

# Guanine-specific DNA Repair after Treatment of Mouse Skin Cells with *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine

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## SUMMARY

The incorporation of [<sup>3</sup>H]thymidine and [<sup>3</sup>H]deoxyguanosine into DNA was studied during DNA repair in mouse skin cells treated with the skin tumor initiator *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. At high, toxic levels of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, repair (incorporation of the precursor into DNA which had not replicated) was demonstrated with both precursors. At lower, less toxic doses of the carcinogen, repair could not be demonstrated with [<sup>3</sup>H]thymidine, but it was clearly demonstrable with [<sup>3</sup>H]deoxyguanosine. Thus, we are apparently observing two kinds of DNA repair, one in which a single base (in this case, guanine) replaces a base lost by chemical or enzymatic depurination and the second in which more than one base is replaced, indicating synthesis of longer stretches of DNA after extensive enzymatic excision. The guanine-specific repair shown at relatively nontoxic dose levels of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine may be more relevant to the survival of cells than the repair demonstrated with [<sup>3</sup>H]thymidine at higher doses.

## INTRODUCTION

The methylating agent MNNG<sup>1</sup> is carcinogenic for the digestive tract of rats, inducing mainly squamous cell carcinomas of the forestomach (13) and adenocarcinomas of the glandular stomach (14). Injection s.c. of MNNG into rats led to fibrosarcomas at the injection site (15). A single application of MNNG to mouse skin has recently been reported to initiate the process of skin tumor formation (3).

MNNG has a short half-life in culture medium (7) and reacts rapidly with cellular DNA to produce 7-methylguanine, 3-methyladenine, and 6-*O*-methylguanine (6). MNNG is toxic to mammalian cells in culture (11) and inhibits the synthesis of nucleic acids and proteins (2). Treatment of cultured mammalian cells with MNNG induces DNA repair synthesis ([<sup>3</sup>H]thymidine incorporation into nonreplicating DNA), characterized by the insertion of about 100 bases for each methyl group eliminated from DNA (12).

We have reported the guanine-specific repair of DNA (incorporation of only [<sup>3</sup>H]deoxyguanosine into nonreplicating DNA) in primary cultures of mouse skin cells treated with relatively low doses of the proximate skin carcinogen and

skin tumor initiator, BPL (5). At higher, very toxic levels of BPL, repair could be demonstrated with pyrimidine precursors as well. In order to determine whether another skin carcinogen active in cultured mammalian cells (4) induced guanine-specific repair, MNNG was studied in the skin cell culture system. We have examined the effects of a range of dose levels of MNNG on [<sup>3</sup>H]thymidine incorporation into DNA and on DNA repair synthesis with either [<sup>3</sup>H]thymidine or [<sup>3</sup>H]deoxyguanosine as precursor.

## MATERIALS AND METHODS

**Chemicals and Radiochemicals.** [*methyl*-<sup>3</sup>H]Thymidine and [8-<sup>3</sup>H]deoxyguanosine (both 5 Ci/mmol) were obtained from Amersham/Searle, Arlington Heights, Ill. MNNG was purchased from Aldrich, Milwaukee, Wis. BrUdR was obtained from Sigma Chemical Co., St. Louis, Mo. Instagel was purchased from Packard Instrument Co., Downers Grove, Ill. Cesium chloride was purchased from Harshaw Chemical Co., Solon, Ohio.

**Cell Culture.** Skin cell cultures containing both epidermal cells and fibroblasts were prepared from full-thickness skins of 1- to 5-day-old Swiss mice (NIH General Purpose, Bethesda, Md.), as described by Yuspa *et al.* (16). Cells were plated at  $2.5 \times 10^6$  cells/ml in 150-mm plastic Petri dishes in Medium 199 containing 11% fetal calf serum (Flow Laboratories, Rockville, Md.) and 1% antibiotic-antimycotic mixture (Grand Island Biological Co., Grand Island, N. Y.). The cells were washed thoroughly with PBS at Day 1 and were given fresh medium at Days 1 and 3.

**Demonstration of DNA Repair.** To distinguish the incorporation of a DNA precursor during repair from incorporation during DNA replication, one must separate replicating and nonreplicating DNA. To accomplish this, we have utilized BrUdR as a density label. When both BrUdR and a tritiated deoxyribonucleoside are present in the culture medium during normal, semiconservative DNA replication, tritium is found only in the heavy, BrUdR-containing strand of hybrid-density DNA (with 1 light and 1 heavy strand). Any tritium found in light, unreplicated DNA (with both strands of normal density) indicates incorporation of the precursor during DNA repair synthesis. Separation of replicating DNA (hybrid density) from nonreplicating DNA (normal density) was accomplished by centrifugation in cesium chloride buoyant density gradients, as described below.

In experiments of similar design, Roberts *et al.* (12) demonstrated that not only was combining [<sup>3</sup>H]thymidine or [<sup>3</sup>H]deoxycytidine with cold BrUdR equivalent to using

<sup>1</sup> The abbreviations used are: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; BPL,  $\beta$ -propiolactone; BrUdR, 5-bromo-2'-deoxyuridine; PBS, phosphate-buffered saline (in g/liter: NaCl, 8; KCl, 0.2; Na<sub>2</sub>HPO<sub>4</sub>, 1.15; KH<sub>2</sub>PO<sub>4</sub>, 0.2; MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.1; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.1); PCA, perchloric acid.

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[<sup>3</sup>H]BrUdR in assessing repair, but it also provided a more sensitive assay for estimating low levels of repair.

**DNA Extraction and Fractionation on Cesium Chloride Gradients.** DNA was isolated by a phenol procedure (16), dissolved in 10 ml cesium chloride solution (1.68 g/ml, pH 7.2, 0.015 M EDTA), and centrifuged in a Beckman Model L2-65B ultracentrifuge in a 50 Ti rotor for 40 to 65 hr at 45,000 rpm. After fractionation, aliquots of each 0.5-ml fraction were used to determine the absorbance at 260 nm and the radioactivity by scintillation counting. Aliquots of 25 to 200  $\mu$ l were counted in 10 ml Instagel plus 1 ml distilled water.

**Repair Experiments.** One to 2 hr before treatment with MNNG, the old medium was removed and thymine-free Medium 199, pH 7.3, with BrUdR ( $6 \times 10^{-6}$  M) was added to the cells. MNNG was dissolved in absolute ethanol and added to the cultures to give the appropriate concentration of MNNG and 1% ethanol. Controls were treated with 1% ethanol. After 1 hr, the medium was removed, the cells were washed with PBS, and fresh thymine-free Medium 199 containing both  $6 \times 10^{-6}$  M BrUdR and either [<sup>3</sup>H]thymidine or [<sup>3</sup>H]deoxyguanosine was added. After either 3 or 16 hr, the cells were harvested and the DNA was isolated and fractionated as described above. In the 16-hr experiments, a 1- $\mu$  Ci/ml level of the tritiated precursor was used; in the shorter experiment, the level was 10  $\mu$ Ci/ml.

**Toxicity Experiments.** Cultures were treated with MNNG in complete Medium 199 for 1 hr (Hr 0 to 1). The cells were incubated with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/ml) at 1 to 2, 18 to 19, 25 to 26, or 41 to 42 hr after MNNG.

**DNA Hydrolysis for Specific Activity Determination.** After incorporation of [<sup>3</sup>H]thymidine, the cells were washed with PBS, scraped with a polyethylene scraper, and centrifuged at 2000 rpm at 0 to 4°. The pellet was then washed 3 times with 2% (v/v) PCA and once with 95% ethanol. The precipitate was suspended in 0.3 N KOH and incubated at 37° for 1 hr. After cooling and adding concentrated PCA to a final concentration of 5% (v/v), the RNA hydrolysate was decanted. The precipitate was washed once with 2% PCA, and the DNA was hydrolyzed in 5% PCA at 90° for 20 min. An aliquot of the DNA hydrolysate was used for determining the amount of DNA by the diphenylamine reaction; a 2nd aliquot was counted in 10 ml of Instagel to estimate radioactivity. The specific activity is expressed as cpm/ $\mu$ g DNA.

**Chromatography.** Appropriate peaks from cesium chloride gradients were dialyzed against 3 changes of 0.01 M Tris-HCl, pH 7.5, to remove cesium chloride. The DNA was hydrolyzed to the nucleoside level with pancreatic DNase, snake venom phosphodiesterase, and bacterial alkaline phosphatase (8), and was chromatographed on paper in butanol-boric acid-NH<sub>3</sub> (1). The UV-absorbing spots were eluted with 1 ml 1 N HCl and counted in 10 ml Instagel.

## RESULTS

**Toxicity: Inhibition of DNA Synthesis and Decrease in Amount of DNA.** Treatment of cultures of skin cells for 1 hr with levels of MNNG of 1, 5, or 25  $\mu$ g/ml inhibited the incorporation of [<sup>3</sup>H]thymidine into DNA at 2 hr. Reductions with MNNG were to 80% of the control with 1  $\mu$ g/ml, 35% of

the control with 5  $\mu$ g/ml, and 4% of control with 25  $\mu$ g/ml (Chart 1A). By 19 to 26 hr, a somewhat greater inhibition was seen with all 3 doses, with a complete inhibition of DNA synthesis at the highest dose. No recovery was seen by 42 hr.

The amount of DNA extracted per Petri dish was not affected 2 hr after MNNG treatment (Chart 1B). However, by 26 hr, the level of DNA per dish was reduced to 69% of the control with 5  $\mu$ g MNNG per ml and to 31% of the control with 25  $\mu$ g MNNG per ml. These levels of DNA per dish were maintained at 42 hr.

An impression of the extent of toxicity in MNNG-treated cultures was made by observing the reduction in confluence by phase contrast microscopy. Control cultures were confluent throughout the experiment. Considerable cell killing (greater than three-fourths reduction in confluence) was evident 42 hr after a 1-hr treatment with 25  $\mu$ g MNNG per ml. With 5  $\mu$ g MNNG per ml, the cells were about 50% confluent with foci which appeared to be unaffected by the toxic effects of MNNG. Only slight toxicity was noted with 1  $\mu$ g MNNG per ml. Thus, the early inhibition of DNA synthesis and the subsequent reduction in the level of DNA per dish may be taken as quantitative indicators of the toxicity noted visually.

**Guanine-specific Repair after MNNG Treatment.** Since the dose of 5  $\mu$ g MNNG per ml inhibited DNA replication to about the same extent as a guanine-specific repair-inducing dose of BPL (5), this dose was selected to investigate guanine-specific repair. This level of MNNG for 1 hr, followed by an overnight incubation in medium containing 1  $\mu$ Ci [<sup>3</sup>H]thymidine per ml, gave no evidence for DNA repair, although the height of the replicating peak of radioactivity was reduced to about one-third of the control level (Chart 2, A and B). The data shown in Chart 2 are from the 1st centrifugation of DNA in a cesium chloride gradient. After the absorbance peak of light-density DNA was rebanded twice, a small tritium peak was found associated with this unreplicated DNA in both MNNG-treated and control cul-

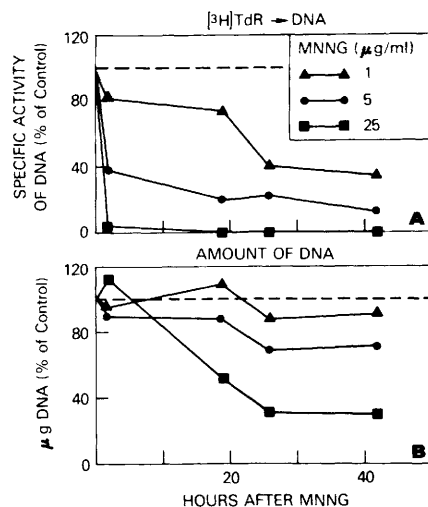


Chart 1. Effect of MNNG on incorporation of [<sup>3</sup>H]thymidine ([<sup>3</sup>H]TdR) into DNA (A) and amount of DNA per Petri dish (B). Skin cell cultures were treated with 1, 5, or 25  $\mu$ g MNNG per ml for 1 hr, washed with PBS, and given fresh medium. [<sup>3</sup>H]Thymidine (1  $\mu$  Ci/ml) was added for 1 hr before harvest at the times indicated. The specific activity of DNA in controls was 1042 cpm/ $\mu$ g DNA at 26 hr, with 142  $\mu$ g DNA per dish.

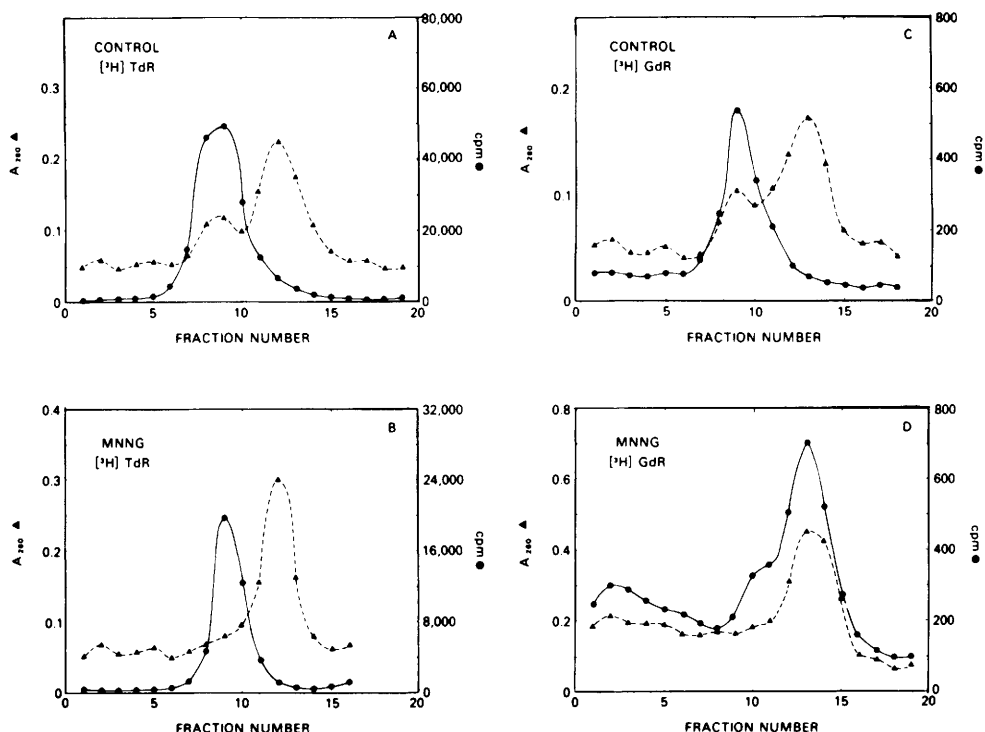


Chart 2. Incorporation of [ $^3\text{H}$ ]thymidine ( $[^3\text{H}]$ TdR) or [ $^3\text{H}$ ]deoxyguanosine ( $[^3\text{H}]$ GdR) into DNA of control or MNNG-treated mouse skin cell cultures. Cells were treated as described in "Materials and Methods" under "Repair Experiments." Treatment for 1 hr with 5  $\mu\text{g}$  MNNG per ml was followed by a 16-hr incubation in thymine-free Medium 199 containing [ $^3\text{H}$ ]thymidine or [ $^3\text{H}$ ]deoxyguanosine (1  $\mu\text{Ci}/\text{ml}$ ) and BrUdR. After DNA isolation and centrifugation in a cesium chloride gradient, the samples were fractionated. The  $A_{260}$  and the cpm in 0.1-ml aliquots of each fraction are plotted. The bottom of the tube is to the left. The peak on the left is replicating DNA; the peak on the right is nonreplicating DNA. A, ethanol-treated controls, [ $^3\text{H}$ ]thymidine; B, MNNG-treated cultures, [ $^3\text{H}$ ]thymidine; C, ethanol-treated controls, [ $^3\text{H}$ ]deoxyguanosine; D, MNNG-treated cultures, [ $^3\text{H}$ ]deoxyguanosine.

tures. In the MNNG-treated cells, the specific activity (cpm/ $A_{260}$ ) of this peak was reduced to 25 to 30% of that found in the controls, a reduction similar to the extent of inhibition of DNA replication by this dose of MNNG. Thus, this peak appears to be associated with replication in both control and MNNG-treated cultures, not with repair. A similar result was found in BPL-treated skin cells (5).

When [ $^3\text{H}$ ]deoxycytidine was utilized as precursor, no repair synthesis could be detected with 5  $\mu\text{g}$  MNNG per ml (data not shown). However, with [ $^3\text{H}$ ]deoxyguanosine as precursor, a large peak of tritium appeared coincident with the light, unreplicated absorbance peak of DNA in MNNG-treated cells (Chart 2, C and D). Thus, DNA repair could be demonstrated with [ $^3\text{H}$ ]deoxyguanosine but not with [ $^3\text{H}$ ]thymidine or [ $^3\text{H}$ ]deoxycytidine after treatment of mouse skin cells for 1 hr with 5  $\mu\text{g}$  MNNG per ml. Repair could also be demonstrated with [ $^3\text{H}$ ]deoxyguanosine but not with [ $^3\text{H}$ ]thymidine at doses of MNNG of 1 and 2.5  $\mu\text{g}/\text{ml}$  (data not shown).

**Repair with [ $^3\text{H}$ ]Thymidine after High Levels of MNNG.** Since DNA repair had been demonstrated with [ $^3\text{H}$ ]thymidine after MNNG treatment of other cell types (12), we examined repair with [ $^3\text{H}$ ]thymidine after a 1-hr treatment with 5, 25, or 50  $\mu\text{g}$  MNNG per ml. Due to the toxicity of the higher doses, this experiment was terminated after a 3-hr exposure to [ $^3\text{H}$ ]thymidine (10  $\mu\text{Ci}/\text{ml}$ ). Again no repair was indicated after treatment with 5  $\mu\text{g}/\text{ml}$  (Chart 3B). At 25  $\mu\text{g}/\text{ml}$ , a shoulder of tritium counts was seen coincident with the peak of unreplicated DNA (Chart 3C); a clear-cut

repair peak was found after the highest MNNG dose (Chart 3D).

**Chromatography of [ $^3\text{H}$ ]Deoxyguanosine-labeled Nucleic Acid Hydrolysates.** In order to establish that the tritium found in the unreplicated peak of DNA after treatment with 5  $\mu\text{g}$  MNNG per ml followed by [ $^3\text{H}$ ]deoxyguanosine was indeed incorporated into the guanine of DNA, the unreplicated DNA peak was hydrolyzed and chromatographed (1). The chromatography system used, butanol-borate- $\text{NH}_2$ , yields clear separation of the purines. With 3 separate samples, more than 99% of the tritium found in the UV-absorbing spots representing the 4 nucleosides was associated with deoxyguanosine. A typical result was the following. Of a total of 2100 cpm associated with the 4 deoxyribonucleoside spots, 2095 cpm were found in deoxyguanosine, 4 cpm were found in deoxycytidine, none in deoxyadenosine, and 1 cpm was found in thymidine. In 3 samples of replicating DNA from control cultures labeled with [ $^3\text{H}$ ]deoxyguanosine, 96.9, 97.8, and 99.5% of the tritium was associated with deoxyguanosine. Thus, the specificity of guanine incorporation in guanine-specific repair synthesis is at least as great as that found in DNA replication.

## DISCUSSION

We have compared [ $^3\text{H}$ ]thymidine and [ $^3\text{H}$ ]deoxyguanosine as precursors for DNA repair synthesis after MNNG treatment. With increasing doses of MNNG, quite different

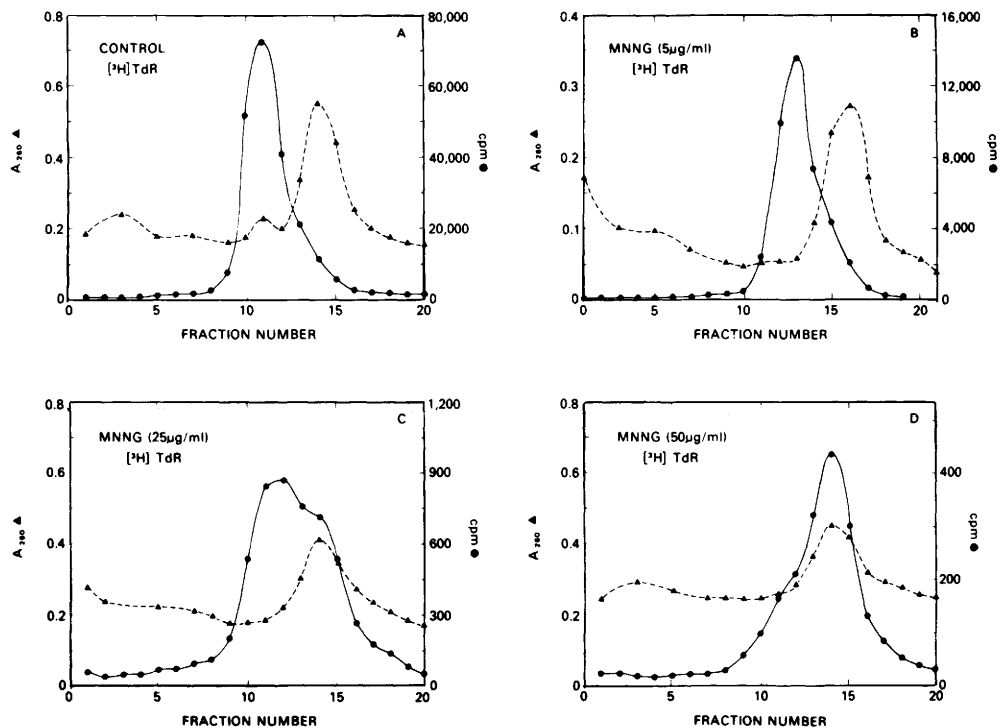


Chart 3. Incorporation of [ $^3\text{H}$ ]thymidine ( $^3\text{H}$ ]TdR) into DNA of control and MNNG-treated mouse skin cell cultures. Experimental conditions are similar to those described in the legend to Chart 2. Treatment for 1 hr with 1% ethanol (A, control), or levels of MNNG of 5  $\mu\text{g}/\text{ml}$  (B), 25  $\mu\text{g}/\text{ml}$  (C), or 50  $\mu\text{g}/\text{ml}$  (D) was followed by a 3-hr incubation in thymine-free medium 199 containing [ $^3\text{H}$ ]thymidine (10  $\mu\text{Ci}/\text{ml}$ ) and BrUdR. The  $A_{260}$  and the cpm in 0.1-ml aliquots of each fraction are plotted.

responses were found with the 2 precursors. In order to detect DNA repair synthesis with [ $^3\text{H}$ ]thymidine (Chart 3), it was necessary to treat the cells with high, toxic levels of MNNG (25 to 50  $\mu\text{g}/\text{ml}$ , Chart 1). At a dose of 5  $\mu\text{g}/\text{ml}$ , repair could not be demonstrated with [ $^3\text{H}$ ]thymidine or [ $^3\text{H}$ ]deoxycytidine but was shown clearly with [ $^3\text{H}$ ]deoxyguanosine as precursor (Chart 2). Thus, with MNNG, as with BPL (5), a deoxyguanosine-specific repair mechanism was demonstrated at a dose of MNNG below that required for repair with [ $^3\text{H}$ ]thymidine or [ $^3\text{H}$ ]deoxycytidine. This repair mechanism, found at levels of MNNG that were toxic to perhaps one-half of the cells (Chart 1), may be more relevant to cell survival than the repair demonstrated with [ $^3\text{H}$ ]thymidine at higher, very toxic levels of carcinogen.

Peterson *et al.* (9) have recently demonstrated single-strand breaks in the DNA of mouse fibroblasts treated with MNNG at a level of 0.5  $\mu\text{g}/\text{ml}$  for 2 hr, a dose that killed less than 20% of the cells. From a comparison of the extent of alkylation and the number of single-strand breaks, they concluded that repair occurred only after loss of 7-methylguanine from the DNA. Since we have detected guanine-specific repair at doses of MNNG as low as 1  $\mu\text{g}/\text{ml}$ , it may be reasonable to suggest that this repair and the MNNG-induced single-strand breaks demonstrated by alkaline sucrose gradients (9) are manifestations of the same repair process. However, guanine-specific repair, in which a single base apparently replaces a base lost by depurination, appears to be distinct from repair in which longer stretches of DNA are synthesized after extensive enzymatic excision (10, 12) or in which only a few bases are replaced (10).

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