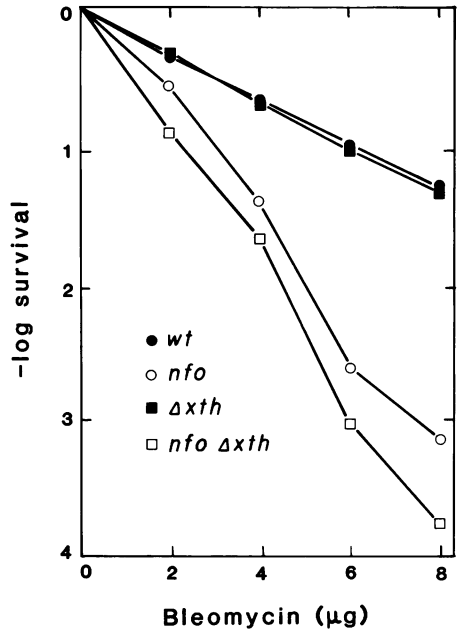

FIG. 5. Sensitivity of mutants to bleomycin. The strains used were those listed in the legend to Fig. 2.

the gradient beyond which growth ceased was taken as a measure of the sensitivity of the strain. Although cruder than colony counting, the test was easier to perform, and it was more sensitive than the conventional spot test, which generates an exponential gradient. Figure 6 shows a typical gradient plate measuring the effect of t-BuO_2H on a full set of eight congenic strains. Other agents were similarly analyzed (Table 6). The results of these screening tests were similar to those of the corresponding survival curves (Fig. 2, 4, and 5) and directed the choice of strains that were studied in the latter experiments.

The studies of Fig. 2 through 5 were performed on derivatives of strain AB1157, which ws chosen only because so many similar studies had been done on it in the past. A spontaneous *xth* deletion was used because it was certain to be the tightest mutation, although it was not known what effect neighboring deleted genes might have on the results. (An *nth* deletion [6, 40] was not used, however, because it

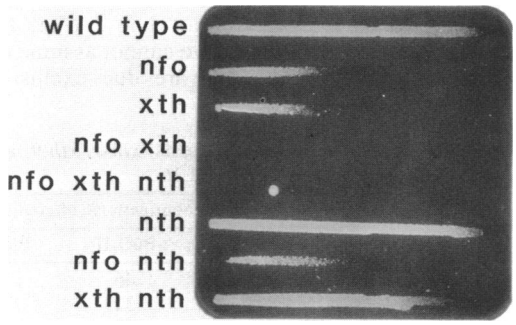

FIG. 6. Gradient plate test for sensitivity to t-BuO_2H. The agar (50 ml) contained a total of 0.9 μl of a 70% solution of t-BuO_2H distributed in a gradient from left to right. The length of a line of cell growth is a measure of the strain's resistance to the agent. The strains used (top to bottom) were AB1157, RPC500, BW9109, RPC501, BW535, BW372, BW534, and BW434.

TABLE 6. Gradient plate sensitivity tests: *nfo*, Δ*xth* and *nth* alleles in strain AB1157

Strain	Relevant genotype	Zone of growth (% of gradient)		
		MMS	*t*-BuO_2H	Bleomycin
AB1157	Wild type	75	>90	86
RPC500	*nfo*	67	40	56
BW9109	Δ*xth*	44	40	85
RPC501	*nfo* Δ*xth*	<10	<10	52
BW535	*nfo* Δ*xth nth*	13	<10	>90[b]
BW372	*nth*	>90	>90	>90
BW534	*nfo nth*	89	46	>90
BW434	*nth* Δ*xth*	67	81	>90

[a] The gradient plates (50 ml) contained 25 μl of MMS, 0.9 μl of 70% *t*-BuO_2H or 17 μg of bleomycin (1.7 U/mg); 100% is equal to 90 mm (Fig. 6).
[b] Bleomycin resistance is specified by the Tn*5*-derived *Kan* segment of *nth-1*::*Kan* (B. Weiss, unpublished data).

affected growth rate.) We attempted to see whether some of the above results could be duplicated in another host strain and with other *xth* mutations. Accordingly, strain Hfr KL16 was used, together with two tight, nitrosoguanidine-induced *xth* mutations. The results (Table 7) essentially confirmed those of the other studies: e.g., the *nfo xth* double mutants were quite sensitive to MMS, *t*-BuO_2H, and bleomycin.

Although not shown, the gamma-ray survival curves obtained with *xth-1* derivatives of AB1157 were similar to those shown in Fig. 3 for the corresponding Δ*xth* strains. At a dose of 18 kilorads, for example, the survival of an *xth-1* mutant was 2.4×10^{-2}, or about that of the wild type, whereas the survival for an *xth-1 nfo-1* double mutant was 4.4×10^{-4}.

DISCUSSION

In this work, we have examined the properties of strains bearing an insertion mutation that affects the production of endonuclease IV. Although insertions usually produce tight mutations, our mutant had considerable residual EDTA-resistant AP endonucleolytic activity (about 25%). This activity could not be attributed to endonuclease III, the only other known EDTA-resistant endonuclease of *E. coli*. Whereas this residual activity appears to be large with respect to that of endonuclease IV, it represents a very small amount of the total cellular AP endonuclease activity, about 85 to 90% of which is due to exonuclease III (24, 42). One future use of the mutants will be to identify and characterize this residual AP endonuclease activity, which may not be associated with an altered endonuclease IV.

Insertion mutations may exert polar effects on other genes of an operon. Because we do not know what type of transcriptional units are involved, we cannot assume that the effects of *nfo* and *nth* insertions are due exclusively to deficiencies of endonuclease IV or endonuclease III. Eventually, we must perform complementation tests with smaller plasmids that specify only these gene products. It is nevertheless apparent, for whatever reason, that the *nfo-1::kan* is a new mutation affecting DNA repair.

TABLE 7. Gradient plate sensitivity tests: *xth-1*, *xth-9*, and *nfo* alleles in Hfr KL16

Strain	Relevant genotype	Zone of growth (% of gradient)[a]		
		MMS	*t*-BuO_2H	Bleomycin
Hfr KL16	Wild type	84	46	73
BW540	*nfo*	79	32	57
BW295	*xth-1*	64	32	68
BW296	*xth-9*	65	30	81
BW541	*nfo xth-1*	31	<10	38
BW542	*nfo xth-9*	12	<10	31

[a] The gradient plates (50 ml) contained 38 μl of MMS, 0.9 μl of 70% *t*-BuO_2H, or 25 μg of bleomycin (1.7 U/mg); 100% is equal to 90 mm (Fig. 6).

TABLE 8. Summary of results

Agent	Increased lethality		
	nfo	*xth*	*nfo xth* > *nfo* and *xth*[a]
dut mutation	No	Yes	Yes
MMS	Yes	Yes	Yes
Mitomycin C	Yes	Yes	No
Gamma rays	No	No	Yes
UV	No	No	?
H_2O_2	No	Yes	Yes
t-BuO_2H	Yes	Yes	Yes
Bleomycin	Yes	No	?

[a] Is the sensitivity of the double mutant greater than that of both single mutants?

Endonuclease IV has an AP endonucleolytic activity that resembles that of exonuclease III: it cleaves 5′ to the AP site. Endonuclease IV, however, has less than 10% of the activity of exonuclease III in crude extracts. We expected, therefore, that the repair deficiencies of *nfo* mutants would not be as great as those of *xth* mutants and that they might be manifest only in the presence of an *xth* mutation. These expectations were largely borne out by our results (Table 8). The addition of an *nfo* mutation increased the sensitivity of an *xth* mutant to the lethal effects of a *dut* mutation, MMS, gamma rays, *t*-BuO_2H, and bleomycin. The simplest explanation for this effect is that the gene products have a similar function in repair, i.e., as 5′ AP endonucleases. It is also possible, however, that other activities may be equally if not more important. The following additional functions have been proposed for exonuclease III: (i) removal of DNA-3′-phosphates and phosphoglycolates produced by oxygen radical generators like bleomycin (13, 28) and ionizing radiation (16, 17); (ii) removal of 3′-terminal base-free sugars (38) such as those generated by the 3′ AP endonuclease activity of endonuclease III and by nonenzymatic β-elimination reactions (22); (iii) an antiligase activity (14); and (iv) endonucleolytic activity at urea-*N*-glycoside residues (20), such as those readily produced by oxidation. It is also possible, that endonuclease IV may have other undiscovered activities; its molecular weight appears to be near those of exonuclease III and endonuclease III, which are multifunctional enzymes.

Two unexpected findings suggested that endonuclease IV is not entirely redundant: the *nfo* mutant was more sensitive than the *xth* mutants to *t*-BuO_2H and to bleomycin. Thus, these agents may produce a class of lesions recognized by endonuclease IV but not by exonuclease III. Alternatively, either enzyme might be selectively induced by different agents, a possibility that is yet to be explored.

In these studies, an *nth* mutation did not increase the sensitivity of any strain to the agents tested. In most cases, it appeared to have a slightly protective effect, which was previously noted with respect to H_2O_2 sensitivity (6). It is possible that the enzyme may exist solely for its glycosylase activity; its AP endonuclease activity may be of little value or perhaps even deleterious. By cleaving 3′ to an AP site, it leaves a poor primer for DNA polymerase I (38); the resulting 3′-terminal base-free sugar would first have to be removed by another enzyme. Although it is the prototype of

an X-ray repair enzyme, no vital role has yet been demonstrated for endonuclease III in any repair pathway.

The isolation and characterization of these mutants represents only a preliminary study. The mutants can best be used to permit the accumulation and subsequent identification of unrepaired lesions and of intermediates in DNA repair, a strategy that has already been used with great success to delineate pathways of intermediary metabolism.

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