

## Effect of Bis( $\beta$ -chloroethyl)sulfide (BCES) on Base Mismatch Repair of DNA in Monkey Kidney Cells<sup>1</sup>

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Effects of Bis( $\beta$ -chloroethyl)sulfide (BCES) on Base Mismatch Repair of DNA in Monkey Kidney Cells. FAN, L., AND BERNSTEIN, I. A. (1991). *Toxicol. Appl. Pharmacol.* **111**, 233-241. Sulfur mustard, bis( $\beta$ -chloroethyl)sulfide (BCES), a bifunctional alkylating agent, is a vesicant whose mode of action involves interference with the integrity of cellular DNA. Alkylation of DNA is responsible for some of the biological effects of BCES in tissue. Another possible mechanism by which BCES could exert its toxic effect is interference with high fidelity repair of damaged DNA. This study evaluated the possible effects of BCES on the repair of specific errors, i.e., mismatched bases, in the DNA. Heteroduplex (ht) DNA, formed between two temperature-sensitive mutants of SV40 virus, tsA239 and tsA255, each having a different point mutation in the gene for large T antigen, was used to study the effect of BCES on mismatched base repair in African green monkey kidney (AGMK) cells. AGMK cells were exposed to dilute solutions of BCES in methylene chloride (MC) prior to cationic lipofection with ht DNA. In order for the cells to produce wild type (wt) SV40 DNA at a nonpermissive temperature (41°C), repair of at least one of the two mismatches in the DNA had to occur. It was observed that (a) as the concentration of BCES was increased, a proportionally longer delay in the appearance of wt DNA at 41°C was observed in treated cells transfected with ht DNA as compared with cultures exposed to MC alone and then transfected with ht DNA, (b) there was no such effect in exposed AGMK cells transfected with wt DNA, (c) wt and ht DNA were transfected at similar rates in unexposed cells, and (d) BCES did not affect the rate of transfection of wt cells. These observations are consistent with the hypothesis that BCES affects mismatched base repair. © 1991 Academic Press, Inc.

Sulfur mustard, bis( $\beta$ -chloroethyl)sulfide (BCES), a bifunctional alkylating agent, has mutagenic (Fox and Scott, 1980; Wheeler, 1962), teratogenic, carcinogenic, and vesicant (blistering) properties (Auerbach and Robson, 1946; Heston, 1953), which makes it a good

model chemical for studying the nature of vesication in cutaneous keratinocytes. Alkylation of DNA is responsible for some of the biological effects in tissue exposed to BCES (Ross, 1962). The mode of vesication involves interference with the integrity of cellular DNA (Papirmeister *et al.*, 1985). The cytotoxic action of BCES could include the inhibition of DNA synthesis (Roberts *et al.*, 1986; Ku, 1987; Ribeiro, 1988; Vaughan *et al.*, 1988) which may result from the existence of unrepaired interstrand crosslinks in the DNA that interfere with template function (Brookes and Lawley, 1963; Connors, 1975).

Recovery of the rate of DNA synthesis is associated with the removal (probably spon-

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taneous and enzymatic) of diguanyl crosslinks and monofunctional alkylation products (Roberts, 1978). Repair synthesis is responsible for the loss of alkylation products in both bacterial (Schjelderup *et al.*, 1972) and mammalian cells (Roberts *et al.*, 1968; Roberts *et al.*, 1971).

The structural integrity of DNA is compromised when monolayer cultures of keratinocytes are exposed to low levels of BCES—probably by the insertion of single strand breaks (Ribeiro, 1988). Although single strand breaks can be repaired (Sorscher and Conolly, 1989), and all breaks may indeed be repaired after low-level exposure (Ribeiro, 1988), the epidermal cultures do not survive (Ku and Bernstein, 1988). It is possible that in such exposed cells, repair of single strand breaks occurs but the integrity of the DNA is not completely restored. Perhaps repair is inefficient and the DNA is not repaired with sufficiently high fidelity to maintain viability. Cells may die because of the presence of an informational error in the DNA related to development.

Mismatch repair, which recognizes and processes base pairing errors within the DNA helix, seems to play a major role in error-free repair. Interference with mismatch repair could lead to the presence of errors in the DNA and might explain the failure of exposed keratinocytes to survive. The objective of this study was to determine the effect of BCES on base mismatch repair of heteroduplex (ht) DNA in African green monkey kidney (AGMK) cells, a permissive host for the virus.

## METHODS

*Cell culture.* BSC-1 and BSC-40 lines of AGMK were obtained from William Brockman.<sup>4</sup> BSC-40 cells were derived from the BSC-1 line by selection for growth at 40°C after long incubation (Brockman *et al.*, 1973). BSC-1 and BSC-40 lines of AGMK cells were routinely maintained

on MEM10<sup>5</sup> at 37°C in a humidified 5% CO<sub>2</sub>-95% air environment. For adaptation, BSC-40 cells were incubated at 41°C for a day before subcultivation (Brockman *et al.*, 1973).

*SV40 virus strains.* Wild type (wt) strain 776 of SV40 virus was also obtained from Dr. Brockman. Two temperature-sensitive mutants of SV40 virus, tsA239 and tsA255 (Chou and Martin, 1974), were acquired from Robert G. Martin. Both mutants have a C to G transversion in the gene for large T antigen. The two sites are separated by 86 bases. Each point mutation results in the substitution of a cysteinyl residue for a tryptophanyl residue in a large T antigen protein which is sufficiently temperature labile that the protein is nonfunctional at 41°C while being functional at 33°C. Large T antigen binds at the SV40 origin of replication and promotes the replication of the SV40 genome. A functional large T antigen is absolutely necessary for viral DNA replication. Replication of viral DNA cannot occur at the nonpermissive temperature, 41°C, after infection with either mutant (Chou and Martin, 1974). Viral stocks were prepared as described by Clark and Hanawalt (1984) and stored in small aliquots at -70°C. Plaque assays as described by Cooper (1967) were performed to determine the titer of SV40 viral stocks.

*Preparation of supercoiled viral DNA.* Confluent BSC-1 cells were infected at 2 plaque-forming units/cell with wt, tsA239, or tsA255 SV40 virus in MEM2.<sup>5</sup> The cells were harvested by lysis according to Hirt (1967). Cellular chromosomal DNA was precipitated in 1 M NaCl. The supernatant solution was treated with RNase (DNase free) and protein was removed by extraction with phenol (Danna and Nathans, 1971). The upper aqueous phase was collected by centrifugation and viral DNA was precipitated in ethanol (Danna and Nathans, 1971). The pellet was dried in vacuum and was dissolved in 1× TE buffer, pH 7.4.<sup>6</sup>

Supercoiled DNA was further purified by centrifugation to equilibrium in a gradient of cesium chloride (Radloff *et al.*, 1967). Ethidium bromide was removed by extraction with isopropanol. Dialysis was carried out overnight at 4°C against 0.1× TE buffer, pH 7.4. To quantify the amount of purified DNA, spectrophotometric readings at 260 and 280 nm (Maniatis *et al.*, 1982) were made using a Varian Cary 219 spectrophotometer. An absorbance of

<sup>5</sup> Complete MEM10 was made by supplementing Eagle's Minimum Essential Medium (MEM; Hyclone Laboratories, Logan, UT) with 10% fetal bovine serum (FBS; Flow Laboratories, MacLean, VA), 100 IU/ml penicillin G, and 100 µg/ml streptomycin. MEM2 contained 2% FBS and the same concentrations of penicillin and streptomycin as in MEM10. To avoid losing samples because of fungal contamination during experiments, 0.25 mg/ml Fungizone was also added.

<sup>6</sup> 1× TE buffer consisted of 10 mM Tris-HCl, pH 7.4, and 1 mM EDTA, pH 8.0 (Maniatis *et al.*, 1982); 0.1× TE buffer was a 1:10 dilution of 1× TE.

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1.00 corresponded to approximately 50  $\mu\text{g/ml}$  for double-stranded DNA. The ratio between the readings at 260 and 280 nm ( $A_{260}/A_{280}$ ) provided an estimate of the purity of DNA. Pure preparations of DNA have been reported to have  $A_{260}/A_{280}$  of 1.8 (Maniatis *et al.*, 1982).

**Agarose gel electrophoresis.** Agarose gel electrophoresis was performed with a Bio-Rad DNA subgel electrophoresis system in  $1\times$  TBE<sup>7</sup> containing ethidium bromide at 0.5  $\mu\text{g/ml}$  (Maniatis *et al.*, 1982). The desired concentration of agarose gel was made in the same solution. DNA samples were loaded in gel-loading buffer ( $1\times$ ) (Maniatis *et al.*, 1982). Fluorescent bands which result from the binding of ethidium bromide to DNA and appear under irradiation from long wave uv light served to locate the nucleic acid on the electrophoretic gel.

**Preparation of heteroduplex DNA.** Heteroduplex DNA with two mismatched base pairs within the gene for the large T antigen was constructed from tsA239 and tsA255 of SV40 virus essentially as described by Peden and Pipas (1985). The steps in the process were monitored by electrophoresis using 1% agarose gels.

First, supercoiled DNA from tsA239 was linearized with *EcoRI* restriction endonuclease and supercoiled DNA from tsA255 was linearized with *BamHI* restriction endonuclease (Maniatis *et al.*, 1982). Restriction enzymes were removed by extraction, in sequence, with phenol, a 1:1 mixture of phenol and chloroform, and chloroform (Maniatis *et al.*, 1982).

Second, *EcoRI*-digested tsA239 DNA was mixed with *BamHI*-digested tsA255 DNA in 9 parts of double-distilled  $\text{H}_2\text{O}$ , denatured by addition of 1 part of 1 M NaOH (final concentration, 0.1 M) and incubation at 37°C for 10 min, and renatured by addition of 2 parts of neutralizing solution (2:1, v/v, 1 M Tris-HCl, pH 7.2:1 M HCl) followed by incubation at 61°C. This mixture was cooled slowly to 4°C. The DNA was precipitated by addition of 24 parts of absolute ethanol and recovered by centrifugation. The DNA was then dissolved in  $1\times$  TE buffer, pH 7.4, and electrophoresed. The yield was 70% after denaturation, 40% after renaturation, and 30% overall, as determined spectrophotometrically. A parallel preparation was carried out with wt DNA.

**Determination of BCES concentration.** Since the solvent vehicle, methylene chloride (MC), of a stock solution of BCES (U.S. Army Medical Institute of Chemical Defense) was highly volatile, the actual concentrations of BCES used were determined by including a trace level of [<sup>14</sup>C]BCES (U.S. Army Medical Institute of Chemical Defense) when the stock solution was prepared and counting the radioactivity in a small aliquot (15  $\mu\text{l}$ ) of the stock solution in 5 ml of toluene-based scintillation solution, immediately before each experiment.

**Exposure of cells to BCES.** Because BCES has a short half-life in aqueous medium and the number of dishes to be exposed at the same time had to be limited, one experiment with several concentrations of BCES was divided into several subexperiments. A single dose of BCES was employed in each subexperiment and the whole process of diluting and adding was completed in 2 min. To avoid possible variation, the same batches of ht DNA and confluent BSC-40 cells at the same number of passages were always used.

A small aliquot was taken from a stock solution of BCES (10 mg/ml) in MC and diluted with PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (PBS-CM) (0.8% NaCl, 0.02% KCl, 0.115%  $\text{Na}_2\text{HPO}_4$ , 0.02%  $\text{KH}_2\text{PO}_4$ , 0.01%  $\text{CaCl}_2$ , and 0.01%  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) to make a working stock solution. Dilutions were made again when an aliquot of this working stock solution was delivered to the dishes where a definite amount of PBS-CM was present. The exposure of confluent BSC-40 cells to BCES was done at 35°C for 30 min in 5%  $\text{CO}_2$ :95% air. Since the stock solution of BCES was in MC and MC had been shown (data not presented) to have an adverse effect on mismatch repair, the concentration of MC in all dishes including the solvent controls was adjusted to that of the MC present at the highest dose of BCES, i.e., 0.00625% in PBS-CM (vol/vol). BSC-40 cultures were washed with warm PBS-CM both prior to and after exposure to BCES. BSC-40 cells were then allowed to recover in MEM10 for 1 hr at 35°C before further processing.

**Lipofection and detection of new viral DNA.** Cationic liposome-mediated transfection (lipofection) was used to introduce SV40 viral DNA into BSC-40 cells (Felgner and Holm, 1989). DNA and lipofectin reagent [1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE) in membrane-filtered water (Bethesda Research Laboratories, Gaithersburg, MD)] were diluted in 1.5 ml of Opti-MEM I, separately to avoid precipitation, and combined. Confluent cultures of BSC-40 cells were washed with warm Opti-MEM I<sup>8</sup> immediately before 3 ml of the DNA/lipofectin complex in Opti-MEM I was added to each dish. After incubation at 37°C for 24 hr, 3 ml of warm MEM20<sup>9</sup> was added to arrest the transfection and the cells were transferred to a 41°C incubator.

Cells were radiolabeled at 37°C for 8 hr with [<sup>3</sup>H]thymidine (82 Ci/mmol, Amersham, Arlington Heights, IL) in MEM10 (2  $\mu\text{Ci/ml}$ ) every 8 hr for 96 hr and harvested after lysis (Hirt, 1967). The viral DNA was isolated by salt precipitation of chromosomal DNA and

<sup>8</sup> 2-Mercaptoethanol was added to Opti-MEM I immediately prior to use to enhance the stability of the medium.

<sup>9</sup> MEM20, which was used to arrest lipofection, contained 20% FBS, 200 units/ml penicillin G, 200 mg/ml streptomycin, and 0.5 mg/ml Fungizone.

<sup>7</sup> TBE buffer consisted of 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA, pH 7.6 (Maniatis *et al.*, 1982).

purified by digestion with RNase, extraction with phenol, and precipitation with ethanol as stated above. After sedimentation by centrifugation, the DNA was washed with cold 80% ethanol and dried in vacuum. The pellet was dissolved in a small amount of DNA standard solution which contained nonradioactive supercoiled wt DNA and gel-loading buffer in double-distilled H<sub>2</sub>O.

Purified DNA samples were applied to 1% agarose gels and electrophoresis was carried out at 60 V in 1× TBE buffer, pH 7.6, which contained 0.5 μg/ml ethidium bromide. The gel was then sliced. Each gel slice was melted and digested overnight at 60°C in TS-2 and the [<sup>3</sup>H] was counted in toluene-based scintillation cocktail (0.5% PPO and 0.05% dimethyl-POPOP).

The band of supercoiled DNA was identified on the agarose gel by coincidence of radioactivity in newly synthesized material and fluorescence (under long wavelength ultraviolet light) of nonradioactive standard wt supercoiled DNA. By this method, new supercoiled SV40 viral DNA was found to appear approximately at gel slice 16. Three micrograms of DNA and 30 μl of lipofectin gave an optimal production of new supercoiled SV40 DNA.

## RESULTS

*Construction of ht DNA.* Figure 1 is a photograph of a 1% agarose gel electrophoretic pattern of samples taken from the various steps through the construction procedure. Linear single-stranded DNA migrated fastest with supercoiled or superhelical (Form I) DNA following. Linear double-stranded (Form III) DNA ran third fastest and nicked circular double-stranded (Form II) DNA ran most slowly.

During renaturation, some strands of tsA239 probably annealed to strands from tsA255 since they are homologous except for 2 of 5243 base pairs. Because the mutants were linearized by cutting the strands at different sites, annealing between strands from the different mutants generated nicked circular molecules or multimers. In contrast, reannealing of strands from the same mutant yielded the original linear molecules. A concentration of 1 μg/ml of DNA and a renaturation time of 2 hr at 61°C were determined to be optimal, to avoid formation of circular multimers and to favor the formation of ht DNA, and these conditions were subsequently used in making ht DNA.

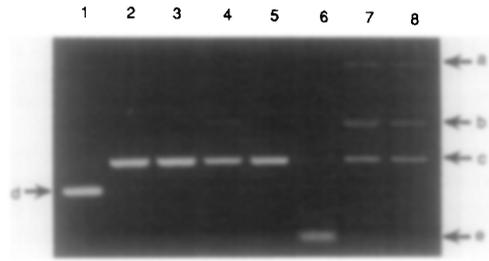


FIG. 1. Analysis of the preparation of heteroduplex DNA by gel electrophoresis on 1% agarose. tsA239 and tsA255, each bearing a single mutation in the T-Ag gene, were linearized with *EcoRI* and *BamHI*, respectively. The linearized DNA strands were mixed, denatured, and renatured. Wild type (wt) DNA was treated similarly as a comparison. Lane 1, Form I DNA of wt, tsA239, or tsA255. Lane 2, wt *EcoRI*. Lane 3, wt *BamHI*. Lane 4, tsA239-*EcoRI*. Lane 5, tsA255-*BamHI*. Lane 6, wt *EcoRI*, wt *BamHI*, tsA239-*EcoRI*, or tsA255-*BamHI* after a long storage time (2 weeks). Lane 7, wt *EcoRI* and wt *BamHI* mixed, denatured, and renatured. Lane 8, tsA239-*EcoRI* and tsA255-*BamHI* mixed, denatured, and renatured. Arrows indicate: a, origin of gel; b, Form II DNA (nicked circular double-stranded); c, Form III DNA (linear double-stranded); d, Form I DNA (supercoiled); and e, linear single-stranded DNA.

Denaturation and renaturation of linear strands from *EcoRI*-digested wt DNA and *BamHI*-digested wt DNA yielded only homoduplex molecules, in either linear or circular forms.

Ligation of nicks generated during the preparation of ht DNA was not carried out because ligation in the host has been reported to be very efficient when DNA molecules are transfected into vertebrate cells (Wake *et al.*, 1984).

*Effects of BCES on base mismatch repair and viral DNA replication.* To study the effect of BCES on mismatch repair in BSC-40 cells, the confluent cultures were first exposed for 30 min at 35°C to 0.1, 0.5, 1.0, or 5.0 μM BCES in PBS-CM. After the cultures were allowed to recover in MEM10 for 1 hr, transfection of cells with ht DNA was performed. Repair of either one of the mismatched base pairs in the gene that codes for large T antigen in ht DNA was necessary before the replication of the whole viral DNA could be initiated at

41°C. The mismatch repair that occurred in the culture at 41°C was monitored by observing the formation of tritiated supercoiled viral DNA during the periods when [<sup>3</sup>H]thymidine was present in the medium. At the same time, in order to answer the question of whether BCES affected the replication of repaired heteroduplex molecules rather than mismatch repair per se, some cultures of exposed BSC-40 cells were transfected with homoduplex molecules formed between *Eco*RI-digested wt DNA and *Bam*HI-digested wt DNA. In this case, mismatch repair was not a prerequisite for the replication of viral DNA at 41°C. Each experiment was repeated once and similar results were observed.

Figure 2 presents three sets of representative data in three graphs (A, B, C) showing the effects of exposure to 0.5, 1.0, and 5.0 μM BCES, respectively, on mismatch repair (i.e., when ht DNA was transfected) in BSC-40 cells. Figure 2D shows the effect of exposure to 5 μM BCES on viral replication per se (i.e., when wt DNA was transfected). No effect was observed on mismatch repair after exposure to 0.1 μM BCES (data not shown). The data shown in 2D are similar to those seen for replication of viral DNA in cells exposed to 0.1, 0.5, and 1.0 μM BCES and transfected with wt DNA (data not shown). Since subexperiments employing the same dose of BCES were done in sequence over a period of several days, the numbers and ages of the cells in the cultures utilized were somewhat different. For this reason a control was included for each time point in every experiment. Each time point represents the total <sup>3</sup>H in cpm for two gel slices at the band of new supercoiled DNA. The time of delay in the appearance of new viral DNA which occurred after an exposure of BCES was determined by extrapolation of the lines manually drawn for the control and experimental data observed after 40 hr.

The intercepts at the abscissa in Figs. 2A, 2B, and 2C were significantly (statistical treatment not shown) different for exposed and nonexposed cells. As the level of exposure to the mustard was increased, the intercept

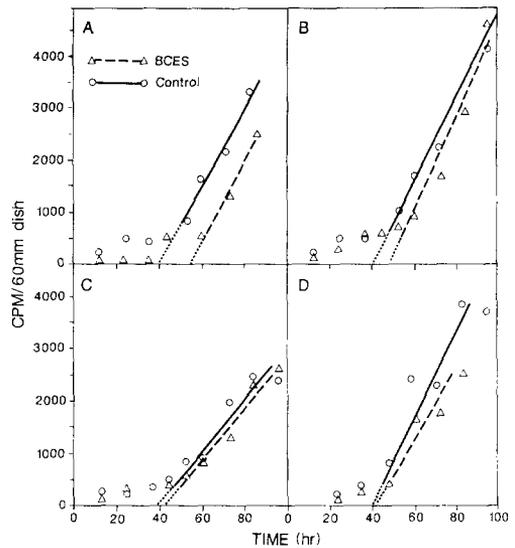


FIG. 2. Time course of adverse effects of bis( $\beta$ -chloroethyl)sulfide on mismatch repair. Confluent cultures were exposed to various doses (A and D, 0.5  $\mu$ M; B, 1.0  $\mu$ M; C, 5.0  $\mu$ M) of BCES/MC in PBS-CM for 30 min at 35°C. For each time point, control cultures were prepared. In all cases, the medium was adjusted to 0.00625% (v/v) with respect to MC. After exposure, cultures were washed with PBS-CM. Transfection of ht (tsA239/tsA255) (A, B, C) or wt (D) DNA was performed by lipofection at 37°C for 24 hr after cultures were allowed to recover in MEM10 for 1 hr at 35°C. Cultures were moved to 41°C and were radiolabeled every 8 hr with [<sup>3</sup>H]thymidine (2  $\mu$ Ci/ml) for 8 hr until 96 hr post-transfection. Viral DNA was isolated and purified. Gel electrophoresis on agarose was performed and the <sup>3</sup>H in each gel slice was counted. Each point represents the total counts of two gel slices at the band of Form I supercoiled DNA.

showed a longer delay in the appearance of new viral DNA as compared with control. While no delay was observed after exposure to 0.1 μM of BCES (data not shown), about 5, 10, and 15 hr of delay, respectively, were seen after 0.5, 1.0, and 5.0 μM of BCES. However, the rate of appearance of new viral DNA was not markedly different in exposed cells as compared with control cells once the period of delay in exposed cells had passed.

There was no difference in the intercepts of the curves in Fig. 2D although the rate at which new viral DNA appeared might have been slightly lower in the exposed cells.

*Demonstration that both mutants were not leaky at the nonpermissive temperature (41°C).* The results shown in Fig. 3 clearly indicate that under conditions which led to the appearance of new viral DNA at 41°C in cells that had been transfected with ht DNA no viral DNA appeared in cells transfected with homologous DNA of either tsA239 or tsA255.

## DISCUSSION

The enzymatic repair of mismatched bases in DNA (Lai and Nathans, 1974, 1975) has been identified in bacteria, fungi, and mammalian cells (Folger *et al.*, 1985; Hare and Taylor, 1985; Glazer *et al.*, 1987; Modrich, 1989; Lahue *et al.*, 1989). The existence of this repair system made possible the technique of marker rescue for mapping mutants by localizing the mutational sites to the nucleotide sequences contained within the active fragment of wt virus (Mantei *et al.*, 1975). Independent correction of mismatched base pairs can occur if the mismatches are separated by 13 or more base pairs (Lai and Nathans, 1975). This technique was also used to establish the proximity of two mutations. Much effort has been expended to elucidate the molecular mechanism of mismatch repair in *Escherichia coli* and mammalian cells although the details of the process are still not clear.

Enzymatic repair of alkylated DNA appears to involve excision of the alkylated bases leading to the presence of apurinic and apyrimidinic sites in the nucleic acid (cf., Sancar and Sancar, 1988). This step is followed by endonucleolytic and exonucleolytic activities to complete the excision of the damaged nucleotide and DNA polymerase and ligase activities to insert an undamaged nucleotide. Apparently, the presence of apurinic sites can lead to the presence of base mismatches in the DNA (Schaaper *et al.*, 1983), so the mismatch repair system could be especially important for the survival of a cell exposed to an alkylating agent such as BCES. BCES-mediated damage to the mismatch repair system might

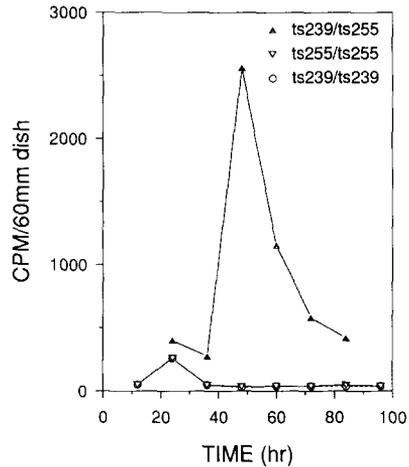


FIG. 3. Time-course of production of new viral DNA at 41°C in BSC-40 cells which were transfected at 37°C with homoduplex DNA of tsA239/tsA239 or tsA255/tsA255 or ht DNA tsA239/tsA255. Homoduplex DNA of tsA239/tsA239 was prepared by denaturing and renaturing together *Eco*RI-digested and *Bam*HI-digested tsA239. Homoduplex DNA of tsA255/tsA255 was formed in the same manner. Transfection was performed by lipofection at 37°C for 24 hr. Cultures were moved to 41°C and were radiolabeled every 12 hr with [<sup>3</sup>H]thymidine for 12 hr until 96 hr post-transfection. Ht DNA of tsA239/tsA255 was used as a positive control. Viral DNA was isolated and purified. Gel electrophoresis on agarose was performed and the <sup>3</sup>H in each gel slice was counted. Each point represents the total counts of two gel slices at the band of Form I DNA.

explain why an exposed culture of keratinocytes was reported (Ku and Bernstein, 1988) to show abnormal loss of viability under conditions in which repair of gross damage to cellular DNA had occurred (Ribeiro, 1988). Although the presence of crosslinks in DNA would be expected to cause cytotoxicity, it appears unlikely that residual crosslinks can explain the demise of a culture 7 days after exposure to 1 μM BCES when excision of diguanyl adducts (i.e., crosslinks) is known to occur, the acute loss of gross structural integrity of the DNA is restored within 24 hr after exposure to the mustard, and DNA replication resumes in about 65% of the cells by 3 days postexposure.

Mismatch repair in AGMK cells was documented (Brown and Jiricny, 1988; Brown *et*

*al.*, 1989; Jiricny *et al.*, 1988) as very efficient. Many types of base/base mismatches have been shown to be corrected in the CV-1 line of AGMK, albeit with different efficiencies and specificities (Brown and Jiricny, 1988). Mismatches consisting of G/G and C/C were corrected with 92% and 66% efficiencies, respectively.

This appears to be the first report in which mismatch repair has been used as an *in vitro* system for evaluating toxicity and for elucidating mechanisms of toxic action. Despite the sensitivity of the nucleoid sedimentation assay for the detection of DNA damage (Ribeiro, 1988) such as single strand breaks in the DNA and of the alkaline DNA unwinding assay for the level of double-stranded DNA (Sorscher and Conolly, 1989), these analytical procedures are not specific for any particular mechanism of repair and cannot distinguish between the different lesions from which single strand breaks result. The technique employed in the present investigation utilized a viral probe with two known mismatched bases thereby allowing investigation of a highly specific repair function.

The viral probe used in this study was a mixture of heteroduplex and homoduplex DNA molecules in circular and linear form, respectively. Because of the different sites at which the DNA of the two mutant forms of SV40 virus were linearized, annealing between strands from the two different mutants resulted in circular molecules with one single strand break (nick) at a different site on each strand. In contrast, reannealing of strands from the same mutant yielded the original linear molecules. All the heteroduplex molecules which were formed had two transversions in their DNA. One of the mismatched bases was on each of the two strands. In the homoduplex molecules of either mutant DNA, there were no base mismatches. Therefore, only the heteroduplex molecules were active in the mismatch repair assay. In confirmation of this point, transfection with homoduplex molecules from either mutant resulted in no synthesis of new viral DNA (Fig. 3). The dem-

onstration that neither mutant was leaky at the nonpermissive temperature also argues against the possibility that repair occurred by the recombination mechanism rather than by base mismatch.

The formation of wt virus from ht DNA molecules at a nonpermissive temperature could occur as a result of the repair of either mismatch since each of the two mismatched bases was on different strands of the DNA separated by 86 bases and a correction of either mismatched base would result in one strand having the correct sequence for wt DNA.

The dose-responsive increase in the delay before new viral DNA appeared (i.e., as indicated by the position of the intercept with the abscissa in Figs. 2A, 2B, and 2C) in cells exposed to BCES and then transfected with ht DNA suggested that the repair process was sensitive to the mustard. The absence of an effect in exposed cells transfected with wt DNA confirmed that the repair process rather than the replication process was the target of BCES. This conclusion is strengthened by the observations that transfection of wt and ht DNA occurred in the same length of time (data not shown) and new viral DNA appeared in the same time after transfection with wt DNA whether or not the cells were exposed to BCES (Fig. 2D).

In drawing the lines whose extrapolations to the abscissa define the time when new viral DNA was first observed (Fig. 2), the data obtained prior to 40 hr after the time of transfection were ignored. This was done because no viral DNA was detected by fluorescence at the early time points, suggesting that the low level of  $^3\text{H}$  found at these times represented "spillover" from an adjacent unidentified radioactive gel band which appeared early after transfection.

The mechanism by which BCES works to affect mismatch repair in BSC-40 cells is presently unknown. Perhaps, it does so by interfering with recognition of mismatched base pairs or by decreasing the fidelity of repair synthesis.

The BSC-40 line of AGMK cells has a fibroblast origin and is a natural host of SV40 virus replicating the viral DNA in large quantity. It was for this reason that this cell line was used for the present study. However, vesication in skin exposed to BCES involves epithelial cells. In order to verify that interference with mismatch repair plays a role in BCES-mediated cutaneous vesication, it is necessary to apply the same viral technology to cultures of primary human keratinocytes which are semipermissive for SV40 virus.

## REFERENCES

- AUERBACH, P., AND ROBSON, J. M. (1946). Chemical production of mutations. *Nature (London)* **157**, 157.
- BROCKMAN, W. W., LEE, T. N. H., AND NATHANS, D. (1973). The evolution of new species of viral DNA during serial passage of simian virus 40 at high multiplicity. *Virology* **54**, 384-397.
- BROOKES, P., AND LAWLEY, P. D. (1963). Effects of alkylating agents on T2 and T4 bacteriophage. *Biochem. J.* **89**, 138-144.
- BROWN, T. C., AND JIRICNY, J. (1988). Different base/base mispairs are corrected with different efficiencies and specificities in monkey kidney cells. *Cell* **54**, 705-711.
- BROWN, T. C., ZBINDEN, I., CERUTTI, P. A., AND JIRICNY, J. (1989). Modified SV40 for analysis of mismatch repair in simian and human cells. *Mutat. Res.* **220**, 115-123.
- CHOU, J. Y., AND MARTIN, R. G. (1974). Complementation analysis of simian virus 40 mutants. *J. Virol.* **13**, 1101-1109.
- CLARK, J. M., AND HANAWALT, P. C. (1984). Replicative intermediates in UV-irradiated simian virus 40. *Mutat. Res.* **132**, 1-14.
- CONNORS, T. A. (1975). Mechanisms of action of 2-chloroethylamine derivatives, sulfur mustard, epoxides and aziridines. In *Handbook of Experimental Pharmacology: Antineoplastic and Immunosuppressive Agents II* (A. C. Sartorelli and D. G. Johns, Eds.), pp. 18-34. Springer-Verlag, Berlin.
- COOPER, P. D. (1967). The plaque assay of animal viruses. *Methods Virol.* **3**, 243-311.
- DANNA, K., AND NATHANS, D. (1971). Specific cleavage of simian virus 40 DNA by restriction endonuclease of *Hemophilus influenzae*. *Proc. Natl. Acad. Sci. USA* **68**, 2913-2917.
- FELGNER, P. L., AND HOLM, M. (1989). Cationic liposome-mediated transfection. *Focus* **11**, 21-25.
- FOLGER, K. R., THOMAS, K., AND CAPECCHI, M. R. (1985). Efficient correction of mismatched bases in plasmid heteroduplexes injected into cultured mammalian cell nuclei. *Mol. Cell. Biol.* **5**, 70-74.
- FOX, M., AND SCOTT, D. (1980). The genetic toxicology of nitrogen and sulfur mustard. *Mutat. Res.* **75**, 131-168.
- GLAZER, P. M., SARKAR, S. N., CHISHOLM, G. E., AND SUMMERS, W. C. (1987). DNA mismatch repair detected in human cell extracts. *Mol. Cell. Biol.* **7**, 218-241.
- HARE, J. T., AND TAYLOR, D. (1985). One role of DNA methylation in vertebrate cells is strand discrimination in mismatch repair. *Proc. Natl. Acad. Sci. USA* **82**, 7350-7354.
- HESTON, W. R. (1953). Occurrence of tumors in mice injected subcutaneously with sulfur mustard and nitrogen mustard. *J. Natl. Cancer Inst.* **14**, 131-140.
- HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**, 365-369.
- JIRICNY, J. B., RUDKIN, B., BROWN, T. C., AND CORMAN, N. (1988). Correlation between the substrate specificity of a HeLa mismatch binding protein and the efficiency of mismatch repair in vivo. In *Mechanism and Consequences of DNA Damage Processing* (E. Friedberg and P. Hanawalt, Eds.), UCLA Symp. Mol. Cell. Biol., New Series, Vol. 83, pp. 273-277. Liss, New York.
- KU, W. W. (1987). Bis-(2-chloroethyl)sulfide (BCES)-induced changes in proliferation and differentiation using primary cultures of cutaneous keratinocytes from the newborn rat. Doctoral Thesis. The University of Michigan. Ann Arbor, MI.
- KU, W. W., AND BERNSTEIN, I. A. (1988). Bis-(2-chloroethyl)sulfide (BCES)-induced changes in epidermal cell homeostasis in vitro. *Toxicol. Appl. Pharmacol.* **95**, 397-411.
- LAHUE, R. S., AU, K. G., AND MODRICH, P. (1989). DNA mismatch repair in a defined system. *Science* **245**, 160-164.
- LAI, C. J., AND NATHANS, D. (1974). Mapping temperature-sensitive mutants of simian virus 40: Rescue of mutants by fragments of viral DNA. *Virology* **60**, 466-475.
- LAI, C. J., AND NATHANS, D. (1975). A map of temperature-sensitive mutants of simian virus 40. *Virology* **66**, 70-81.
- MANIATIS, T., FRITSCH, E. F., AND SAMBROOK, J. (1982). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- MANTEI, N., BOYER, H. W., AND GOODMAN, H. M. (1975). Mapping simian virus 40 mutants by construction of partial heterozygotes. *J. Virol.* **16**, 754.
- MODRICH, P. (1989). Methyl-directed DNA mismatch correction. *J. Biol. Chem.* **264**, 6597-6600.
- PAPIRMEISTER, B., GROSS, C. L., MEIER, H. L., PETRALI, J. P., AND JOHNSON, J. B. (1985). Molecular basis for mustard-induced vesication. *Fundam. Appl. Toxicol.* **5**, S134-S149.

- PEDEN, K. W. C., AND PIPAS, J. M. (1985). Site-directed mutagenesis of the simian virus 40 large T-antigen gene: Replication-defective amino acid substitution mutants that retain the ability to induce morphological transformation. *J. Virol.* **55**, 1-9.
- RADLOFF, R., BAUER, W., AND VINOGRAD, J. (1967). A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: The closed circular DNA in HeLa cells. *Proc. Natl. Acad. Sci. USA* **57**, 1514-1521.
- RIBEIRO, P. L. (1988). The repair of bis-(2-chloroethyl) sulfide (BCES)-induced DNA alkylation damage and its possible role in the survival of exposed primary monolayer cultures of rat cutaneous keratinocytes. Thesis. The University of Michigan, Ann Arbor, MI.
- ROBERTS, J. J. (1978). The repair of DNA modified by cytotoxic, mutagenic and carcinogenic chemicals. *Adv. Radiat. Biol.* **9**, 211-435.
- ROBERTS, J. J., CRATHORN, A. R., AND BRENT, T. P. (1968). Repair of alkylated DNA in mammalian cells. *Nature (London)* **218**, 970-972.
- ROBERTS, J. J., PASCOE, J. M., SMITH, B. A., AND CRATHORN, A. R. (1971). Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. II. Non-semiconservative DNA synthesis (repair synthesis) in HeLa and Chinese hamster cells following treatment with alkylating agents. *Chem. Biol. Interact.* **3**, 49-68.
- ROBERTS, J. J., SCOTT, F. D., ORMEROD, M. G., AND RAWLINGS, C. J. (1986). The unique sensitivity of Walker rat tumor cells to bifunctional agents is associated with a failure to recover from inhibition of DNA synthesis and increased chromosome damage. *Mutat. Res.* **166**, 169-181.
- ROSS, W. C. J. (1962). *Biological Alkylating Agents*. Butterworths, London.
- SANCAR, A., AND SANCAR, G. B. (1988). DNA repair enzymes. *Annu. Rev. Biochem.* **57**, 29-67.
- SCHAAPER, R. M., KUNKEL, T. A., AND LOEB, L. A. (1983). Infidelity of DNA synthesis associated with bypass of apurinic sites. *Proc. Natl. Acad. Sci. USA* **80**, 487-491.
- SCHJLDERUP, A., ZARINS, P., AND SMITH-KIELLAND, I. (1972). Repair replication in different strains of *E. coli* after treatment with alkylating agents and its dependence on protein synthesis. *Biochim. Biophys. Acta.* **262**, 269-274.
- SORSCHER, D. H., AND CONOLLY, R. B. (1989). Pretreatment of primary rat cutaneous epidermal keratinocyte culture with a low concentration of MNNG: Effect on DNA cross-linking measured in situ after challenge with bis-2-chloroethyl sulfide. *J. Toxicol. Environ. Health* **27**, 367-379.
- VAUGHAN, F. L., ZAMAN, S., SCAVARELLI, R., AND BERNSTEIN, I. A. (1988). Macromolecular metabolism of a differentiated rat keratinocyte culture system following exposure to sulfur mustard. *J. Toxicol. Environ. Health* **23**, 507-518.
- WAKE, C., GUDEWICZ, T., PORTER, T., WHITE, A., AND WILSON, J. (1984). How damaged is the biologically active subpopulation of transfected DNA? *Mol. Biol.* **4**, 387-398.
- WHEELER, G. P. (1962). Studies related to the mechanisms of action of cytotoxic alkylating agents: A review. *Cancer Res.* **22**, 651-673.