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Repair Response of *Escherichia coli* to Hydrogen Peroxide DNA Damage

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The repair response of *Escherichia coli* to hydrogen peroxide-induced DNA damage was investigated in intact and toluene-treated cells. Cellular DNA was cleaved after treatment by hydogen peroxide as analyzed by alkaline sucrose sedimentation. The incision step did not require ATP or magnesium and was not inhibited by *N*-ethylmaleimide (NEM). An ATP-independent, magnesium-dependent incorporation of nucleotides was seen after the exposure of cells to hydrogen peroxide. This DNA repair synthesis was not inhibited by the addition of NEM or dithiothreitol. In *dnaB*(Ts) strain CRT266, which is thermolabile for DNA replication, normal levels of DNA synthesis were found at the restrictive temperature (43°C), showing that DNA replication was not necessary for this DNA synthesis. Density gradient analysis also indicated that hydrogen peroxide inhibited DNA replication and stimulated repair synthesis. The subsequent reformation step required magnesium, did not require ATP, and was not inhibited by NEM, in agreement with the synthesis requirements. This suggests that DNA polymerase I was involved in the repair step. Furthermore, a strain defective in DNA polymerase I was unable to reform its DNA after peroxide treatment. Chemical cleavage of the DNA was shown by incision of supercoiled DNA with hydrogen peroxide in the presence of a low concentration of ferric chloride. These findings suggest that hydrogen peroxide directly incises DNA, causing damage which is repaired by an incision repair pathway that requires DNA polymerase I.

Hydrogen peroxide is known to have several effects on DNA including inhibiting transforming activity and decreasing UV-light absorption and the melting temperature (18, 24). Peroxide can release all four bases from DNA and cleave the DNA backbone (16, 18). The exact nature of the DNA adduct, if any, formed by hydrogen peroxide is not yet known, but investigators have related the damage caused by this agent to that caused by near-UV light (9, 19) or X rays (21).

recA (2, 4), *polA* (2, 4), *xthA* (7), and *polC* (M. Hagensee and R. Moses, unpublished data) mutants are more sensitive to the lethal effects of peroxide than are normal cells. In addition, Ananthaswamy and Eisenstark (2) have shown that hydrogen peroxide causes incisions of DNA in wild-type *E. coli* as analyzed by alkaline sucrose sedimentation. Recently, other investigators (11, 17) have reported that hydrogen peroxide, as well as other hydroperoxides, can relax supercoiled DNA. Finally, Demple and Halbrook (6) have reported that, after a sublethal dose of peroxide, cells can resist killing by a subsequent dose of peroxide. This induced protection mechanism was found to be independent of the SOS response since it was still present in *recA* strains. The repair mechanism by which *E. coli* removes DNA damage caused by hydrogen peroxide, as well as the molecular basis for the induced response, has not been determined.

We show that the incision of DNA in the cell occurred after exposure of the cell to hydrogen peroxide. This was followed by reformation of high-molecular-weight DNA after removal of the hydrogen peroxide. After cell exposure to hydrogen peroxide, ATP-independent DNA synthesis was seen. DNA polymerase III requires ATP for replicative synthesis and is inhibited by *N*-ethylmaleimide (NEM) (15). Thus, the synthesis seen after H_2O_2 exposure was most likely dependent on DNA polymerase I. Confirming this, the synthesis was missing in a strain lacking a functional DNA polymerase I. Although DNA polymerase III is needed for optimal cell survival after H_2O_2 (unpublished data), DNA polymerase I accounted for the observable repair synthesis. Density gradient analysis demonstrated that hydrogen peroxide inhibited DNA replication and stimulated repair synthesis. Finally, hydrogen peroxide, in the presence of a low concentration of ferric chloride, could incise supercoiled DNA, indicating that peroxide can chemically break DNA.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are given in Table 1. They were previously described (4). HM1 is a spontaneous *polC*$^+$ revertant from HS432 (*polA1 polB100 polC1026 pcbA1*).

Materials. Growth media were purchased from Difco Laboratories. Hydrogen peroxide was obtained from Fisher Scientific Co. [^{3}H]thymidine and [α-^{32}P]dTTP were purchased from ICN Pharmaceuticals, Inc. The NACS-37 column was purchased from Bethesda Research Laboratories, Inc.

Alkaline sucrose gradients. The alkaline sucrose gradient assays of whole cells were performed by the method of Dorson et al. (9). Cells were prelabeled with [^{3}H]thymidine (20 μCi/ml, 35 Ci/mmol) and grown in L broth, at 37°C, to an optical density at 600 nm of 1.0 to 1.5 (6×10^8 to 9×10^8 cells per ml). Cells were collected by centrifugation and suspended in 50 mM phosphate buffer, pH 7.4. The cells were then exposed to hydrogen peroxide for 10 min at 37°C, collected by centrifugation, and washed. The hydrogen peroxide reaction was stopped by the addition of catalase (0.25 mg/ml). The cells were then suspended in M9 medium and allowed to repair at 37°C for 4 h.

Samples (50 μl each) were taken at intervals, and 2 volumes of 200 mM Tris–40 mM EDTA (pH 8.0) were added. Of this solution, 50 μl was then layered onto 5 to 20% alkaline sucrose gradients, as described by McGrath and Williams (14), containing an upper layer of 200 μl of lysing

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TABLE 1. Bacterial strains

Strain	Genotype or phenotype	Source
W3110	Wild type	J. Cairns
HM1	*polA1 polB100 leu his*	This laboratory
P3478	*polA1*	J. Cairns
CRT266	*dnaB*(Ts)	Y. Hirota

solution (0.5 M NaOH, 1% Sarkosyl [CIBA-GEIGY Corp.], 4 mM EDTA). Samples were allowed to lyse for 20 min and then were spun in an SW50.1 rotor (Beckman Instruments, Inc.) at 25,000 rpm for 2 h at 20°C.

Gradients were fractionated by puncturing the bottoms of the tubes and collecting 8-drop fractions on Whatman 3MM filter paper rolls. Each roll was then treated with successive 10-min washes of 10% trichloroacetic acid (TCA)–0.1 M sodium pyrophosphate, 5% TCA, and 0.01 N HCl. The rolls were then dried and prepared for scintillation counting. The results were expressed as proportions of the total counts in each fraction.

Toluene treatment. Cells were toluene treated as described by Moses and Richardson (15). Cells were grown in L broth to an optical density at 600 nm of 0.9 to 1.0 (6×10^8 cells per ml), collected by centrifugation, and suspended in 1/25 volume of cold 50 mM phosphate buffer, pH 7.4. The cells were then exposed to 1% toluene and slowly shaken for 10 min at room temperature. The cells were again collected by centrifugation, and the cell pellet was washed with cold phosphate buffer. The cells were then suspended in 1/25 of the original volume of 50 mM phosphate buffer, and a replication assay was performed to verify the results of the toluene treatment.

Repair synthesis assay on toluene-treated cells. The repair synthesis assay was performed by the method of Ross et al. (20). The standard reaction mixture of 300 μl contained 67 mM potassium phosphate (pH 7.4), 13 mM $MgCl_2$, 1.3 mM ATP, and 33 μM concentrations of the four deoxyribonucleotides. [^{3}H]dTTP (20 to 50 cpm/pmol) or [α-^{32}P]dTTP (20 to 50 cpm/pmol) was added. Hydrogen peroxide was used at 70 mM for 10 min before the addition of the other components, followed by the addition of 0.25 mg of catalase per ml. When present, other components and concentrations (mM) were as follows: EDTA, 27; NEM, 13.3; nicotinamide mononucleotide (NMN), 5.3; dithiothreitol, 1.3; and ATP, 1.3. Reactions were begun by the addition of 10^9 toluene-treated cells which were incubated at 37°C for 30 min. The reaction was stopped by the addition of 3 ml of 10% TCA–0.1 M sodium pyrophosphate, and the tubes were placed on ice for 10 min. The TCA precipitate was collected by filtration through Whatman GF/C filters (diameter, 24 mm) and washed two times with 3 ml of 10% TCA–0.1 M sodium pyrophosphate followed by 5 ml of 0.01 M hydrochloric acid. The filters were dried, and radioactivity was determined.

Density gradient analysis. Cesium chloride density gradient analyses were performed by the method of Dorson and Moses (8). Cells were prelabeled with [^{3}H]thymidine (20 μCi/ml, 35 Ci/mmol) and grown to an optical density at 600 nm of 0.9. Cells were toluene treated as described above, and DNA synthesis (600 μl final volume) was assayed in a reaction containing 67 mM potassium phosphate (pH 7.4), 13 mM $MgCl_2$, 1.3 mM ATP, and 33 μM of dATP, dGTP, bromodeoxyuridine triphosphate instead of dTTP, and [^{32}P]dCTP (3,000 Ci/mmol). The reaction was started by the addition of 10^9 toluene-treated cells which were incubated at 37°C for 30 min. The reaction was stopped by the addition of 20 μl of 0.1 mM NEM and 2 ml of NET buffer (0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris hydrochloride [pH 8.0]). Cells were collected by centrifugation at 8,000 rpm for 10 min, and the pellet was suspended in 0.2 ml of 1/10-volume NET buffer. The cells were lysed by the addition of 40 μl of 10% Sarkosyl plus 40 μl of pronase (1 mg/ml) and incubated at 60°C for 2 h. The DNA was then vortexed vigorously to shear the DNA, and 65% $CsCl_2$ (made in 1/10 NET buffer) was added to a density of 1.725 g/cm^2. The gradients were spun at 37,000 rpm for 40 h at 25°C. Fractions were pumped from the bottoms of the tubes, TCA precipitated, filtered, washed with cold 10% TCA–0.1 M sodium pyrophosphate plus cold 0.01 N HCl, and prepared for scintillation counting. The data are presented as the percentages of the total counts recovered.

Preparation of supercoiled φX174 DNA. φX174 was grown as previously described (19), and supercoiled DNA was isolated by using the NACS-37 column procedure of Bethesda Research Laboratories.

Nicking assay. The filter retention assay was performed, and the extent of incision was calculated by the method of Kuhnlein et al. (12). The reaction mixture (100 μl total volume) consisted of 0.1 M Tris [pH 8.0], 10 mM $MgCl_2$, and 0.1 to 0.5 μg of supercoiled φX174 (approximately 2,000 cpm per assay). Ferric chloride was added at a final concentration of 100 μM, and hydrogen peroxide was added at a final concentration of 44 mM. This mixture was incubated at 37°C for 15 min, and the reaction was stopped by the addition of 0.5 ml of 0.01% sodium dodecyl sulfate. The reaction mixture was then denatured, renatured, and filtered as described previously (19).

RESULTS

Incision and reformation in cells. The repair response of *E. coli* to hydrogen peroxide was studied in cells by alkaline sucrose sedimentation analysis. The response of a wild-type cell line, W3110, to a challenge of 117 mM hydrogen peroxide at 37°C is illustrated in Fig. 1. After a 10-min exposure, the DNA was of lower molecular weight, indicating that incision had occurred. This could have been caused by production of a strand break directly or by production of an alkali-sensitive site which was cleaved upon exposure to alkali. After the inactivation of peroxide by the addition of catalase and a subsequent 4-h incubation at 37°C, the DNA returned to a high molecular weight, demonstrating its reformation. The extents of incision and reformation shown in this experiment were slightly greater than previously reported (2), but our incubations were at 37°C rather than 25°C. It was not clear from this experiment whether hydrogen peroxide cleaved the DNA directly or whether the DNA was broken by enzymatic incision after the formation of a peroxide-induced DNA adduct.

DNA synthesis after exposure of cells to hydrogen peroxide is ATP-independent. Cells were permeabilized by toluene treatment and exposed at 37°C to increasing concentrations of hydrogen peroxide, and the rate of DNA synthesis (37°C) after this treatment was measured. Wild-type cells, before the addition of hydrogen peroxide, showed an ATP-dependent replicative synthesis (Fig. 2) which was inhibited by NEM, characteristic of DNA polymerase III synthesis (20). Upon the addition of hydrogen peroxide, these cells showed an ATP-independent synthesis which was not inhibited by NEM. In cases where ATP-dependent replicative synthesis was high, a decrease to the level of ATP-independent synthesis was noted with increasing hydrogen

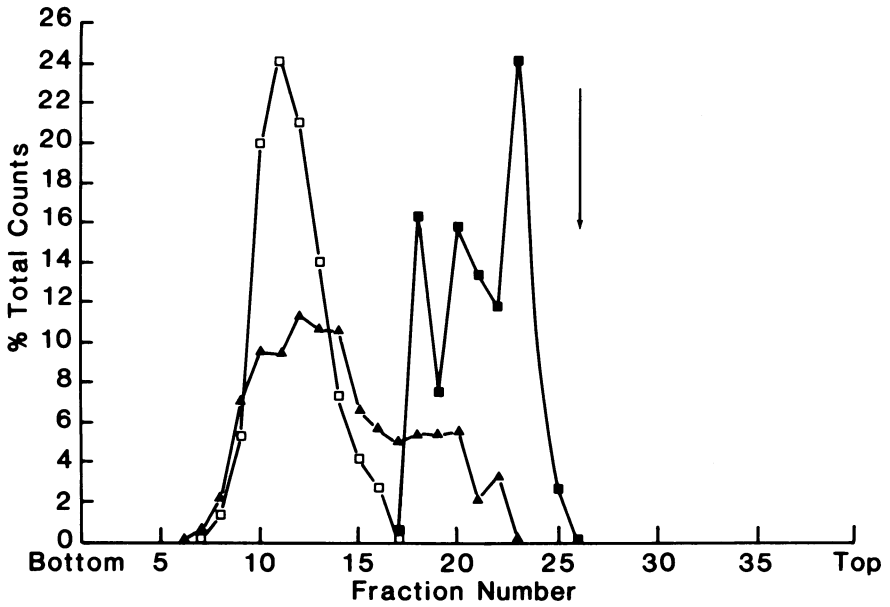

FIG. 1. Alkaline sucrose sedimentation analysis of wild-type *E. coli* W3110 cells after exposure to hydrogen peroxide. Cells were exposed to 117 mM hydrogen peroxide for 10 min and then allowed to repair for 4 h as described in Materials and Methods. □, Untreated cells; ■, 10-min exposure; ▲, 4 h postexposure. Arrow, Sedimentation of ϕX174 RFI (53S). The profile of the sedimentation indicates 482 single-strand breaks per genome after treatment and 18 remaining after repair (8).

peroxide (Fig. 2 and 3). The rate of synthesis was maximal at a peroxide concentration of around 250 mM, indicating saturation of damage. The addition of ATP slightly decreased the incorporation seen, possibly due to activation of nucleases. The properties of this DNA synthesis are given in Table 2. The response was dependent on divalent cations since no response was seen when magnesium was left out of the reaction mixture or EDTA was added. The synthesis was not inhibited by the addition of dithiothreitol or NEM and was stimulated slightly by the DNA ligase inhibitor NMN. Furthermore, this increase in DNA synthesis was not seen in strains HM1 (Fig. 2) and P3478 (data not shown), which lack a functional DNA polymerase I. These properties are consistent with the functioning of DNA polymerase I and DNA ligase in a repair pathway which is independent of ATP-dependent replication.

DNA synthesis after peroxide treatment is repair synthesis. Strain CRT266 contains a *dnaB*(Ts) mutation which renders it thermolabile for DNA replication. This strain was toluene treated and exposed to hydrogen peroxide at the permissive (32°C) and restrictive (43°C) temperatures. ATP-dependent replicative synthesis was shut off at 43°C as seen in Fig. 3. However, DNA synthesis did occur after the exposure of cells to hydrogen peroxide even under conditions in which

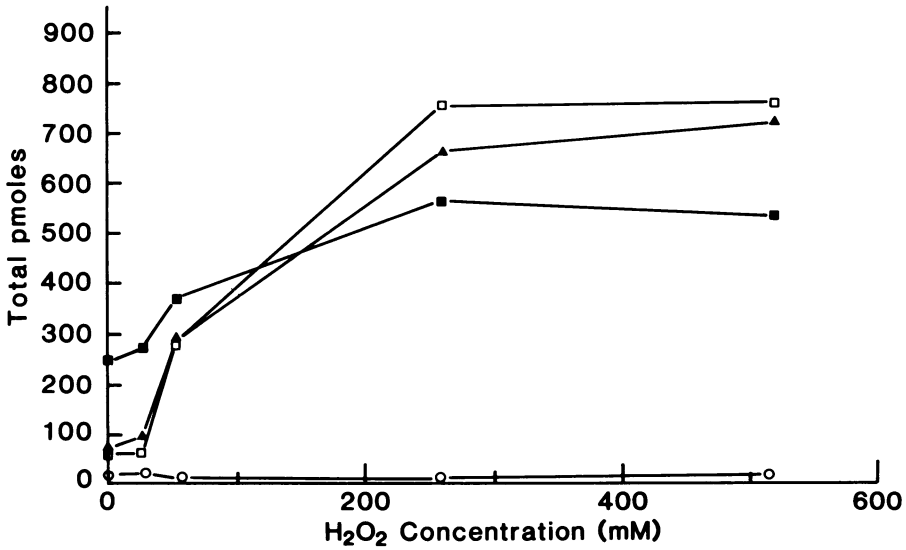

FIG. 2. Repair synthesis in toluene-treated *E. coli* cells. The reaction was performed as described in Materials and Methods. Total represents picomoles of nucleotides incorporated during a 30-min incubation. □, −ATP; ■, +ATP; ▲, +NEM; ○, HM1 (*polA1*) −ATP. □, ■: Incubation at 32°C; ○, △: incubation at 43°C.

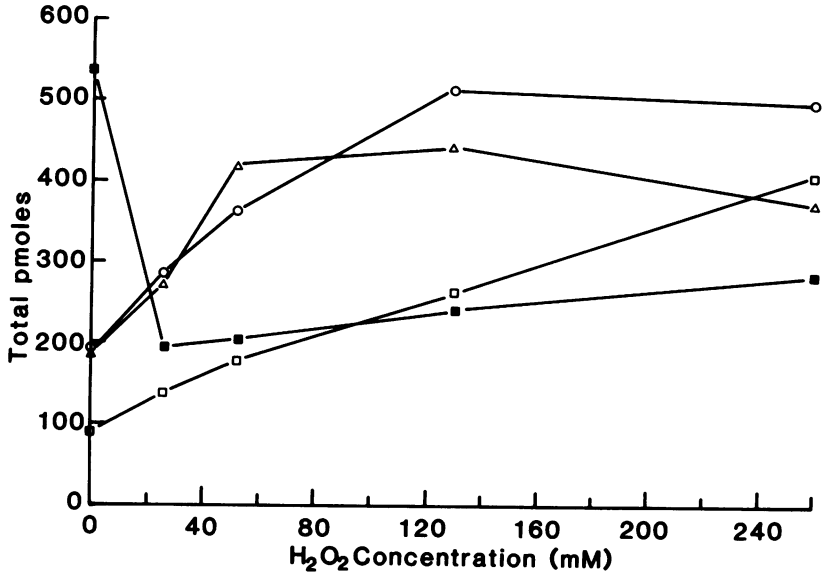

FIG. 3. Repair synthesis in toluene-treated CRT266 [*dnaB*(Ts)] cells. Conditions were as described in Materials and Methods. □, −ATP, 32°C; ■, +ATP, 32°C; ○, −ATP, 43°C; △, +ATP, 43°C.

DNA replication was absent, indicating that this DNA synthesis was caused by DNA repair. The decrease in ATP-dependent synthesis at 32°C suggests an inhibition of replication followed by increasing repair synthesis with increasing hydrogen peroxide concentrations. The increase in nonreplicative synthesis was about the same at 32 and 43°C, although the background of repair was higher at 43°C.

To confirm these findings, DNA from toluene-treated wild-type cells was analyzed by density gradient centrifugation. A profile from cells with no addition of peroxide under conditions allowing replication (37°C) is illustrated in Fig. 4A. The parental DNA was of low density with a newly replicated peak of DNA present at a hybrid density around fraction 19. The addition of hydrogen peroxide to this assay at 37°C (Fig. 4B) inhibited DNA replication and stimulated a large amount of DNA synthesis at the parental density. This confirmed that the peroxide-induced DNA synthesis was repair synthesis and demonstrated that hydrogen peroxide inhibited DNA replication.

Reformation does not require ATP. Alkaline sucrose sedimentation of DNA from toluene-treated cells was performed to determine the requirements for incision and reformation after treatment of the cells by hydrogen peroxide. A typical response to hydrogen peroxide by wild-type cells is shown in Fig. 5A. After a 10-min exposure at 37°C, incision of the DNA was observed. The DNA was reformed to a high molecular weight within 60 min after removal of the hydrogen peroxide (37°C); thus, the response appeared the same as in intact cells. Incision was not inhibited by the omission of magnesium or ATP or by the addition of EDTA, NEM, or NMN (Table 3). These properties suggest that hydrogen peroxide breaks the DNA by direct chemical means, although enzymatic means such as endonuclease III action were not ruled out conclusively.

TABLE 2. Properties of repair synthesis after exposure to 250 mM hydrogen peroxide

Reaction components	Total dNTPs incorporated (picomoles)
Complete[a]	946
+ATP	595
+NEM	780
−Mg	71
+EDTA	65
+DTT	941
+NMN	1,037

[a] The complete reaction consisted of 67 mM KPO_4 (pH 7.4), 13 mM $MgCl_2$, 33 μM of the four dNTPs, [^{3}H]dTTP (final specific activity, 20 to 50 cpm/pmol), and 10^9 toluene-treated cells for 30 min at 37°C. DTT, Dithiothreitol.

The requirements for reformation are given in Table 3. Reformation of the DNA required magnesium and was inhibited by EDTA. This step did not require ATP and was not inhibited by NEM. However, no reformation was seen when the DNA ligase inhibitor NMN was added. These properties indicated the involvement of DNA polymerase I as well as DNA ligase in the repair response of hydrogen peroxide-induced DNA damage and were in agreement with the requirements for synthesis noted above. The NEM resistance of the synthesis and reformation indicated that DNA polymerase I is the major contributor to the synthesis-reformation phase of repair.

Reformation is not seen in cells lacking DNA polymerase I. Strain HM1 lacks a functional DNA polymerase I. This strain was toluene treated and exposed to hydrogen peroxide. Incision was normal in this strain; however, no reformation was seen (Fig. 5B). The same results were obtained with a second *polA1* strain, P3478 (data not shown). This was consistent with a requirement for a functional DNA polymerase I for repair of peroxide-induced DNA damage.

Incision of supercoiled DNA. Incision of supercoiled DNA by hydrogen peroxide was measured by a filter retention assay (12). Hydrogen peroxide, in the presence of a low concentration of ferric chloride, could incise supercoiled DNA, producing 95.7% of the control retained (3.3 breaks per molecule). Hydrogen peroxide or ferric chloride alone did not cleave the DNA (17 and 15% of the DNA were retained, respectively). Copper could substitute for the iron requirement; however, cobalt or zinc could not. These data

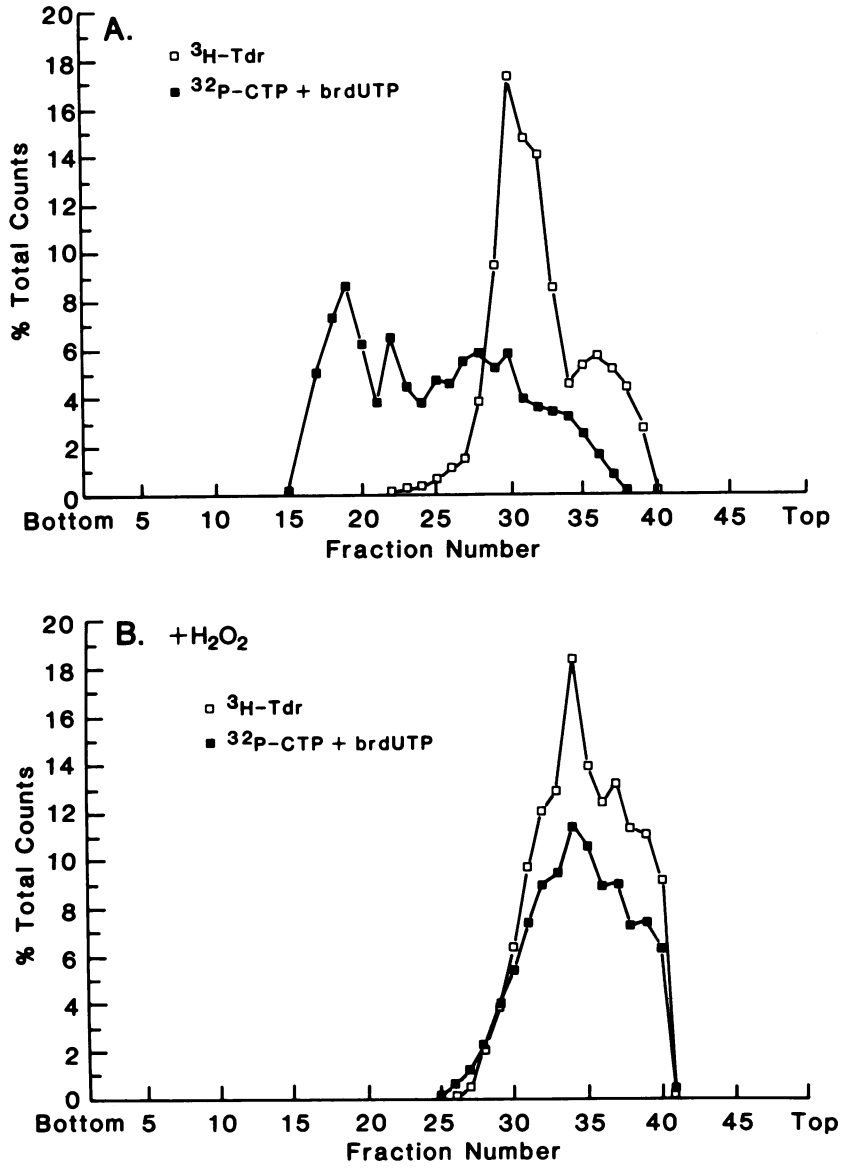

FIG. 4. Cesium chloride density analysis of toluene-treated W3110 cells. (A) Untreated cells. □, ^{3}H-labeled parental DNA, total counts 8,400; ■, ^{32}P-labeled, newly synthesized DNA, total counts 280,000. (B) Hydrogen peroxide (260 mM)-treated cells. □, ^{3}H-labeled parental DNA, total counts 15,000; ■, ^{32}P-labeled, newly synthesized DNA, total counts 180,000. Density of light peak (parental DNA), 1.726; density of hybrid peak, 1.765.

demonstrated that hydrogen peroxide can cleave DNA chemically with the addition of a heavy metal such as iron or copper and were consistent with published results (11, 17).

DISCUSSION

Investigators have related the damage produced by hydrogen peroxide to that produced by near-UV light (10, 22). In particular, near-UV irradiation of L-tryptophan produces hydrogen peroxide (1), and these two agents have been found to work synergistically (10, 22, 26). Furthermore, the adaptive response seen by treating cells with a sublethal concentration of hydrogen peroxide can also be produced by near-UV irradiation (22). Near-UV irradiation has also been shown to produce breaks in DNA (21), inhibit replication gap closure, and inhibit repair of X-ray-induced, single-strand breaks (25). The active agent in these cellular responses has been postulated to be hydrogen peroxide, and it is thought that near-UV lights, X rays, and hydrogen peroxide share common repair mechanisms. The data presented here demonstrated that hydrogen peroxide, by itself, could produce breaks in the DNA. DNA was incised after hydrogen peroxide treatment in whole cells as well as permeable cells in a process requiring no cofactors such as ATP or Mg for the incision event. The mechanism of this incision probably resulted from a modified Haber-Weiss reaction (3, 13, 23).

Hydrogen peroxide damage stimulated DNA repair synthesis in wild-type *E. coli* cells. DNA synthesis that does not require ATP and is not inhibited by NEM is caused by DNA polymerase I (9). On the other hand, DNA polymerase III repair synthesis requires ATP and is inhibited by NEM (20). The repair synthesis seen in this study was characteristic of

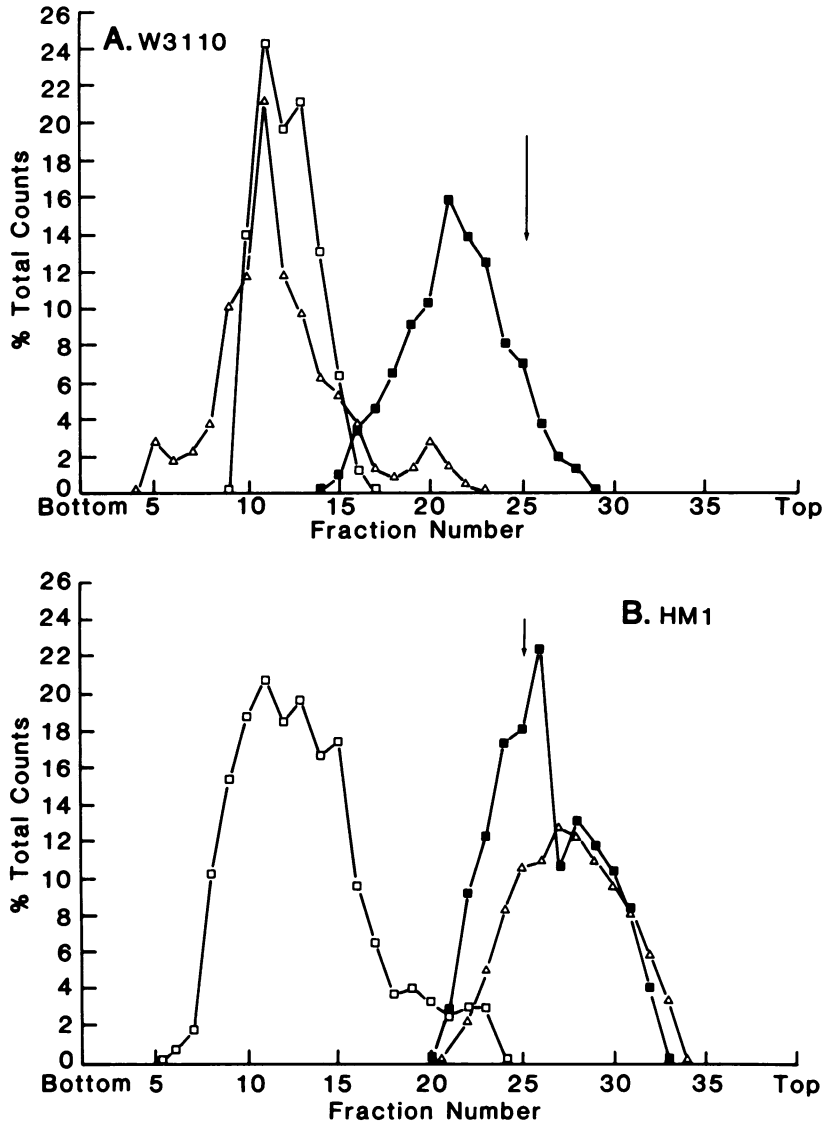

FIG. 5. Alkaline sucrose sedimentation of toluene-treated *E. coli* cells after treatment with hydrogen peroxide. (A) W3110. (B) HM1 (*polA1*). Cells were toluene treated, exposed to 70 mM hydrogen peroxide for 10 min, and then allowed to repair for 1 h as described in Materials and Methods. □, Untreated cells; ■, 10-min exposure; △, 1-h postexposure. Arrows denote the sedimentation of φX174 RFI (53S). The profiles indicate 482 single-stranded breaks per genome in strain W3110 after treatment, with none remaining after repair; 695 breaks are indicated in HM1 after treatment, with 776 postincubation.

TABLE 3. Requirements for incision and reformation of DNA from toluene-treated *E. coli* cells after exposure to 70 mM hydrogen peroxide

Reaction components	DNA incised	DNA reformed (%)[b]
Complete[a]	+	>90
−ATP	+	>90
−Mg	+	0
+EDTA	+	0
+NEM	+	>90
+NMN	+	0

[a] The complete reaction consisted of 67 mM KPO_4 (pH 7.4), 13 mM $MgCl_2$, 1.3 mM ATP, and 10^9 toluene-treated cells.

[b] >90, >90% reformation to high-molecular-weight DNA. 0, No significant change of peak from incised control.

DNA polymerase I synthesis and was not present in strains lacking a functional DNA polymerase I. In addition, reformation of high-molecular-weight DNA was not inhibited by the addition of NEM and was not present in *polA1* strains. This indicates that DNA polymerase I was the major contributor to peroxide-induced synthesis and subsequent reformation. The results presented here do not rule out a role in the response for DNA polymerase III but do demonstrate a strict requirement for DNA polymerase I.

To be determined is whether DNA polymerase I can synthesize directly after peroxide-induced incision or whether additional cellular components are required for terminus modification before polymerase I-dependent synthesis. These cellular components could be exonuclease III, DNA polymerase III, or both, since strains lacking these enzymes are quite sensitive to hydrogen peroxide (7; unpublished data).

Cells with a temperature-sensitive mutation for DNA polymerase III are sensitive to the lethal effects of peroxide at the restrictive temperature and cannot reform high-molecular-weight DNA after peroxide treatment (unpublished data). From the data reported here, no synthesis role for DNA polymerase III was seen. DNA polymerase III may be required for a small amount of synthesis which was not detectable in the repair synthesis assay used. On the other hand, DNA polymerase III may function in the repair of hydrogen peroxide-induced DNA damage in a role outside actual synthesis, perhaps by providing a terminus modification function for subsequent DNA polymerase I synthesis.

ACKNOWLEDGMENTS

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