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Repair of *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine-Induced DNA Damage by ABC Excinuclease

BENNETT VAN HOUTEN* AND AZIZ SANCAR

Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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***Escherichia coli* has several overlapping DNA repair pathways which act in concert to eliminate the DNA damage caused by a diverse array of physical and chemical agents. The ABC excinuclease which is encoded by the *uvrA*, *uvrB*, and *uvrC* genes mediates both the incision and excision steps of nucleotide excision repair. Traditionally, this repair pathway has been assumed to be active against DNA adducts that cause major helical distortions. To determine the level of helical deformity required for recognition and repair by ABC excinuclease, we have evaluated the substrate specificity of this enzyme by using DNA damaged by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. ABC excinuclease incised methylated DNA in vitro in a dose-dependent manner in a reaction that was ATP dependent and specific for the fully reconstituted enzyme. In vivo studies with various alkylation repair-deficient mutants indicated that the excinuclease participated in the repair of DNA damage induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.**

Genetic and biochemical studies have shown that the ABC excinuclease of *Escherichia coli* acts on a wide spectrum of DNA adducts (5, 26). The enzyme is composed of three subunits, i.e., the UvrA (M_r, 103,874), UvrB (M_r, 76,118), and UvrC (M_r, 66,038) proteins, and repairs damaged DNA by incisions on both sides of the modified nucleotide(s) in an ATP-dependent reaction (17, 29). In the case of UV-light-induced pyrimidine dimers and 6-4 photoproducts, the excinuclease hydrolyzes the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ to the diadducts (17, 29). Cisplatin diadducts are removed by hydrolysis of the eighth and fourth phosphodiester bonds 5′ and 3′ to the damaged nucleotides, respectively (1). Nucleotide monoadducts caused by *N*-acetoxy-*N*-acetylaminofluorene and psoralen are removed by hydrolysis of the eighth (5′) and fifth (3′) phosphodiester bonds on either side of the modified nucleotide (16, 24, 25). It has been suggested that this dual-incision mechanism in which the enzyme acts at a distance from the DNA adducts enables the enzyme to repair a wide variety of bulky-type lesions which would otherwise interfere with the binding of the enzyme were the incisions to occur at the site of DNA damage (17, 25).

Since ABC excinuclease apparently recognizes damage-induced helical distortions rather than the DNA adduct itself, it is of interest to determine the minimum helical perturbation which is necessary for enzyme recognition and incision. The alkyl-nitrosoguanidines are a convenient series of compounds for delineating the substrate specificity of ABC excinuclease because they react with DNA in a well-characterized manner, transferring alkyl groups of various sizes and shapes to preferred sites on DNA. The major alkylation products produced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) are N^7-methylguanine, methylphosphotriesters, O^6-methylguanine, and N^3-methyladenine (19). Methylphosphotriesters and O^6-methylguanine are repaired by the alkyl transferase encoded by the *ada* gene. The two 3-methyladenine DNA glycosylases (Tag I and Tag II) encoded by the *tag* and *alkA* genes repair N^3-methyladenine (Tag I and Tag II) as well as minor alkylation products such as N^3-methylguanine (Tag II) (20). The repair mechanisms for longer or branched alkyl-base adducts are not well understood at present. It is possible that alkyl groups of sufficient size or shape create adducts that constitute a substrate for the ABC excinuclease. Previous in vivo studies by Warren and Lawley (27) implicated the ABC excinuclease in the removal of O^6-ethylguanine but not O^6-methylguanine, while studies by Todd and Schendel (23) indicated that nucleotide excision repair prevented both the mutagenic and toxic effects only of alkyl groups larger than two carbons but had no effect on mutagenicity of or killing by MNNG or *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine. More recently, Chambers et al. (3) reported that O^6-methylguanine was 40-fold less mutagenic and O^6-butylguanine was 8-fold more mutagenic in a *uvrA* mutant than for a *uvrA*$^+$ strain. In this study, we investigated the effect of ABC excinuclease in vitro on DNA treated with MNNG. We found that the purified enzyme incised DNA damaged by MNNG, and we present in vivo data suggesting that ABC excinuclease contributes to cell survival after MNNG treatment but that this contribution is obscured in wild-type cells by the DNA glycosylases that repair the major lethal adduct, N^3-methyladenine, of MNNG.

MATERIALS AND METHODS

Chemical reagents. MNNG was purchased from Sigma Chemical Co., St. Louis, Mo. A stock solution of MNNG was prepared by first dissolving the compound in dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) and then diluting the solution with sodium acetate (30 mM, pH 5.0) to a final concentration of 50 mM (approximately 30% dimethyl sulfoxide). Portions were stored at −70°C.

Bacterial strains and cell survival studies. The strains of *E. coli* K-12 used in this study are listed in Table 1. The *uvrA*::Tn*10* mutation was introduced into several alkylation repair-deficient mutants by P1 transduction from strain CGSC 6661 (*uvrA*::Tn*10*). This strain was obtained from R. Lloyd via B. J. Bachmann. For cell survival studies, the cells were grown in Luria broth to late log phase (2×10^8 to 4×10^8 cells per ml), collected by centrifugation, washed, and suspended in phosphate-buffered saline. Portions (1.0 ml) were treated with the alkylating agents for 10 min at 37°C.

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Genotype	Source or reference
AB1157	Wild type	CGSC[a]
AB1886	As AB1157, but *uvrA6*	CGSC
UNC1158	As AB1157, but *uvrA*::Tn*10*	This work
BS24	As AB1157, but *ada-5*	Sedgwick (18)
UNC25	As BS24, but *ada uvrA*::Tn*10*	This work
BK2114	*tag*	Karran et al. (8)
UNC2115	As BK2114, but *tag uvrA*::Tn*10*	This work
BK2106	*ada tag*	Karran et al. (8)
UNC2107	As BK2106, but *ada tag uvrA*::Tn*10*	This work
MV1174	As AB1157, but *his*$^+$ *alkA*	M. Volkert
UNC1175	As MV1174, but *alKA1 uvrA*::Tn*10*	This work
MV1199	As BK2114, but *tag alkA*::λpSG1	M. Volkert
UNC1199	As MV1199, but *tag alkA*::λpSG1 *uvrA*::Tn*10*	This work
MV1198	As BK2106, but *ada tag alkA*::λpSG1	M. Volkert
UNC1198	As MV1198, but *ada tag alkA*::λpSG1 *urvA*::Tn*10*	This work

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

The cells were then chilled on ice, collected by centrifugation, washed, and plated on Luria agar after appropriate dilutions.

Substrates. Radiolabeled pBR322 DNA was prepared as described previously (16), and the superhelical form was purified by two successive ethidium bromide-CsCl density gradient centrifugations. The tritiated DNA used in the incision assay had a specific activity of 1.5×10^5 cpm/μg. The alkylated substrates were prepared by treating 1.5 μg of [^{3}H]-labeled pBR322 in a 100-μl reaction mixture containing 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 10 mM dithiothreitol, 0.1 mM EDTA, and 0 to 10 mM MNNG. After a 1-h incubation at 37°C, the samples were chilled on ice and dialyzed overnight against 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, and 0.1 mM EDTA (two changes) at 4°C. After dialysis, a portion of each sample was used to determine (by scintillation counting) the amount of DNA recovered to normalize the amount of DNA used in each incision assay.

Incision assay. The incision assay measured the conversion of covalently closed superhelical DNA to the open circular form by ABC excinuclease. The reaction mixture (50 μl) contained 50 mM Tris hydrochloride (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 2.0 mM ATP, 10 mM dithiothreitol, 50 μg of bovine serum albumin per ml, and, unless otherwise indicated, 50 fmol of [^{3}H]-labeled pBR322, 60 fmol of UvrA, 313 fmol of UvrB, and 180 fmol of UvrC proteins (22). After incubation at 37°C for 20 min, the reaction was stopped by the addition of 10 μl of 5× loading mixture (2.5% sodium dodecyl sulfate, 0.05% bromophenol blue, and 0.25% xylene cyanol in 50% glycerol). After being heated at 65°C for 5 min, one-half of the sample was loaded onto a 1% agarose gel, and the DNA was separated by electrophoresis. The nicked and superhelical forms of DNA were located by ethidium bromide staining, and the stained bands were cut out. The agar was dissolved in perchloric acid. After neutralization with NaOH, a scintillant was added to the solution, and the radioactivity in the gel slices corresponding to superhelical and open circular DNA was quantitated. The average number of incisions per plasmid was determined from the fraction of superhelical DNA.

RESULTS

Incision of MNNG-damaged DNA by ABC excinuclease. Figure 1 shows the results of increasing doses of MNNG on the numbers of ABC excinuclease-induced DNA incisions. We obtained a linear dose response with MNNG concentrations of up to 7.5 mM. It was not feasible to carry out the experiments with higher MNNG concentrations, as higher doses of the chemical introduced an unacceptable level of scissions into the plasmid. Although the number of adducts produced at different doses was not determined, according to Lawley and Thatcher (10), who used treatment conditions similar to those we used, 3 mM MNNG should produce 50 to 100 adducts per plasmid molecule. Since 50 adducts per molecule (2.5 pmol) were far in excess of the amount of ABC

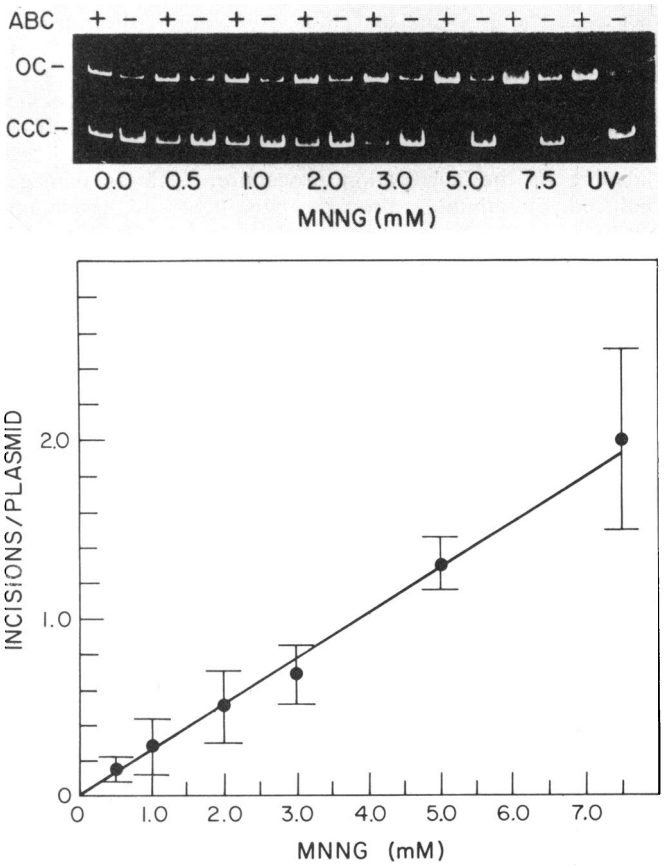

FIG. 1. Incision of MNNG-damaged DNA by ABC excinuclease. (Top) Ethidium bromide-stained agarose gel containing MNNG-damaged [^{3}H]-labeled pBR322 DNA which had been either digested (+) or not digested (−) with ABC excinuclease. OC, Open circular DNA; CCC, covalently closed circular DNA. The UV-irradiated sample received a 125-J/m^2 fluence of 254-nm light, which produced approximately 10 photoproducts per plasmid. (Bottom) The number of ABC excinuclease-specific incisions was calculated from the amount of covalently closed circular DNA which remained after digestion (sample calculations, see Results). Each data point represents the mean ± standard error of three to five separate experiments. The reaction conditions were the same as those described in Material and Methods, except that 120 fmol of UvrA protein was used.

excinuclease (~0.12 pmol) in the reaction mixture and since the excinuclease did not turn over under our assay conditions (2, 6), it appears that the major MNNG adducts were probably not the substrate for ABC excinuclease but that the substrate is in fact a minor adduct that increases in a dose-dependent manner within the range used. To better understand the lesion(s) which is a substrate for the excinuclease, a number of control experiments were conducted. The results of these experiments (Table 2) are as follows.

(i) ABC excinuclease apparently cleaved nondamaged DNA in an ATP-dependent manner. At least two possible explanations can be offered for this action. First, the enzyme preparation used in this study may have contained a multisubunit ATP-dependent endonuclease which was active only when the three subunit fractions of ABC excinuclease were mixed. No double-strand-specific ATP-dependent endonuclease is known to occur in *E. coli*. The only ATP-dependent endonuclease which has been identified in this bacterium is exonuclease V (RecBC nuclease), which requires at least a 6-nucleotide-long single-stranded region to act as an endonuclease and, thus, could not be the cause of the nonspecific incision observed in our experiments (15). A more likely explanation for the nonspecific incision may be that ABC excinuclease recognizes and incises at specific DNA substructures created by superhelicity (e.g., B-to-Z transition) or at a type of DNA damage inherent to the labeling or the purification procedure, such as damage induced by ethidium bromide plus light or by tritium decay.

(ii) The UvrA protein preparation used in our experiment introduced a low but reproducible level of nicks in MNNG-damaged DNA. Since MNNG treatment causes a low level of depurination, these incisions were probably caused by an apurinic endonuclease. Whether this activity is intrinsic to the UvrA protein or, more likely, due to a contaminating apurinic endonuclease is not clear at present.

(iii) The extent of ABC excinuclease incision of DNA damaged by 5 mM MNNG was comparable to that for UV-irradiated DNA (approximately 10 photoproducts per plasmid). To determine the number of ABC excinuclease specific incisions for UV-treated DNA (Table 2), three types of nonspecific DNA breakage had to be subtracted from the total number of DNA breaks. These breaks included preexisting DNA breaks (reaction 15 [0.67 incisions]), the ABC specific incisions (ATP dependent) for nondamaged DNA (reaction 4 [1.06 incisions] minus 5 [0.78 incisions] = 0.28 incisions), and ABC nonspecific incisions (ATP independent) for nondamaged DNA (reaction 5 [0.78 incisions] minus reaction 1 [0.68 incisions] = 0.10 incisions). The number of ABC specific incisions for UV-damaged DNA was 1.07, or 2.12 − (0.67 + 0.28 + 0.10).

To determine the number of ABC excinuclease specific incisions for MNNG-treated DNA (Table 2), three types of nonspecific DNA breakage had to be subtracted from the total number of DNA breaks. These breaks included preexisting and MNNG-induced DNA breaks (reaction 6, [0.87 incisions]), ABC specific incisions (ATP dependent) for nondamaged DNA (reaction 4 [1.06 incisions] minus reaction 5 [0.78 incisions] = 0.28 incisions), and ABC nonspecific incisions (ATP independent) for MNNG-damaged DNA (reaction [1.10 incisions] minus reaction 6 [0.87 incisions] = 0.23 incisions. The number of ABC specific incisions for MNNG-damaged DNA was 0.84, or 2.22 − (0.87 + 0.28 + 0.23).

Since MNNG produces a wide spectrum of DNA lesions (19), we were interested in determining whether all of these lesions or just a particular one was the cause of ABC excinuclease-mediated plasmid incision. To determine this, we used terminally labeled MNNG-treated DNA fragments as substrate, an approach which was used previously in determining the specific incision sites of DNA damaged by UV irradiation (17), psoralen (16, 24, 25), acetylamino fluorene (16), or cisplatin (1). We failed to observe specific incisions introduced by ABC excinuclease by this method because the treatments necessary for analyzing DNA on sequencing gels introduced DNA breaks at the sites of MNNG modification (mainly at N^7-methylguanine), resulting in a high level of background nicking (data not shown).

TABLE 2. Incision of damaged pBR322 DNA by ABC excinuclease[a]

Reaction no. and components[b]	DNA damage	Incisions per plasmid (mean ± SD)[c]
1. pBR322	Nondamaged	0.68 ± 0.06
2. + A	Nondamaged	0.69 ± 0.07
3. + A, C	Nondamaged	0.69 ± 0.05
4. + ABC	Nondamaged	1.06 ± 0.21
5. + ABC − ATP	Nondamaged	0.78 ± 0.03
6. pBR322	Plus MNNG (5 mM)	0.87 ± 0.03
7. + A	Plus MNNG (5 mM)	1.06 ± 0.03
8. + B	Plus MNNG (5 mM)	0.83 ± 0.04
9. + C	Plus MNNG (5 mM)	0.86 ± 0.07
10. + A, B	Plus MNNG (5 mM)	1.07 ± 0.06
11. + A, C	Plus MNNG (5 mM)	1.04 ± 0.09
12. + B, C	Plus MNNG (5 mM)	0.84 ± 0.07
13. + ABC	Plus MNNG (5 mM)	2.22 ± 0.49 (0.84)[d]
14. + ABC − ATP	Plus MNNG (5 mM)	1.10 ± 0.07
15. pBR322	Plus UV light (125 J/m^2)	0.67 ± 0.05
16. + A, C	Plus UV light (125 J/m^2)	0.68 ± 0.06
17. + ABC	Plus UV light (125 J/m^2)	2.12 ± 0.10 (1.07)[d]

[a] The incision reaction was performed as described in Materials and Methods.
[b] A, B, and C refer to the UvrA, UvrB, and UvrC proteins, respectively.
[c] Calculated from the percentage of supercoiled DNA remaining in three to five experiments.
[d] Actual number of ABC excinuclease specific incisions.

Role of ABC excinuclease in cell survival after MNNG treatment. While our studies failed to reveal the specific MNNG-induced lesion that constitutes a substrate for ABC excinuclease, they clearly showed that the enzyme acts on MNNG damage. Yet, previous in vivo studies failed to demonstrate any effect of nucleotide excision repair on MNNG- or *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine-induced cell killing (23). We reasoned that this discrepancy might be due to the existence of very efficient repair pathways for alkylation damage (26) which would mask the relatively small contribution of nucleotide excision repair. We therefore tested the killing effects of MNNG in *uvrA*$^+$ and *uvrA* mutant strains that were deficient in alkylation repair by alkyl transferase or DNA glycosylases (3-methyladenine DNA glycosylases I and II) or both (Fig. 2, Table 3). In agreement with previous reports (23, 27), the *uvrA* mutation alone had little or no effect on survival of MNNG damage of otherwise wild-type cells. However, in alkylation repair-deficient strains, which are unable to repair the major lethal adduct, N^3-methyladenine, the *uvrA* mutation clearly increased sensitivity to MNNG. We thus conclude that nucle-

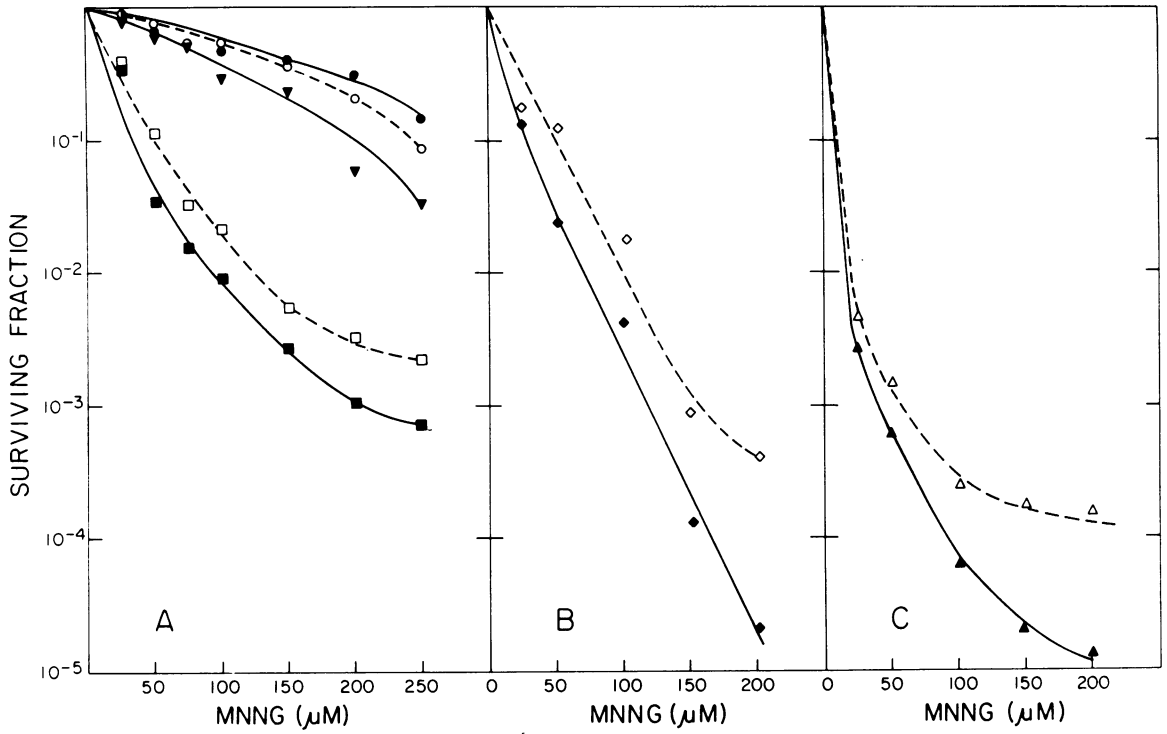

FIG. 2. Effects of the *uvrA* mutation on cell survival after MNNG treatment. The *uvrA*::Tn*10* mutation (closed symbols) was introduced into various alkylation repair-deficient strains (open symbols), and the relative survival after various doses of MNNG was determined. Symbols: (A) ○, AB1157 (wild type); ●, UNC1158 (*uvrA*); ▼, AB1886 (*uvrA6*); □, BK2106 (*tag ada*); ■, UNC2107 (*tag ada uvrA*); (B) ◇, MV1199 (*alkA tag*), ◆, UNC1199 (*alkA tag uvrA*); (C) △, MV1198 (*ada alkA tag*); ▲, UNC1198 (*ada alkA tag uvrA*). Each value is the mean of three to four separate experiments.

otide excision repair contributes to MNNG resistance in *E. coli*.

DISCUSSION

We examined the substrate specificity of ABC excinuclease by using DNA damaged by MNNG, which form adducts with both the bases and the phosphates of DNA. Using incision data, we found that MNNG treatment induced some structural change in the DNA which could be recognized and incised by the ABC excinuclease. These data in conjunction with the survival studies suggest a role for ABC excinuclease in the repair of MNNG-induced DNA damage in vivo. This effect on survival was not observed previously because *E. coli* has two efficient alkylation repair mechanisms (alkyltransferase and DNA glycosylase) that obscure the minor contribution of nucleotide excision repair pathway. Consistent with our findings, a recent study done by a transfection assay with ϕX174 DNA containing either O^6-methylguanine or O^6-butylguanine at a unique location revealed that the products of the *uvr* genes were involved in processing these two DNA adducts (3). It was reported that a mutation in the *uvrA* gene caused an increase in the number of O^6-butylguanine-induced mutations and a decrease in the number of O^6-methylguanine-induced mutations. However, the effect of these adducts on ϕX174 bacteriophage survival was not reported, and therefore, a direct comparison between the results of that study and those reported in this paper cannot be made.

The data presented here raise two related questions. First, what type of helix distortion is necessary for ABC excinuclease recognition and incision, and second, what methylated base is the substrate for the excinuclease? Our results indicate that, contrary to earlier generalizations (5), ABC excinuclease acts on small DNA adducts as well as on bulky-type DNA adducts, suggesting that relatively subtle structural changes in DNA may be sufficient to make it a substrate for the excinuclease. However, it is known that the distortions created by base mismatches or by extrahelical

TABLE 3. Relative survival of various combinations of alkylation repair-deficient strains of *E. coli*

Strain	Genotype	Strain survival (S/S_0) at 150 μM MNNG[a]	Relative survival of *uvrA* mutant[b]
AB1157	Wild type	3.7×10^{-1}	
AB1886	*uvrA*	2.5×10^{-1}	0.67
UNC1158	*uvrA*	4.3×10^{-1}	1.16
BS24	*ada*	1.7×10^{-2}	
UNC25	*ada uvrA*	1.35×10^{-2}	0.80
BK2114	*tag*	1.0×10^{-1}	
UNC2115	*tag uvrA*	4×10^{-2}	0.40
MV1174	*alkA*	9.5×10^{-2}	
UNC1175	*alkA uvrA*	1.37×10^{-1}	1.44
BK2106	*ada tag*	5.3×10^{-3}	
UNC2107	*ada tag uvrA*	2.8×10^{-3}	0.52
MV1199	*alkA tag*	8.8×10^{-4}	
UNC1199	*alkA tag uvrA*	1.06×10^{-4}	0.12
MV1198	*alkA ada tag*	1.14×10^{-4}	
UNC1198	*alkA ada tag uvrA*	2.0×10^{-5}	0.18

[a] S, Survival of MNNG-treated strain; S_0, survival of nontreated control.
[b] Ratio of relative survival of a strain with a *uvrA* mutation to that of a strain without the mutation.

insertions of 1, 3, or 4 bases are not sufficient for ABC excinuclease cleavage (21). With regard to the detection and repair of methylated bases, it is interesting to note that the excision repair epistasis group of *Saccharomyces cerevisiae* has been shown to act on N^6-methyladenine (6). Our attempts to identify the specific methylated base responsible for ABC excinuclease incision were equivocal because of the high level of chemically induced breaks observed with MNNG-damaged DNA on sequencing gels.

In addition to making methylphosphotriesters, MNNG is known to alkylate almost all of the atoms of the bases in DNA, with N^7-guanine (72%), N^3-adenine (11%), and O^6-guanine (7%) making up the majority of the lesions (19). The *ada* gene codes for a protein which removes methyl groups from the *O*-6 of guanine and *O*-4 of thymine as well as from the *S* stereoisomer of methylphosphotriester (13, 28). The Ada protein also stimulates the synthesis of a number of other repair genes, one of which is *alkA*. The *alkA* gene encodes 3-methyladenine DNA glycosylase II (Tag II) (4, 8, 9, 11–14). This enzyme removes N^3-methyladenine as well as N^7-methylpurines and O^2-methylpyrimidines, while Tag I (which is not regulated by the Ada protein) acts exclusively on N^3-methyladenine, which is believed to be the site of the main lethal lesion caused by MNNG treatment (8, 9, 12).

We do not believe that the substrate for ABC excinuclease in MNNG-treated DNA is N^7-methylguanine, O^6-methylguanine, or N^3-methyladenine because we obtained a dose response with the MNNG concentrations that produce these lesions far in molar excess of ABC excinuclease subunits. (ABC excinuclease does not turn over in the absence of DNA polymerase I plus helicase II [2, 7]). Furthermore, preliminary experiments with a substrate containing O^6-methylguanine at a defined position failed to detect ABC excinuclease incisions at this DNA adduct (B. Van Houten, M. Topal, and A. Sancar, unpublished observation).

The MNNG survival studies revealed that strains completely deficient in 3-methyladenine DNA glycosylase (*alkA tag* double mutant) were extremely sensitive to MNNG killing and that this sensitivity was further increased by a mutation in the *uvrA* gene. While this enhanced sensitivity to the killing effect of MNNG is consistent with 3-methyladenine as a substrate for ABC excinuclease, the survival data could also be explained by an assumption that a minor alkylation adduct (which is the substrate for the excinuclease) contributes more significantly to the lethality of MNNG when the pathways repairing the main lethal adduct (N^3-methyladenine) are eliminated.

One MNNG-induced DNA adduct which may be a substrate for ABC excinuclease is N^1-methyladenine. This adduct is produced with relatively low frequency by alkylating agents (19) and is not repaired by methyl transferase or glycosylases. Furthermore, methylation at the N-1 of adenine disrupts Watson-Crick base pairing and, therefore, is expected to produce a considerable helical distortion, which seems to be the common characteristic of ABC excinuclease substrates. Another potential MNNG-induced ABC excinuclease substrate is an apurinic site which is produced as a result of spontaneous hydrolysis of the *N*-glycosidic bond of N^7-methylguanine (19). Such apurinic sites are likely to distort the helix significantly and may create binding sites for ABC excinuclease. Experiments with uniquely modified substrates containing either an N^1-methyladenine or an apurinic site are required to determine whether these structures are recognized by ABC excinuclease.

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