Acta Medica Okayama

Volume 43, *Issue* 2 1989 *Article* 2

APRIL 1989

Studies on bleomycin-induced repair DNA synthesis in permeable mouse ascites sarcoma cells.

Shigeru Mori* Shuji Seki†

Takuzo Oda‡

*Okayama University,
†Okayama University,
‡Okayama University,

Studies on bleomycin-induced repair DNA synthesis in permeable mouse ascites sarcoma cells.*

Shigeru Mori, Shuji Seki, and Takuzo Oda

Abstract

To study the mechanism of DNA excision repair, a DNA repair system employing permeable mouse sarcoma (SR-C3H/He) cells was established and characterized. SR-C3H/He cells were permeabilized with a 0.0175% Triton X-100 solution. The permeable cells were treated with 1 mM ATP and 0.11 mM bleomycin, and then washed thoroughly to remove ATP and bleomycin. Repair DNA synthesis occurred in the bleomycin-damaged, permeable SR-C3H/He cells when incubated with ATP and four deoxyribonucleoside triphosphates. The repair nature of the DNA synthesis was confirmed by the BrdUMP density shift technique, and by the reduced sensitivity of the newly synthesized DNA to Escherichia coli exonuclease III. The DNA synthesis was optimally enhanced by addition of 0.08 M NaCl. Studies using selective inhibitors of DNA synthesis showed that aphidicolin-sensitive DNA polymerase (DNA polymerase alpha and/or delta) and DNA polymerase beta were involved in the repair process. The present DNA repair system is thought to be useful to study nuclear DNA damage by bleomycin, removal of the damaged ends by an exonuclease, repair DNA synthesis by DNA polymerases and repair patch ligation by DNA ligase(s).

KEYWORDS: DNA repair, bleomycin, DNA polymerases, permeable cells, mouse ascites cells

---

*PMID: 2471392 [PubMed - indexed for MEDLINE]

Acta Med Okayama 43 (2) 81-88 (1989)

Studies on Bleomycin-Induced Repair DNA Synthesis in Permeable Mouse Ascites Sarcoma Cells

Shigeru Mori*, Shuji Seki and Takuzo Oda

Department of Biochemistry, Cancer Institute, Okayama University Medical School, Okayama 700, Japan

To study the mechanism of DNA excision repair, a DNA repair system employing permeable mouse sarcoma (SR-C3H/He) cells was established and characterized. SR-C3H/He cells were permeabilized with a 0.0175% Triton X-100 solution. The permeable cells were treated with 1 mM ATP and 0.11 mM bleomycin, and then washed thoroughly to remove ATP and bleomycin. Repair DNA synthesis occurred in the bleomycin-damaged, permeable SR-C3H/He cells when incubated with ATP and four deoxyribonucleoside triphosphates. The repair nature of the DNA synthesis was confirmed by the BrdUMP density shift technique, and by the reduced sensitivity of the newly synthesized DNA to *Escherichia coli* exonuclease III. The DNA synthesis was optimally enhanced by addition of 0.08 M NaCl. Studies using selective inhibitors of DNA synthesis showed that aphidicolin-sensitive DNA polymerase (DNA polymerase α and/or δ) and DNA polymerase β were involved in the repair process. The present DNA repair system is thought to be useful to study nuclear DNA damage by bleomycin, removal of the damaged ends by an exonuclease, repair DNA synthesis by DNA polymerases and repair patch ligation by DNA ligase(s).

Key words: DNA repair, bleomycin, DNA polymerases, permeable cells, mouse ascites cells

Cellular DNA is damaged not only by numerous physical and chemical agents from the environment but also by endogenous stresses such as activated oxygen species generated during oxidative metabolism. Damaged DNA is generally repaired accurately, but sometimes inaccurately, by cellular repair systems. Inaccurate repair is thought to play a role in mutagenesis, transformation and carcinogenesis. Studies on DNA repair mechanisms are, therefore, important not only for understanding essential cellular processes of DNA metabolism, but also for resolving mechanisms of carcinogenesis. DNA repair mechanisms in mammalian cells seem to be complex as suggested by the nine complementation groups found in XP cells which are defective in the repair of ultraviolet light-damaged DNA. Subcellular systems are thought to be important for studying such complex repair mechanisms, espe-

*To whom correspondence should be addressed.

cially since genetic approaches to these mechanisms are limited in mammalian cells (refer to Ref. 1 for references concerning relevant subcellular systems).

DNA damage caused by ionizing radiation such as X-ray is known to be complex (2-4). At present it is thought that repair mechanisms of DNA damaged by ionizing radiation are too complicated to be studied analytically. Bleomycin, a radiomimetic antibiotic which generates active oxygen intermediates when bound to DNA in the presence of Fe(II), is known to cause simple DNA damage (5, 6). The major type of damage is the formation of single-strand breaks in which a sugar fragment (glycolate) is linked by an ester bond to a 3'-phosphate group. The same type of damage is known to be induced in DNA by ionizing radiation (3, 7). We have used bleomycin as a DNA-damaging agent to establish a simple DNA repair system for analytical studies of DNA repair processes, and recently established a DNA repair system using bleomycin-pretreated permeable HeLa cells (1). In the present study, a bleomycin-induced repair DNA synthesis system using permeable mouse ascites sarcoma cells was developed and characterized, and the system was compared with the DNA repair system established using permeable HeLa cells.

Materials and Methods

The reagents used in these experiments were obtained from the following sources: [^{3}H]dTTP from Amersham Japan Corp., Tokyo, Japan, ribonucleotides (NTPs) and deoxyribonucleotides (dNTPs) from Seikagaku Kogyou Co., Ltd., Tokyo, 2', 3'-dideoxythymidine 5'-triphosphate (ddTTP) and *E. coli* exonuclease III from P-L Biochemicals, and N-ethylmaleimide from Sigma Chemical Co., St. Louis, MO, USA. Aphidicolin was kindly provided by Dr. A.H. Todd of Imperial Chemical Industries, Macclesfield, Cheshire, UK. Copper-free bleomycin A_2 was kindly provided by Nippon Kayaku Co., Tokyo. Mouse ascites sarcoma (SR-C3H/He) cells and the cultured cell line were obtained as described previously (8). SR-C3H/He cells were cultured in plastic dishes in Eagle's minimal essential medium (MEM 1, Nissui Co., Tokyo) supplemented with 10% bovine serum and 2 mM glutamine in a humidified 5% CO_2-95% air atmosphere. Cells were harvested by treatment with 0.125% trypsin and 0.01% EDTA, and washed 3 times with fresh culture medium.

The following procedures were carried out at 0-4°C unless otherwise indicated.

Preparation of bleomycin-pretreated permeable cells. SR-C3H/He cells were permeabilized by treatment with buffer B (0.25 M sucrose, 10 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0) supplemented with 0.0175% Triton X-100 (Triton-buffer B) (9). The permeable cells were treated with 1 mM ATP and 0.11 mM bleomycin, and then washed thrice with 2-mercaptoethanol-free Triton-buffer B, as described previously (1, 10).

Assay of DNA synthetic activity. Bleomycin-induced, unscheduled DNA synthesis (UDS) was assayed in a mixture (final volume, 0.6 ml; standard assay mixture for bleomycin-induced UDS) containing 2×10^6 bleomycin-pretreated permeable cells, 0.0117% Triton X-100, 0.167 M sucrose, 0.67 mM EDTA, 40 mM Tris-HCl, 5 mM $MgCl_2$, 80 mM NaCl, 2.5 mM ATP, 50 μM dATP, 10 μM dCTP, 50 μM dGTP and 2.5 μM [^{3}H]dTTP at 0.5 Ci/mmol, pH 8.0 at 25°C, essentially as described previously (11). Replicative DNA synthesis was measured in the same assay mixture except that the permeable cells were not pretreated with bleomycin.

Isopyknic centrifugation of DNA labeled with BrdUMP and [^{3}H]dTMP in permeable cells. To distinguish UDS from replicative DNA synthesis, DNA synthesis was performed as described above, except that the concentration of [^{3}H]dTTP was 0.083 μM (50 Ci/mmol), and that bromodeoxyuridine triphosphate (BrdUTP) was added at 8.3 μM. DNA was extracted from 1.6×10^7 labeled cells. Equilibrium sedimentation analysis of BrdUMP-labeled DNA in alkaline CsCl was performed as described previously (10).

Test of exonuclease III sensitivity of synthesized DNA. Exonuclease III sensitivity of DNA syn-

thesized in bleomycin-pretreated permeable cells was measured as described previously (1) except that *in vivo* labeling of DNA with [^{14}C]deoxythymidine was omitted.

Almost all assays were performed in duplicate. Variation in the results for duplicate test was mostly less than 10%.

Results

Properties of DNA synthesis in bleomycin-pretreated permeable SR-C3H/He cells. The time course of [^{3}H]dTMP incorporation was almost linear for 10 min and proceeded thereafter at a diminished rate (Fig. 1). [^{3}H]dTMP incorporation was directly proportional to cell number to 2.5×10^{6} (Fig. 2).

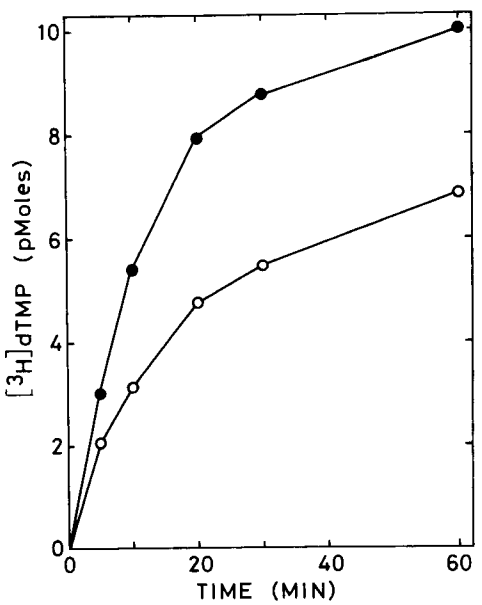

Fig. 1 Time course of [^{3}H]dTMP incorporation into bleomycin-pretreated permeable mouse ascites sarcoma (SR-C3H/He) cells. DNA synthesis in the bleomycin-pretreated permeable cells was assayed in the standard assay mixture for bleomycin-induced UDS(●), or in the standard assay mixture except that ATP was omitted (○), as described in Materials and Methods. The incubation was conducted at 37℃ for the indicated intervals. The data are expressed as pmoles of [^{3}H]dTMP incorporated per 1×10^{6} cells.

ATP enhanced the DNA synthesis 1.5-2 times. The optimum concentration of ATP was 2.5 to 5 mM (12). The DNA synthesis was stimulated by an appropriate concentration of NaCl. The salt stimulation of DNA synthesis was more significant when measured without ATP than with ATP (Fig. 3). Optimal NaCl concentrations were 40 to 80 mM for UDS measured with 2.5 mM ATP, 80 to 150 mM for UDS measured without ATP, and 80 mM for replicative DNA synthesis (Fig. 3). NaCl could be replaced with KCl. In the following experiments, bleomycin-pretreated permeable SR-C3H/He cells were incubated for 30 min in the presence of 80 mM NaCl and 2.5 mM ATP unless otherwise indicated.

DNA polymerases involved in UDS. Aphidicolin, which specifically inhibits DNA polymerases α and δ without affecting DNA

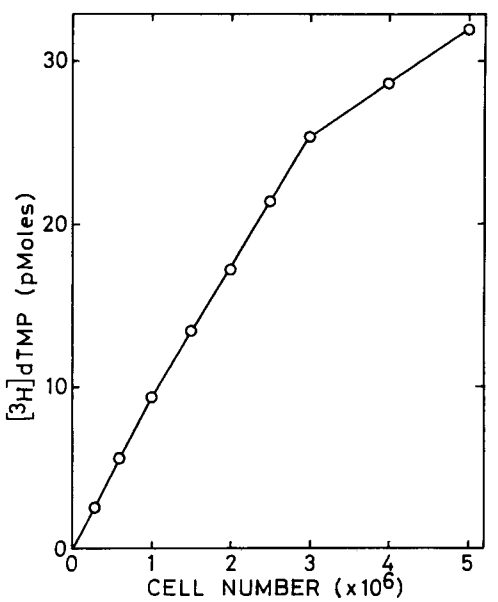

Fig. 2 Effect of permeable cell concentrations on [^{3}H]dTMP incorporation. DNA synthesis was assayed in the standard assay mixture for bleomycin-induced UDS with various concentrations of bleomycin-pretreated permeable cells. The data are expressed as pmoles of [^{3}H]dTMP incorporated per 30 min.

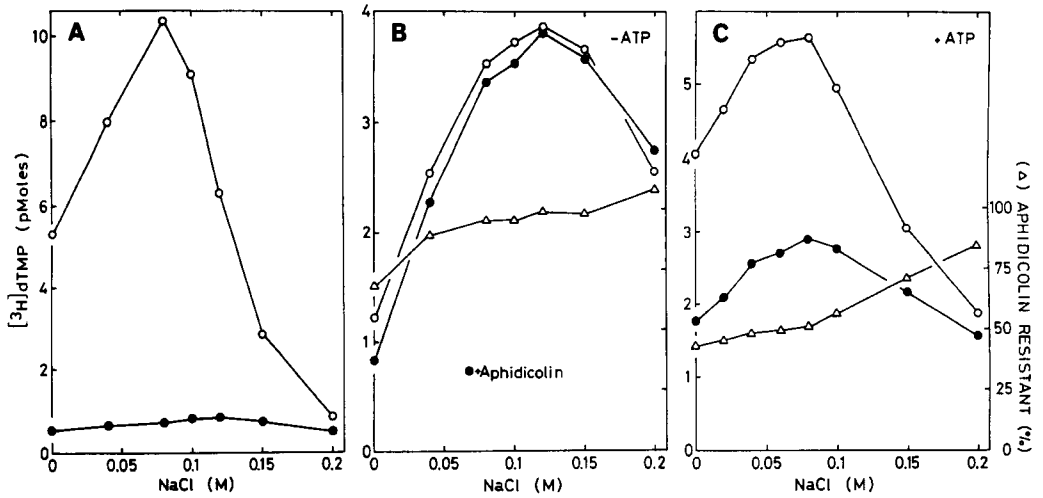

Fig. 3 Effects of varying concentrations of NaCl on bleomycin-induced UDS and replicative DNA synthesis in permeable SR-C3H/He cells. Bleomycin-nontreated (A) or pretreated (B, C) permeable cells were incubated at 37°C for 30 min with reaction mixtures modified from the standard assay mixture described in Materials and Methods. DNA synthesis was measured in the absence (○) or presence (●) of 30 μM aphidicolin. The activity is expressed as pmol [^{3}H]dTMP incorporated per 10^6 cells per 30 min. Triangles (△) show % resistance of DNA synthesis to aphidicolin. A, replicative DNA synthesis; B, bleomycin-induced UDS measured in the absence of ATP; C, bleomycin-induced UDS measured in the presence of 2.5 mM ATP.

polymerases β and γ (13-15), partially inhibited UDS measured in the presence of 2.5 mM ATP (Fig 3C), although UDS measured without ATP was highly resistant to the drug (Fig. 3B). Replicative DNA synthesis was highly sensitive to the drug (Fig. 3A). Fig. 3 also suggests that DNA synthesis increased due to the addition of NaCl at the optimal concentration was mostly aphidicolin-resistant. DNA polymerases involved in UDS measured in the presence of 80 mM NaCl and 2.5 mM ATP were studied further by using selective inhibitors of DNA polymerases (Table 1). The UDS was partially sensitive to 20 μM ddTTP (a selective inhibitor of DNA polymerases β and γ), to 30 μM aphidicolin or to 1 mM N-ethylmaleimide (a selective inhibitor of DNA polymerases α, γ and δ). The UDS was strongly inhibited by the combination of ddTTP and N-ethylmaleimide, or ddTTP and aphidicolin, but not by the combination of aphidicolin and N-ethylmaleimide. The results suggested

Table 1 Effects of various combinations of inhibitors on UDS measured in the presence of 2.5 mM ATP in bleomycin-pretreated, permeable SR-C3H/He cells

Inhibitor[a]			Activity (%)[b]
ddTTP	Aphidicolin	N-ethylmaleimide	
−	−	−	100
+	−	−	22
−	+	−	78
−	−	+	66
+	+	−	11
+	−	+	8
−	+	+	66

a: Where added (+), the concentrations of ddTTP, aphidicolin and N-ethylmaleimide were 20 μM, 30 μM and 1 mM, respectively.

b: Assay was performed in duplicate. Variation in the results for duplicate tests was mostly less than 10%.

that aphidicolin-sensitive DNA polymerase (DNA polymerase α and/or δ) and DNA polymerase β were involved in the UDS.

Nature of DNA synthesized in bleomycin-pretreated permeable SR-C3H/He cells. Newly synthesized DNA in permeable cells

was labeled with BrdUMP and [^{3}H]dTMP. The labeled DNA was analyzed by alkaline CsCl density gradient (isopyknic) centrifugation as described in Materials and Methods (Fig. 4). Although the DNA replicatively synthesized in non-bleomycin-treated permeable cells sedimented at a higher density than normal density bulk DNA (Fig. 4A), DNA newly synthesized in bleomycin-pretreated permeable cells in the absence of ATP (Fig. 4B) or in the presence of 2.5 mM ATP (Fig. 4C), ATP and 30 μM aphidicolin (Fig. 4D) or ATP and 20 μM ddTTP (Fig. 4E) all sedimented at a normal, bulk DNA density. The results indicated that DNA synthesis in bleomycin-pretreated permeable SR-C3H/He cells was unscheduled. It is worth noting that ddTTP-resistant, in other words, DNA polymerase α and/or δ-dependent-DNA synthesis in bleomycin-pretreated permeable SR-C3H/He cells was not replicative but unscheduled (Fig. 4E).

Repair patch ligation in bleomycin-pretreated permeable cells. To investigate whether or not repair patches were completed and ligated during the incubation for UDS in bleomycin-pretreated permeable SR-C3H/He cells, the exonuclease III sensitivity of [^{3}H]dTMP-labeled DNA isolated from permeable cells after the incubation for UDS was

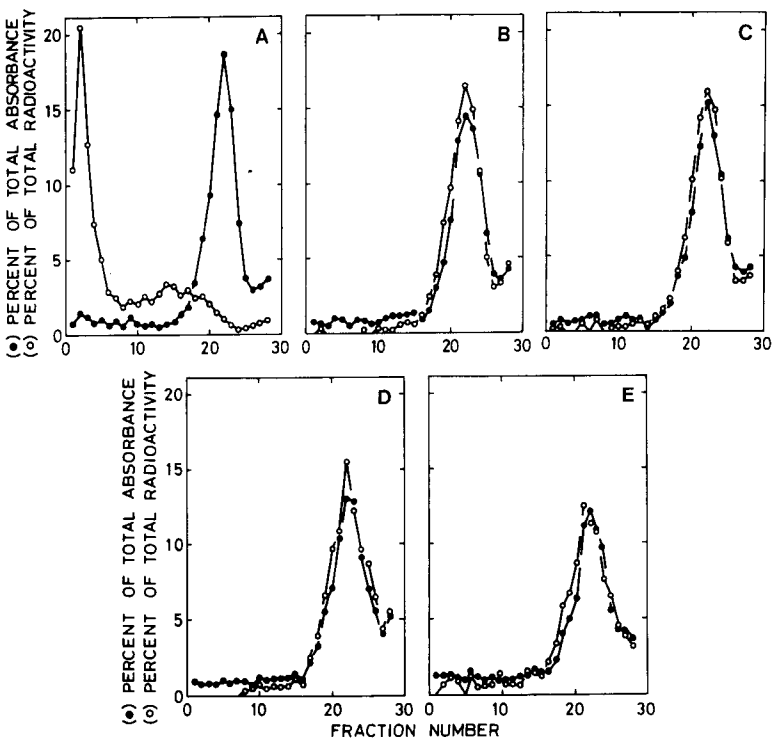

Fig. 4 Isopyknic centrifugation of DNA labeled with BrdUMP and [^{3}H]dTMP in permeable SR-C3H/He cells. Sedimentation is from right to left. ●, % of total absorbance at 260 nm; ○, % of total radioactivity. A, replicative DNA synthesis in permeable cells; B, DNA synthesis measured without ATP in bleomycin-pretreated permeable cells; C, DNA synthesis measured in the presence of 2.5 mM ATP in bleomycin-pretreated permeable cells; D, DNA synthesis measured in the presence of 2.5 mM ATP and 30 μM aphidicolin in bleomycin-pretreated permeable cells; E, DNA synthesis measured in the presence of 2.5 mM ATP and 20 μM ddTTP in bleomycin-pretreated permeable cells.

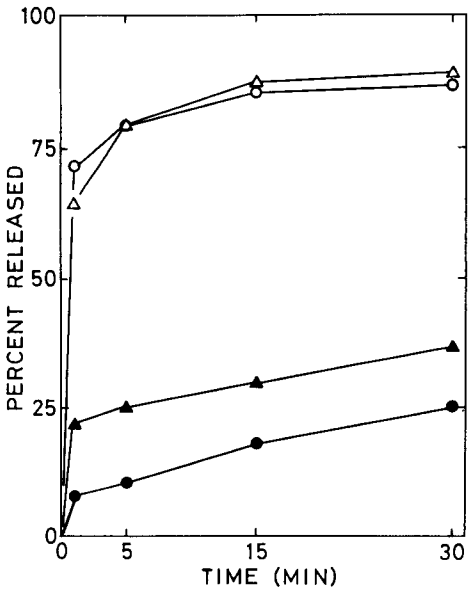

Fig. 5 Effect of ATP on repair patch ligation measured by the sensitivity of newly synthesized DNA to exonuclease III. UDS in bleomycin-pretreated permeable cells was measured at 37℃ for 10 min without ATP (△) or with 2.5 mM ATP (▲), or at 37℃ for 10 min without ATP and then chased for 50 min by the addition of 0.25 mM cold dTTP (○), or by the addition of 0.25 mM cold dTTP and 2.5 mM ATP (●). DNA extracted from the [^{3}H]dTMP-labeled permeable cells was incubated at 37℃ for the indicated intervals with exonuclease III, and the % of ^{3}H-nucleotides that were released from DNA was determined.

studied. Although the DNA synthesized in the absence of ATP was highly sensitive to exonuclease III, the DNA synthesized in the presence of 2.5 mM ATP, or in the absence of ATP and then chased by adding cold dTTP and 2.5 mM ATP was resistant to exonuclease III (Fig. 5). The result indicated that UDS measured in the presence of an appropriate concentration of ATP in bleomycin-pretreated permeable SR-C3H/He cells proceeded to completion and ligation of repair patches.

Discussion

Previously we established an *in vitro* DNA repair system using bleomycin-pretreated permeable HeLa cells (1). In the present paper, a DNA repair system using permeable mouse ascites sarcoma (SR-C3H/He) cells was established and characterized for comparison with the HeLa cell system. SR-C3H/He cells were selected because DNA repair of eukaryotic cells has been studied frequently using both rodent and primate cells, some differences in DNA repair between cells of these animals have been suggested (16), and our previous study (11) suggested a possible difference in the repair polymerase involved in bleomycin-induced UDS between permeable primate cells and rodent cells.

Some differences in UDS existed between SR-C3H/He cells and HeLa cells. The optimal NaCl concentration for UDS measured in the absence of ATP was clearly different between SR-C3H/He cells and HeLa cells. The optimal NaCl concentration was higher in the former (80-150 mM) than in the latter (40-60 mM). Aphidicolin sensitivity of UDS measured in the absence of ATP was also clearly different between SR-C3H/He cells (over 90% resistant) and HeLa cells (about 50% resistant) (11). These differences between bleomycin-pretreated permeable SR-C3H/He cells and HeLa cells were reduced when 2.5 mM ATP was added to the reaction mixture for repair DNA synthesis (Fig. 3, Table 1 and Ref. 1), although aphidicolin sensitivity of UDS measured in the presence of ATP was still lower in SR-C3H/He cells than in HeLa cells. It is known that purified DNA polymerase α, an aphidicolin-sensitive DNA polymerase, is strongly inhibited by 0.1 M KCl (or NaCl), and purified DNA polymerase β is stimulated by the same or even higher concentration of the salt (17). A possible explanation of why the optimal NaCl concentration for the bleomycin-induced UDS in permeable SR-C3H/He cells was reduced along with the appearance

of aphidicolin sensitivity by the addition of 2.5 mM ATP is that the UDS was accomplished by both a salt- and aphidicolin-sensitive DNA polymerase, which was stimulated by ATP (12), and salt-stimulated DNA polymerase β. As a consequence, the optimal salt concentration for UDS measured in the presence of 2.5 mM ATP in bleomycin-pretreated permeable SR-C3H/He cells would be determined by the relative contribution of each type of DNA polymerase. We have still no clear explanation of why the difference mentioned above existed between SR-C3H/He cells and HeLa cells. Considering that ATP is essential to DNA repair (12) and is present in intact cells at millimolar concentrations, and that the salt concentration in intact cells is approximately 0.1 M, we can study DNA repair mechanisms of both rodent and primate cells using the present bleomycin-pretreated permeable cell system at nearly physiological ionic strength and ATP concentration.

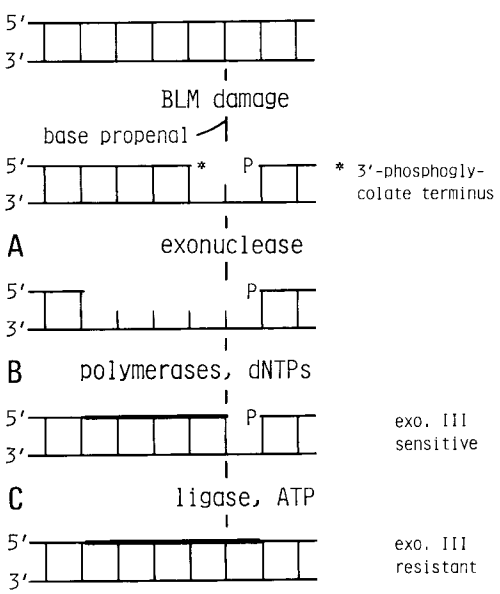

Fig. 6 A schematic representation of bleomycin-damaged DNA and the repair process. When DNA is damaged by bleomycin, base propenals are released, and single-strand breaks with 3′-phosphoglycolate and 5′-phosphate termini are formed. Exo. III sensitive and exo. III resistant indicate that the newly synthesized DNAs are sensitive and resistant to exonuclease III, respectively. A, priming step; B, DNA synthesis step; C, ligation step.

DNA excision repair processes in eukaryotic cells can be divided into four steps: priming (recognition of the DNA-damaged site and incision-excision), repair DNA synthesis, repair patch ligation and chromatin reorganization. DNA damage caused by bleomycin has been studied extensively, and the results indicate that the bleomycin-damaged ends in DNA have mostly phosphoglycolates at their 3′-termini and phosphates at their 5′-termini (5, 6). Bleomycin-induced DNA damage occurred in permeable cells (10). To repair the lesions, the damaged ends must be recognized and modified to short gaps with 3′-hydroxyl termini by a priming factor having an exonucleolytic activity. The presence of this type of exonuclease in permeable SR-C3H/He cells was indicated by the findings that bleomycin-induced repair DNA synthesis occurred actively in the permeable cells. The enzyme was partially purified and characterized (18, 19). Considering this and the results described previously, the present DNA repair system is thought to be useful for analytically studying the priming, repair DNA synthesis and ligation steps shown schematically in Fig. 6.

SR-C3H/He cells are useful for studying DNA repair, because both cultured cells and ascites cells are available, and a large amount of cells can be collected from ascites fluid.

Acknowledgments. The authors wish to thank Mr. T. Nakamura and Ms. T. Yasui for their technical assistance, Nippon Kayaku Co. for providing copper-free bleomycin A_2 and Dr. A.H. Todd of Imperial Chemical Industries for providing aphidicolin. This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1. Seki S and Oda T: DNA repair synthesis in bleomycin-pretreated permeable HeLa cells. Carcinogenesis (1986) **7**, 77-82.
2. Teoule R: Radiation-induced DNA damage and its repair. Int J Radiat Biol (1987) **51**, 573-589.
3. Feingold JM, Masch J, Maio J, Mendez F and Bases R: Base sequence damage in DNA from X-irradiated monkey CV-1 cells. Int J Radiat Biol (1988) **53**, 217-235.
4. Teebor GW, Boorstein RJ and Cadet J: The repairability of oxidative free radical mediated damage to DNA: a review. Int J Radiat Biol (1988) **54**, 131-150.
5. Giloni L, Takeshita M, Johnson F, Iden C and Grollman AP: Bleomycin-induced strand-scission of DNA. Mechanism of deoxyribose cleavage. J Biol Chem (1981) **256**, 8608-8615.
6. Burger RM, Projan SJ, Horwitz SB and Peisach J: The DNA cleavage-mechanism of iron-bleomycin. Kinetic resolution of strand scission from base-propenal release. J Biol Chem (1986) **34**, 15955-15959.
7. Henner WD, Rodoriguez LO, Hecht SM and Haseltine WA: γ Ray induced deoxyribonucleic acid strand breaks: 3' glycolate termini. J Biol Chem (1983) **258**, 711-713.
8. Seki S and Oda T: DNA synthesis in permeable mouse ascites sarcoma cells. Cancer Res (1977) **37**, 137-144.
9. Seki S and Oda T: DNA synthesis in detergent-treated mouse ascites sarcoma cells. Biochim Biophys Acta (1977) **476**, 24-31.
10. Seki S, Ikeda S and Oda T: Effects of nucleoside triphosphates and ferrous ions on bleomycin-induced unscheduled DNA synthesis. Carcinogenesis (1985) **6**, 853-858.
11. Seki S, Ohashi M, Ogura H and Oda T: Possible involvement of DNA polymerase α and β in bleomycin-induced unscheduled DNA synthesis in permeable HeLa cells. Biochem Biophys Res Commun (1982) **104**, 1502-1508.
12. Seki S, Mori S, Nakashima A and Oda T.: Effects of ATP and other nucleotides on DNA repair synthesis in bleomycin-pretreated permeable mouse sarcoma cells. Carcinogenesis (1987) **8**, 1391-1394.
13. Huberman JA: New views of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell (1981) **23**, 647-648.
14. Lee MYWT and Toomey NL: Human placental DNA polymerase δ: Identification of a 170-kilodalton polypeptide by activity staining and immunoblotting. Biochemistry (1987) **26**, 1076-1085.
15. So AG and Downey KM: Mammalian DNA polymerases α and δ: Current status in DNA replication. Biochemistry (1988) **27**, 4591-4595.
16. Lehmann AR and Karran P: DNA repair. Int Rev Cytol (1981) **72**, 101-146.
17. Hubscher U: DNA polymerases in prokaryotes and eukaryotes: Mode of action and biological implications. Experientia (1985) **39**, 1-25.
18. Seki S, Mori S and Oda T: Bleomycin-induced DNA synthesis in a cell-free system using a permeable mouse sarcoma cell extract. Acta Med Okayama (1987) **41**, 195-199.
19. Seki S and Oda T: An exonuclease possibly involved in the initiation of repair of bleomycin-damaged DNA in mouse ascites sarcoma cells. Carcinogenesis (1988) **9**, 2239-2244.

Received December 15, 1988; accepted February 7, 1989