Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 534–538, January 1982
Genetics

Role of DNA repair in mutagenesis of Chinese hamster ovary cells by 7-bromomethylbenz[*a*]anthracene

(mutagen sensitivity/mutation induction/sister chromatid exchange/thioguanine resistance/ouabain resistance)

LARRY H. THOMPSON, KERRY W. BROOKMAN, ANTHONY V. CARRANO, AND LARRY E. DILLEHAY

Biomedical Sciences Division, L-452, Lawrence Livermore National Laboratory, P.O. Box 5507, Livermore, California 94550

Communicated by Richard B. Setlow, September 17, 1981

ABSTRACT **The role of DNA repair in mutagenesis was studied in normal, repair-proficient Chinese hamster ovary cells and in two mutant strains that are deficient in excision repair. By using the mutagen 7-bromomethylbenz[*a*]anthracene (7-BrMeBA) and the technique of alkaline elution of DNA, the mutants were found to be defective at or before the incision step of excision repair. Dose–responses were determined for cell killing, mutation induction at three loci, and sister chromatid exchanges over a survival range of 1.0–0.1 after 7-BrMeBA treatment. The mutants were 5-fold more sensitive to killing than were the normal cells, but the degree of hypersensitivity to mutation induction varied depending on the mutant strain, the genetic marker, and the dose of mutagen. In each instance, the dose–response curve for mutations was essentially linear in the repair-deficient cells. In the normal cells, however, the curves for induced resistance to thioguanine and azaadenine were complex and were curvilinear with increasing slope at low doses. This behavior may be attributable to saturation of the excision repair system. No difference was seen in the efficiency of inducing ouabain-resistant mutations in the repair-deficient cells compared to the normal cells, indicating a qualitatively different behavior of this marker. These results are consistent with excision repair of 7-BrMeBA damage being error-free in Chinese hamster ovary cells. Sister chromatid exchange, another manifestation of DNA damage, also was induced with greater efficiency in the repair-deficient cells.**

An understanding of how cellular DNA repair processes influence mutation frequencies is of paramount importance in learning how environmental insults to genetic material may lead to heritable mutations or cancer. Several human genetic diseases suggest causal relationships between defects in repairing DNA damage and increased probabilities of developing neoplasia (1). The best known syndrome is xeroderma pigmentosum (XP), which has well-documented deficiencies in excision repair in response to UV radiation or certain chemicals (2–6). In culture, fibroblasts from XP individuals show increased sensitivity to killing and mutation induction by various mutagens (4, 6–9), establishing a direct link between mutagenesis and carcinogenesis.

The role of DNA repair in mutagenesis in diverse organisms has been studied with respect to the particular repair pathway acting on the DNA lesions. In bacteria the *uvr* excision repair pathway appears to be an error-free process, and mutagenesis is a consequence mainly of an induced error-prone system, termed "SOS" repair (10, 11). In yeast also, chemical and UV mutagenesis are dependent on the integrity of certain repair systems (12, 13). In mammalian cells the genetic control of mutagenesis is not well understood. Most studies with fibroblasts from XP and normal persons have indicated that the UV excision repair system is essentially error-free (8, 9, 14) although one study presented exceptional results (15). Moreover, enhanced viral mutagenesis in UV-treated host cells has suggested the presence of mutagenic repair (16, 17). In rat hepatoma cells the increase in x-ray-induced mutations under conditions of uncoupling of oxidative phosphorylation has also been interpreted as reflecting error-prone repair (18).

The recent isolation of repair-deficient mutant strains of Chinese hamster ovary (CHO) cells provides a new system in which the involvement of repair in mutagenesis can be quantitatively assessed (19–21). In this report we present evidence that the ability of normal CHO cells to repair DNA damage induced by the mutagen/carcinogen 7-bromomethylbenz[*a*]anthracene (7-BrMeBA) protects them against cytotoxicity, induced mutations, and sister chromatid exchange (SCE). The degree of protection depends on the genetic end point as well as the dose of mutagen; the presence of repair has a qualitative effect on the shape of the dose–response curves for mutations.

MATERIALS AND METHODS

Cells and Culture Conditions. We previously described (22) the origin of a parental repair-proficient strain, AA8, that is functionally heterozygous at the adenine phosphoribosyltransferase (*aprt*) locus. The two repair-deficient mutant strains, UV-5 and UV-20, had undetectable levels of repair replication after UV exposure (19) and were in different complementation classes (21). Culture conditions have been reported (19, 21, 22).

Alkaline Elution of DNA from Filters. Elution was performed as described (23) with modifications. The lysis buffer was 20 mM EDTA/2% $NaDodSO_4$/0.1 M glycine. The elution buffer was as described (23) with the addition of 0.1% $NaDodSO_4$, and filters were polycarbonate with a pore diameter of 0.2 μm (Nuclepore, Pleasanton, CA). Before elution the extract was treated with proteinase K at 0.5 mg/ml (EM Laboratories, Elmsford, NY) in lysis buffer for 1 hr. For scintillation counting the eluted fractions were mixed with 6 vol of Instagel (Packard) containing 0.15% acetic acid. DNA was labeled by adding [2-^{14}C]thymidine (59 mCi/mmol; 50 μCi/ml; 1 Ci = 3.7 $\times$ 10^{-10} becquerels; Amersham) to the cultures at 0.005 μCi/ml 48 hr before mutagen treatment. Three hours before treatment, the cells were incubated in fresh medium without labeled thymidine.

Exposure to Mutagen and Assay of Mutations. Samples of 7-BrMeBA were kindly provided by A. Dipple (Frederick Cancer Research Center, Frederick, MD) and J. A. Mazrimas (Lawrence Livermore National Laboratory); the mutagen was dissolved in dimethyl sulfoxide just prior to addition to the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: 7-BrMeBA, 7-bromomethylbenz[*a*]anthracene; CHO, Chinese hamster ovary; XP, xeroderma pigmentosum; TG^r, 6-thioguanine resistance; AA^r, 8-azaadenine resistance; Oua^r, ouabain resistance; SCE, sister chromatid exchange; APRT, adenine phosphoribosyltransferase; Ara-C, 1-β-D-arabinofuranosylcytosine.

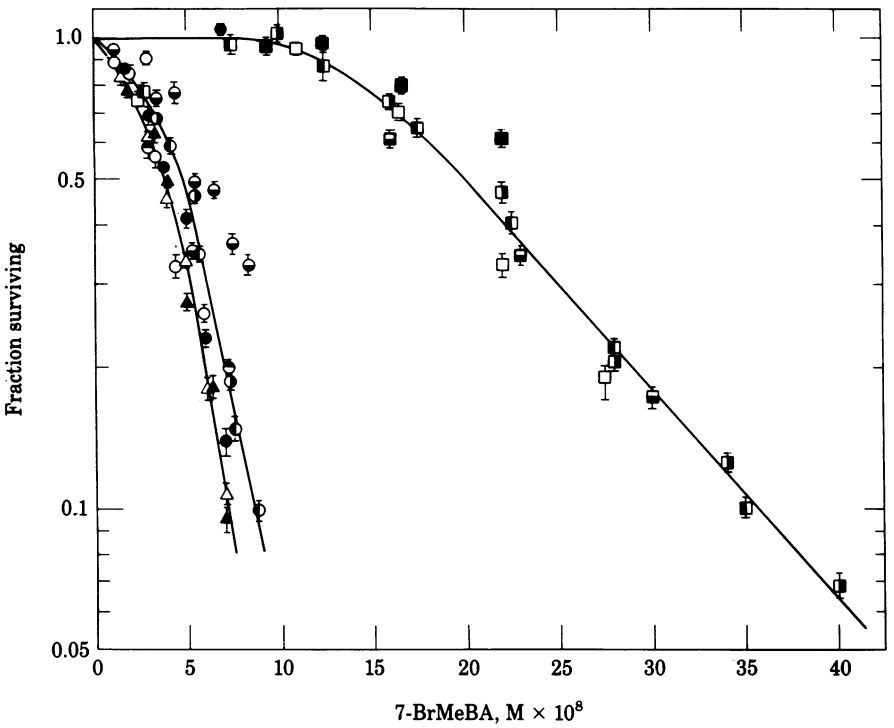

FIG. 1. Survival curves of wild type and mutants UV-5 and UV-20 in response to 7-BrMeBA. Different symbols on each curve represent different experiments; error bars are SEM for colony counts on four replicate dishes. D_0 and extrapolation number, n, were determined from the curves fit by eye. Plating efficiencies ranged from 0.66 to 0.89 and were similar for all three cell lines. Squares, AA8, D_0 = 9.5, n = 4; circles, UV-5, D_0 = 2.2, n = 4; triangles, UV-20, D_0 = 1.7, n = 4.

cultures. To obtain reproducible exposure, cells were treated in suspension (≈ 1.4 × 10^5 cells per ml) at 37°C in complete medium. Rapid mixing in 200-ml spinner flasks was performed on a stirring platform. Because of the short half-life of the 7-BrMeBA reaction (24), the cells were not rinsed after exposure. Cell survival, measured 1 hr after start of exposure, was unaffected by longer incubation.

Cultures in suspension were diluted at 2-day intervals for mutation expression as validated (22) and then plated for viability and mutations at the times indicated. The optimal conditions for detecting thioguanine resistance (TG^r) and azaadenine resistance (AA^r) in AA8 cells were reported (22); ouabain resistance (Oua^r) was assayed at 3 mM ouabain (Sigma) as validated (25). Plating conditions for colonies were: viability, 300 cells per 100-mm dish (four replicates); TG^r and AA^r, 6 × 10^5 cells per dish (six replicates); Oua^r, 1.5 × 10^6 cells per dish (20 or 30 replicates). To minimize sampling errors, the minimum cell count per culture during expression was 5 × 10^6 for AA^r and TG^r and 5 × 10^7 for Oua^r.

Measurement of SCE. Cultures for assay of SCE were aliquots of the same cultures used for mutation induction. Our conditions for SCE analysis have been described (26).

RESULTS

Cytotoxicity of 7-BrMeBA. 7-BrMeBA was chosen as a prototype mutagen for study because it is direct acting and its adducts are known to be repaired by the nucleotide excision repair pathways in both *Escherichia coli* (24) and human cells (3, 4). The principal DNA adducts are formed by its reaction with the exocyclic amino groups of adenine, guanine, and possibly cytosine (24, 27, 28). The two UV-sensitive mutants of CHO cells were hypersensitive to killing by this compound (Fig. 1). As indicated, the D_0 (dose required to produce a decrement in survival of 63% on the exponential region) of each mutant's survival curve was 1/5 to 1/4 the value for the AA8 parental cells. The nonexponential character of the curves for the mutants implies either a multi-hit response or the presence of some repair capacity.

Mutations Affecting the Incision Step of Excision Repair. The technique of alkaline elution of DNA (23) was used to test for a repair defect that might account for the hypersensitivity of the mutants to killing by 7-BrMeBA. The DNA of AA8 cells eluted from the filter much faster after treatment with 7-BrMeBA, and the rate was further enhanced upon incubation with hydroxyurea and 1-β-D-arabinofuranosylcytosine (Ara-C) (Fig. 2). These drugs have been shown to retard the closing of nicks produced by the incision step of repair (29). In marked

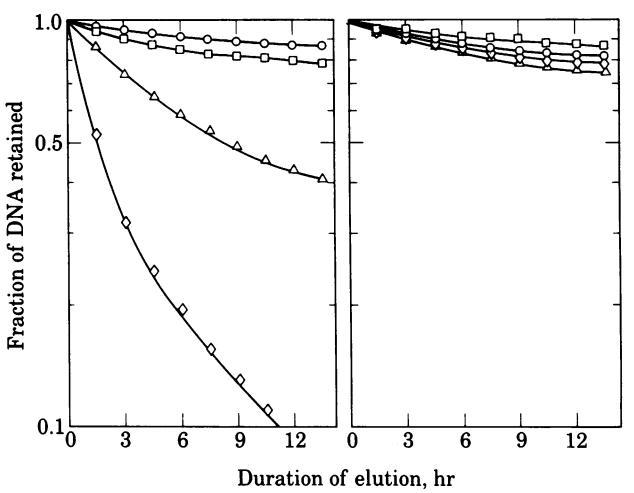

FIG. 2. Alkaline elution of DNA from UV-20 and parental AA8 cells after treatment with 7-BrMeBA. The fraction of DNA remaining on the filter is plotted against elution time. Cells were treated for 5 min at 37°C with 7-BrMeBA at 0.8 μM in suspension. They were then diluted 1:10 into Dulbecco's phosphate-buffered saline at 4°C, centrifuged, and resuspended in fresh medium at 37°C. Cultures were next incubated for 60 min with or without DNA synthesis inhibitors present. ○, Untreated control cells; □, cells treated only with hydroxyurea at 2 mM and Ara-C at 10 μM; △, cells treated only with 7-BrMeBA; ◇, cells treated with 7-BrMeBA, hydroxyurea, and Ara-C. *Left*, AA8; *Right*, UV-20.

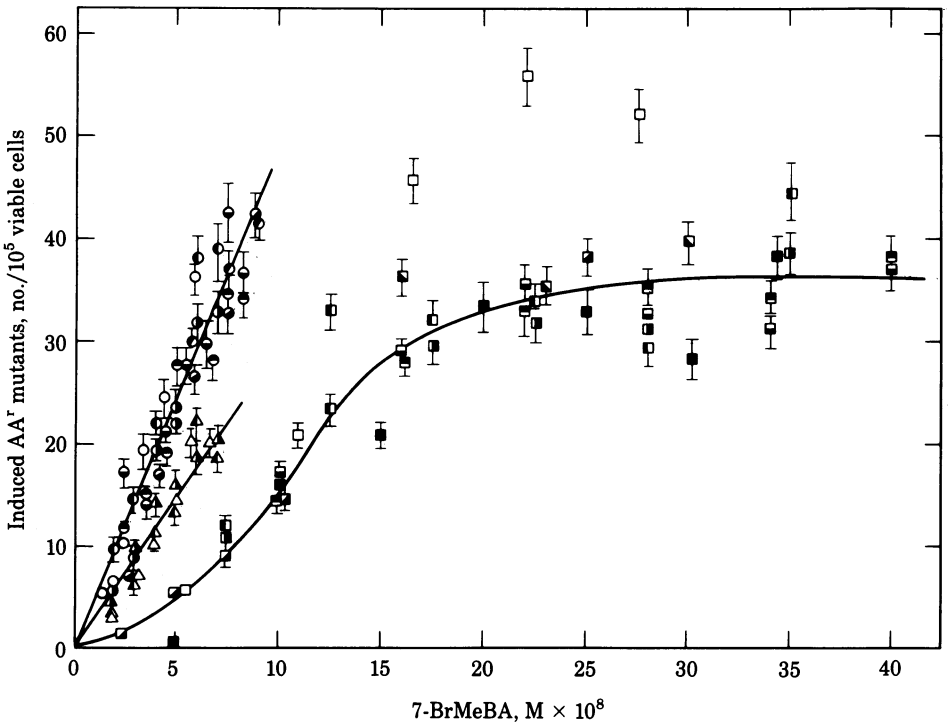

FIG. 3. Dose–response curves for induction of AA^r mutants in AA8, UV-5, and UV-20. Spontaneous mutant frequencies (average of duplicate cultures in each experiment) were subtracted; for each cell line the average values were in the range 2.3 to 3.1 × 10^{-5}. Error bars represent compounded SEM for the colony counts on the mutation and plating efficiency dishes. Symbols at the same dose and shaded on opposite sides represent measurements at 6 and 7 days of expression; otherwise, different symbols represent different experiments plated on day 6. Squares, AA8; circles, UV-5; triangles, UV-20.

contrast to AA8, UV-20 cells showed only a slight, if significant, increase in the rate of elution under either set of post-treatment conditions. Thus, UV-20 does not perform the incision step in response to 7-BrMeBA damage. Mutant UV-5 behaved similarly, and both mutants responded to UV in an analogous manner (results not shown).

Induction of Mutations by 7-BrMeBA. For three genetic markers we measured induced mutations in normal and repair-deficient cells over the survival range 1.0 to 0.1. All three cell lines had identical killing curves for each selecting drug (data not shown). The dose–response curves for induced AA^r mutations at the *aprt* locus are shown in Fig. 3. Whereas the repair-

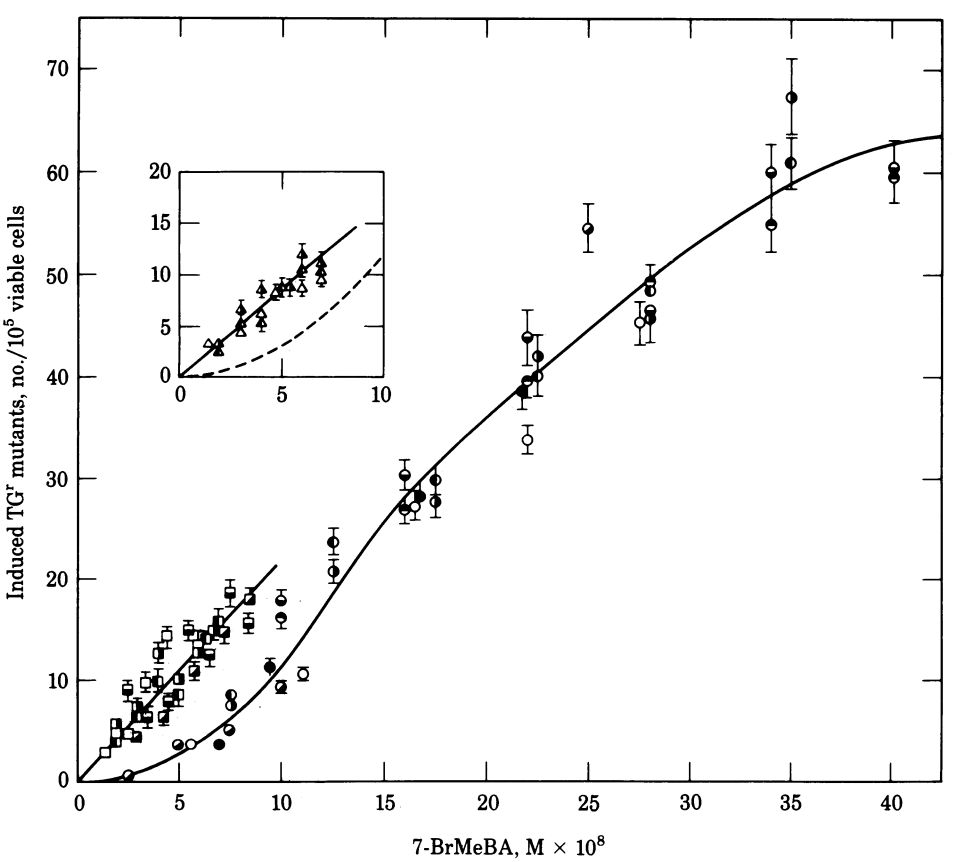

FIG. 4. Dose–response curves for induction of TG^r mutants in AA8, UV-5, and UV-20. The average spontaneous mutant frequencies were 7×10^{-6} (AA8), 1.5×10^{-5} (UV-5), and 3.3×10^{-5} (UV-20). Error bars are as defined in Fig. 3. Different symbols represent different experiments plated after 6 days of expression; in the case of pairs of symbols at a given dose that are shaded on opposite sides, plating was done at both 6 and 7 or 6 and 8 days. Circles, AA8; squares, UV-5; triangles, UV-20. The dashed line in the *Inset* refers to curve for AA8.

deficient strains UV-5 and UV-20 gave linear responses, the parental AA8 cells were less sensitive and had a more complex behavior. At doses up to 120 nM there was a curvilinear response in which the slope increased with dose; then the slope decreased rapidly to produce a plateau at higher doses. The results for TG^r were similar (Fig. 4). Both repair-deficient strains were hypersensitive and had linear responses. The response for AA8 was curvilinear in the low-dose region; at higher doses the frequency continued to increase.

Induced Oua^r mutants, measured in separate experiments using larger cultures, are compared for UV-5 and AA8 in Fig. 5. At doses between 0 and 100 nM (nontoxic doses for AA8) the response of AA8 at this marker was linear; the behavior of UV-5 did not appear to differ significantly. At doses that produced cytotoxicity in AA8, mutation induction in UV-5 was more efficient than at lower doses and continued to increase with dose.

Induction of SCEs by 7-BrMeBA. SCE, a chromosomal alteration that reflects DNA breakage and rejoining (30, 31), was also analyzed (Fig. 6). In AA8 cells, induced SCEs increased linearly with dose and reached a value of 47 SCEs per cell at 400 nM (results not shown for higher doses). Mutant strains UV-5 and UV-20 were significantly more sensitive than AA8 to SCE induction over the same dose range.

DISCUSSION

In this study we investigated the role of DNA repair in chemical mutagenesis by analyzing the response of normal CHO cells and two mutants that are deficient in excision repair of UV damage and the adducts arising from the carcinogen 7-BrMeBA. The data presented here (and unpublished results) indicate that the

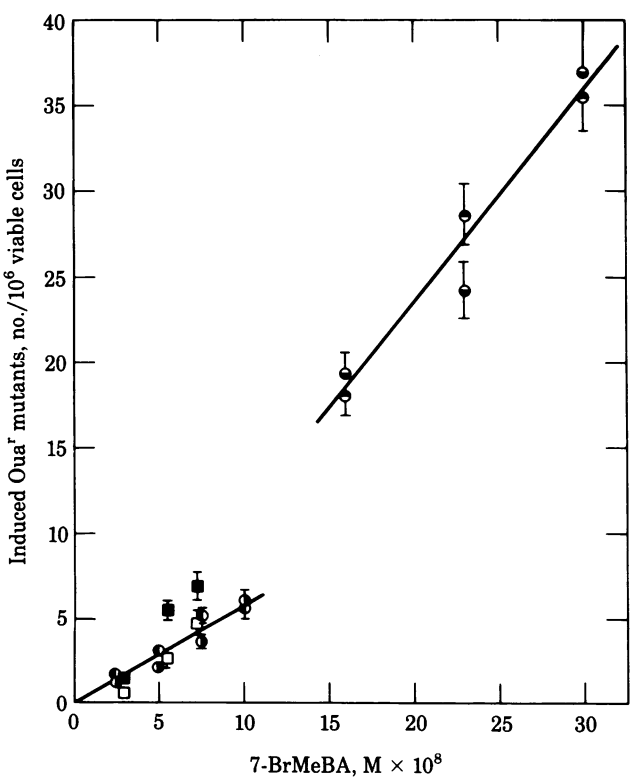

FIG. 5. Dose–response curves for induction for Oua^r mutations in AA8 and UV-5. The average background frequencies that were subtracted were 1.7×10^{-6} for both cell lines. □, ■, Separate experiments with UV-5 plated after 3 days of expression; ◐, ◑, AA8 plated after 2 and 3 days of expression; ◒, ◓, a second experiment with AA8 plated after 2 and 3 days of expression.

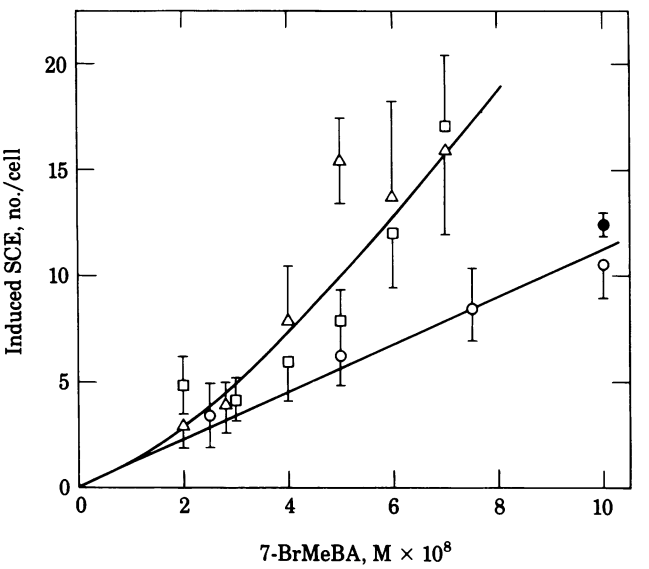

FIG. 6. Induced SCEs as a function of dose of 7-BrMeBA. The baseline SCE frequencies that were subtracted were: AA8, 9.0 SCEs per cell; UV-5, 10.5; UV-20, 7.9. The error bars are SEM for replicate cultures fixed at different times after 7-BrMeBA treatment (26). Circles, AA8; squares, UV-5; triangles, UV-20. The solid circle represents the lowest dose point in a separate experiment.

defects in these mutants lie at or before the incision step, which makes the mutants phenotypically analogous to XP cells (32). The lack of repair after 7-BrMeBA exposure in the mutants provides a qualitative explanation for the hypersensitivity that is observed for cytotoxicity, induced mutations, and SCEs.

Mutant strains UV-5 and UV-20 produced more mutations for the two enzyme-deficiency markers, AA^r and TG^r, than did the parental cells exposed to equal concentrations. This suggests that mutations are more likely to occur if lesions remain unrepaired until DNA replication than if they are acted on by repair. The degree of differential mutagenesis between mutant and parental cells was dependent on the dose of mutagen because the shapes of the dose–response curves differed qualitatively; the difference in mutagenic efficiency was greatest at very low doses. For both genetic markers, mutant UV-20 yielded fewer mutations than did UV-5. This difference may reflect diversity in the nature of the underlying biochemical defects. In this regard, the two mutants were previously found to differ greatly in sensitivity to killing by the DNA crosslinking agent mitomycin C (19).

Induced Oua^r mutations behaved differently from the other two markers. The results for mutant UV-5 compared with normal CHO cells indicated no significant differences in Oua^r mutations in the two cell lines, suggesting that the excision repair process in the normal cell, which is defective in UV-5, does not correct the class of DNA damage that leads to these mutations. Oua^r is thought to arise from base-substitution mutations because resistance is associated with a change in the ouabain-binding site in a functional protein (33). The differing behavior of the Oua^r marker with regard to repair could be due to the fact that these mutations reflect a more restricted class of damage than do AA^r and TG^r mutations. Alternatively, the effect may be specific to this particular marker for reasons relating to the structure of the Na^+,K^+-ATPase gene.

The shapes of the dose–response curves for mutation induction were qualitatively different in the repair-deficient cells compared to parental cells. Whereas the mutant strains had linear responses, with parental cells the curves were complex and

varied with genetic marker. With respect to repair, the most interesting region of the curves is the low-dose range, in which measurable killing of the normal cells does not occur (<120 nM 7-BrMeBA). In this range the efficiency of inducing AA^r and TG^r mutations increased with dose, which suggests that saturation of the excision repair system was occurring. At higher doses the efficiency of mutation production decreased, more noticeably in the case of AA^r (Fig. 3) than TG^r. Because these results were obtained with asynchronous cells, differential sensitivity of the cell cycle phases could govern the curve shapes. For example, the plateau behavior of AA^r might result from preferential killing of a subpopulation that is the most sensitive in terms of both mutagenicity and survival.

An important implication of the curvilinear responses is that, at dose rates approaching zero, there may be proportionately quite high levels of mutagenesis in repair-deficient cells compared with the normal cells. Analogous nonlinear responses have been reported for UV-induced mutations in human fibroblasts (14) and for methylation mutagenesis in V79 hamster cells (34). Although the Oua^r response in the normal CHO cells was linear at low doses, at higher doses there was an increase in slope which somehow may be related to repair.

The repair-deficient strains were also found to be slightly, but significantly, hypersensitive to the induction of SCE by 7-BrMeBA. Thus, excision repair appears to remove lesions capable of forming SCEs in a manner analogous to the repair of certain lesions that are potentially mutagenic (as in the case of AA^r and TG^r). These results strengthen the previously discussed correlations between mutations and SCEs (30, 31).

In summary, we conclude that the excision-repair defects in the mutants UV-5 and UV-20 have differential effects on the three types of genetic end points examined. In no instance was a mutagenesis end point induced to a higher level in the normal CHO cells than in a mutant strain at a given dose. These results therefore are consistent with the repair being error-free in CHO cells. Finally, the enhanced sensitivity of the repair-deficient strains renders them useful in detecting certain environmental mutagens that may be present at limiting concentrations or that may have only marginal activity in the normal CHO cells.

We thank Carolyn Mooney, Joyce Hunt, and Jay Minkler for technical assistance. This work was performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under Contract W-7405-ENG-48.

1. Setlow, R. B. (1978) *Nature (London)* **271,** 713–717.
2. Cleaver, J. E. (1978) in *Metabolic Basis of Inherited Disease,* eds. Stanbury, J. B., Wyngaarden, J. B. & Frederickson, D. S. (McGraw-Hill, New York), 4th Ed., pp. 1072–1095.
3. Slor, H. (1973) *Mutat. Res.* **19,** 231–235.
4. McCaw, B. A., Dipple, A., Young, S. & Roberts, J. J. (1978) *Chem. Biol. Interact.* **22,** 139–151.
5. Ikenaga, M., Takebe, H. & Ishii, Y. (1977) *Mutat. Res.* **43,** 415–427.
6. Heflich, R. H., Hazard, R. M., Lommel, L., Scribner, J. D., Maher, V. M. & McCormick, J. J. (1980) *Chem. Biol. Interact.* **29,** 43–56.
7. Maher, V. M., Birch, N., Otto, J. R. & McCormick, J. J. (1975) *J. Natl. Cancer Inst.* **54,** 1287–1294.
8. Maher, V. M., McCormick, J. J., Grover, P. L. & Sims, P. (1977) *Mutat. Res.* **43,** 117–138.
9. Yang, L. L., Maher, V. M. & McCormick, J. J. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 5933–5937.
10. Witkin, E. M. (1976) *Bacteriol. Rev.* **40,** 869–907.
11. Kimball, R. F. (1978) *Mutat. Res.* **55,** 85–120.
12. Prakash, L. (1974) *Genetics* **78,** 1101–1118.
13. Lawrence, C. W. & Christensen, R. (1976) *Genetics* **82,** 207–232.
14. Maher, V. M., Dorney, D. J., Mendrala, A. L., Konze-Thomas, B. & McCormick, J. J. (1979) *Mutat. Res.* **62,** 311–323.
15. Simons, J. W. I. M. (1979) *Mutat. Res.* **59,** 273–283.
16. DasGupta, U. B. & Summers, W. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2378–2381.
17. Sarasin, A. & Benoit, A. (1980) *Mutat. Res.* **70,** 71–81.
18. Laval, F. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2702–2725.
19. Thompson, L. H., Rubin, J. S., Cleaver, J. E., Whitmore, G. F. & Brookman, K. (1980) *Somatic Cell Genet.* **6,** 391–405.
20. Busch, D. B., Cleaver, J. E. & Glaser, D. A. (1980) *Somatic Cell Genet.* **6,** 407–418.
21. Thompson, L. H., Busch, D. B., Brookman, K., Mooney, C. L. & Glaser, D. A. *Proc. Natl. Acad. Sci. USA* **78,** 3734–3737.
22. Thompson, L. H., Fong, S. & Brookman, K. (1980) *Mutat. Res.* **74,** 21–36.
23. Kohn, K. W., Erickson, L. C., Ewig, R. A. G., & Freidman, C. A. (1976) *Biochemistry* **15,** 4629–4637.
24. Venitt, S. & Tarmy, E. M. (1972) *Biochim. Biophys. Acta* **287,** 38–51.
25. Baker, R. M., Brunette, D. M., Mankovitz, R., Thompson, L. H., Whitmore, G. F., Siminovitch, L. & Till, J. E. (1974) *Cell* **1,** 9–21.
26. Carrano, A. V., Thompson, L. H., Stetka, D. G., Minkler, J. L., Mazrimas, J. A. & Fong, S. (1979) *Mutat. Res.* **63,** 175–188.
27. Dipple, A., Brookes, P., Mackintosh, D. S. & Rayman, M. P. (1971) *Biochemistry* **10,** 4323–4330.
28. Dipple, A. & Roberts, J. J. (1977) *Biochemistry* **16,** 1499–1503.
29. Collins, A. R. S. & Johnson, R. T. (1981) in *DNA Repair, A Laboratory Manual of Research Procedures,* eds. Friedberg, E. C. & Hanawalt, P. C. (Marcel Dekker, New York), Vol. 1, Part B, pp. 341–360.
30. Carrano, A. V., Thompson, L. H., Lindl, P. A. & Minkler, J. L. (1978) *Nature (London)* **271,** 551–553.
31. Carrano, A. V. & Thompson, L. H. (1980) in *Sister Chromatid Exchange,* ed. Wolff, S. (Wiley, New York) in press.
32. Tanaka, K., Sekiguchi, M. & Okada, Y. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 4071–4075.
33. Baker, R. M. (1979) in *Banbury Report 2, Mammalian Cell Mutagenesis: The Maturation of Test Systems,* eds. Hsie, A. W., O'Neill, J. P. & McElheny, V. K. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 237–244.
34. Jenssen, D. & Ramel, C. (1980) *Mutat. Res.* **73,** 339–347.