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Bo Zhang* Shuji Seki† Kosuke Akiyama‡
Ken Tsutsui** Ting Li†† Kazutaka Nagao‡‡

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

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Detection and analyses by gel electrophoresis of cisplatin-mediated DNA damage.*

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Abstract

DNA damage induced by cis-diamminedichloroplatinum (II) (cisplatin: cis-DDP), an anticancer drug, was studied in vitro by monitoring the drug-induced conformational change of pUC18 plasmid DNA, the sensitivity to some restriction enzymes of the damaged DNA and the sequence-dependent termination of DNA synthesis caused by cisplatin. Closed circular, superhelical pUC18 DNA was treated at 37 degrees C for 16 h with various concentrations of cisplatin. Cisplatin-dose-dependent conformational change due to unwinding of the treated DNA was detected by agarose gel electrophoresis. To analyze the base-specificity of the cisplatin damage, the measurement for sensitivity of cisplatin-treated DNA to various types of restriction enzyme and sequence gel analysis of the treated DNA were conducted. The results suggested that cisplatin attacked preferentially the sequence of GG $>$ AG $>$ GNG in the order. In the present assay condition, the cisplatin/DNA nucleotide ratios required for the DNA damage detection were roughly 0.025 for the conformational analysis, 0.001 or more for the restriction enzyme analysis, and less than 0.001 for the sequence gel analysis. By using the present method, it was demonstrated that the cisplatin-mediated DNA damage was inhibited by NaCl, KCl, CaCl2 or MgCl2 at their nearly physiological concentrations, and by reducing agents such as thiourea and 2-mercaptoethanol in the reaction mixture.

KEYWORDS: DNA damage, cisplatin, gel electrophoresis, sequence gel analysis

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Detection and Analyses by Gel Electrophoresis of Cisplatin-Mediated DNA Damage

Bo Zhang[a], Shuji Seki*, Kosuke Akiyama, Ken Tsutsui, Ting Li[a] and Kazutaka Nagao

Department of Molecular Biology, Institute of Cellular and Molecular Biology, Okayama University Medical School, Okayama 700, Japan

DNA damage induced by cis-diamminedichloroplatinum (II) (cisplatin: cis-DDP), an anticancer drug, was studied *in vitro* by monitoring the drug-induced conformational change of pUC18 plasmid DNA, the sensitivity to some restriction enzymes of the damaged DNA and the sequence-dependent termination of DNA synthesis caused by cisplatin. Closed circular, superhelical pUC18 DNA was treated at 37°C for 16h with various concentrations of cisplatin. Cisplatin-dose-dependent conformational change due to unwinding of the treated DNA was detected by agarose gel electrophoresis. To analyze the base-specificity of the cisplatin damage, the measurement for sensitivity of cisplatin-treated DNA to various types of restriction enzyme and sequence gel analysis of the treated DNA were conducted. The results suggested that cisplatin attacked preferentially the sequence of GG > AG > GNG in the order. In the present assay condition, the cisplatin/DNA nucleotide ratios required for the DNA damage detection were roughly 0.025 for the conformational analysis, 0.001 or more for the restriction enzyme analysis, and less than 0.001 for the sequence gel analysis. By using the present method, it was demostrated that the cisplatin-mediated DNA damage was inhibited by NaCl, KCl, $CaCl_2$ or $MgCl_2$ at their nearly physiological concentrations, and by reducing agents such as thiourea and 2-mercaptoethanol in the reaction mixture.

Key words : **DNA damage, cisplatin, gel electrophoresis, sequence gel analysis**

Cisplatin is one of the most widely used anticancer drugs. It is used for testicular, ovarian, bladder, head and neck, and other carcinomas (1, 2). Although it is such a potent anticancer drug, the appearance of the resistant cells and/or its toxicity to several organs often limited the therapeutic value (1, 2). Several significant evidences such as altered DNA repair capacity (3-5), altered accumulation of platinum by the cell and increased intracellular levels of glutathione or metallothionein (2, 6) have been shown in connection with the characteristics of the resistant cells, although the exact, molecular mechanism of the cisplatin resistance has not yet been elucidated.

A large body of evidence indicates that cis-

* To whom correspondence sholud be addressed.
a: Present address: B.Z., Department of Pathology, Beijing Medical University, 100083 Beijing, China: T.L., Department of Pathology, China-Japan Friendship Hospital, 100029 Beijing, China.

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platin exhibits its biological activity by binding to DNA, consequently inhibiting various DNA functions such as DNA replication, transcription and DNA repair (1, 7). Easy methods for the detection and analyses of cisplatin-mediated DNA damage are thought to be important to promote the study of the biological activity of cisplatin.

In the present paper, the cisplatin-induced conformational change of plasmid DNA (8), sensitivity to some restriction enzymes of cisplatin-damaged DNA (9), and sequence dependent termination of DNA synthesis caused by cisplatin (10, 11) were studied in comparison with one another for the detection and analyses of cisplatin-mediated DNA damage, and the methods were applied to study the effects of inorganic salts and reducing agents on cisplatin-mediated DNA damage.

Materials and Methods

Materials. Cisplatin was kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan. Other reagents were purchased from the following sources: agarose from Nippon Gene Co., Ltd., Tokyo, ethidium bromide, NaCl, KCl, $MgCl_2$ and $CaCl_2$ from Nakarai Tesque Inc., Kyoto, restriction endonucleases from Toyobo Co., Ltd., Osaka, T4 polynucleotide kinase from Takara Co., Ltd., Tokyo, 7-deaza sequencing kit (containing 7-deaza-2'-dGTP and Sequenase) from U.S. Biochemical Corp., Ohio, [γ-^{32}P] ATP (7000 Ci/mmol, 160 mCi/ml) from ICN Biochemicals Inc., CA, USA.

Preparation of pUC18 DNA. Growth of HB101 cells (a strain derived from *E. coli*) transformed with pUC18 plasmid, amplification of the plasmid, harvesting and alkaline lysis of the bacteria, and purification of the plasmid DNA were conducted by the large-scale isolation procedure as described by Sambrook *et al.* (12). The DNA preparation about 90% in the superhelical form was further purified by polyethylene glycol (PEG) precipitation method. The resultant DNA was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Cisplatin-treatment of pUC18 DNA. The reaction mixture for the cisplatin-treatment of DNA contained 1 μg pUC18 DNA and cisplatin freshly diluted at an appropriate concentration with TE. In some experiments, various concentrations of reducing agents or inorganic salts were added to the reaction mixture. The final volume of the reaction mixture was adjusted to 20 μl with TE. Cisplatin was always the last addition. The mixture was incubated at 37°C for 16 h in the dark. The amount of cisplatin added to the reaction mixture was expressed as the molar ratio of cisplatin molecules and the nucleotides of pUC18 DNA. The concentrations of DNA nucleotides and cisplatin were 150 μM and 0.15–15 μM, respectively, in the standard incubation mixture.

Digestion with restriction endonucleases. The cisplatin-treated DNA digested with one of various restriction endonucleases in a reaction mixture (final volume: 20 μl) consisting of 10 μl (0.5 μg DNA) of cisplatin-treated DNA solution, 2 μl of a 10-fold-concentrated, appropriate buffer for the restriction enzyme, TE and 1 unit enzyme. The mixture was incubated at 37°C for 2 h.

Agarose gel electrophoresis. Agarose gels were prepared at 1% or 2% in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid and 0.002 M EDTA, pH 8.0) supplemented with or without ethidium bromide (EtdBr) at 0.5 μg/ml (12). The electrophoresis buffer was TBE buffer with or without 0.5 μg/ml EtdBr depending on the gel used. One volume of a six-fold-concentrated gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in distilled water) was added to five volumes of each sample. A portion of the solution containing 0.25 μg DNA was loaded into a slot of a submerged agarose gel. Electrophoresis was performed at 50 volts for 100 min using a Mini Gel Electrophoresis System (Mupid 2) of Cosmo Bio Co., Ltd., Tokyo. When the gel was electrophoresed without EtdBr, it was shaken gently for 60 min in TBE buffer supplemented with EtdBr at 0.5 μg/ml for DNA staining (12–14).

Sequence gel analysis of DNA damage by cisplatin. pUC18 DNA was treated with cisplatin as described above. Two kinds (M4 = 5'-GTTTTCCCAGTCACGAC-3' and P8 = 5'-AGCGGATAACAATTTCACACAGGAAAC-3') of DNA sequencing primers for pUC18 DNA were synthesized on an Applied Biosystems model 380B DNA synthesizer. 5' ends of these primers were labeled with radioactive phosphate by using T4 polynucleotide kinase and [γ-^{32}P] ATP. Briefly, the kination mixture (10 μl) containing 1 μl of 20 μM primer solution, 46 pmoles of [γ-^{32}P] ATP, 1 μl of 10-fold-concentrated T4 kinase buffer (0.7 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$ and 50 mM dithiothereitol) and 10 units of T4 polynucleotide kinase was incubated at 37°C for

was incubated at 37°C for 30 min, and then at 90°C for 3 min to inactivate the enzyme.

For DNA sequencing analysis, 3 μg of cisplatin-treated DNA or control pUC18 DNA was denatured in 0.2 N NaOH solution for 5 min at room temperature, and recovered by ethanol precipitation. The denatured, cisplatin-treated DNA or the control was hybridized with 1 pmole of the ^{32}P-labeled primer by warming at 68°C for 2 min and cooling slowly to room temperature. The primer was extended by 3.25 units of Sequenase (version 2.0; U.S. Biochemical Corp) at 37°C for 10 min in 15.5 μl of the reaction mixture containing 26.9 mM Tris-HCl, pH 7.5, 12.9 mM $MgCl_2$, 32.3 mM NaCl, 13.5 mM dithiothreitol, 40.5 μM each deoxynucleotide and 56.5 μg/ml bovine serum albumin. The reaction was stopped by adding of 11.1 μl of water and 17.7 μl of the DNA sequencing gel loading buffer in the Sequenase kit. The denatured, control DNA hybridized with the labeled primer was incubated for DNA synthesis by the Sanger's dideoxy-termination method (16). Three microliter each of these samples were heated at 100°C for 5 min and electrophoresed on a 6 % sequencing gel.

Results and Discussion

Cisplatin-mediated conformational changes of pUC18 DNA. Cisplatin-mediated conformational changes of pUC18 were observed by agarose gel electrophoresis basically as reported previously (8). The electrophoretic mobility on the agarose gel of cisplatin-treated, superhelical DNA diminished contrary to the increase of the molar ratio of platinum per DNA nucleotide (Fig. 1), as indicating that the degree of negative supercoiling of pUC18 DNA was reduced by the formation of platinum adducts (1, 8. 9). On the contrary, the mobility of nicked open circular DNA increased cisplatin-dose-dependently (Fig. 1). When the molar ratio of cisplatin per DNA nucleotide reached 0.1, the mobility of the cisplatin-damaged superhelical DNA became minimum and almost equal with that of the cisplatin-damaged open circular DNA. Further increase of the molar ratio of cisplatin per DNA nucleotide caused reversely increased mobility of the cisplatin-damaged DNA, as indicating that the higher binding levels of cisplatin induced positive supercoiling (Fig. 1) (1, 13, 17). The progressive unwinding of negative supercoiling of pUC18 DNA and proceeding to positive supercoiling as a function of increasing amounts of bound platinum suggest that its major binding mode, the d (GpG) intrastrand cross-link, contributes to the phenomenon (1, 17).

When the cisplatin-treatment of pUC18 was conducted at 37°C at 0.1 of the molar ratio of cisplatin per DNA nucleotide, the mobility change increased in accordance with the incubation time and reached maximum by 16-hour incubation (Fig. 2). The DNA treated at 0°C with cisplatin for 24 h did not show any change of the mobility. The results indicate that the formation of bifunctional DNA platinum (II) adducts inducing the conformational change depends on the incubation time and temperature. The cisplatin-induced relaxation of pUC18 DNA reached the maximum at 0.1 of the molar ratio of cisplatin per DNA nucleotide and by incubating for 16 h (Fig. 1). The molar ratio of 0.1 and 16 h incubation at 37°C were used in the following experiments,

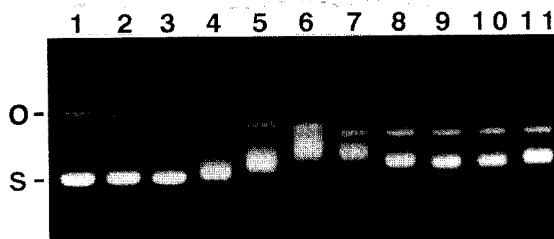

Fig. 1 Effects of varying concentrations of cisplatin on conformation of pUC18 plasmid DNA. The reaction mixture (20 μl in final volume) for cisplatin treatment contained 1 μg pUC18 DNA, varying amounts of cisplatin, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. After having been incubated at 37°C for 16 h in dark, 5 μl (0.25 μg DNA) of the mixture was taken out and mixed with 1 μl of 6-fold-concentrated gel loading buffer. The sample was loaded into a slot of a 1 % agarose gel and electrophoresed at 50 volts for 100 min. The gel was stained with EtdBr (0.5 μg/ml) for 60 min. Channels (1-11) correspond to DNA samples incubated in the molar ratio of cisplatin and nucleotide of pUC18 DNA at 0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1 and 2.5. Abbreviation used: S, superhelical DNA; O, nicked, open circular DNA.

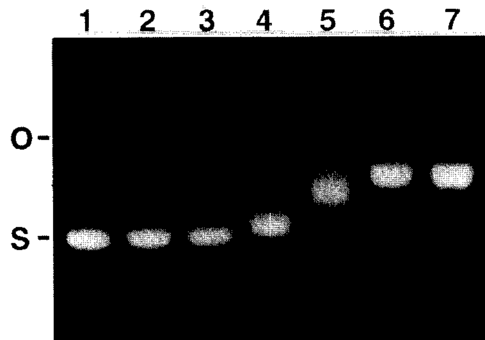

Fig. 2 Platination kinetics of pUC18 DNA measured by the conformational change. The reaction mixture (20 μl in final volume) for platination contained 1 μg pUC18 DNA (150 μM DNA nucleotides), 15 μM cisplatin, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. The mixture was incubated at 37 °C for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 4 h (lane 4), 8 h (lane 5), 16 h (lane 6), 32 h (lane 7). The agarose gel electrophoresis and the DNA staining with EtdBr were performed as described in Materials and Methods and legend to Fig. 1.

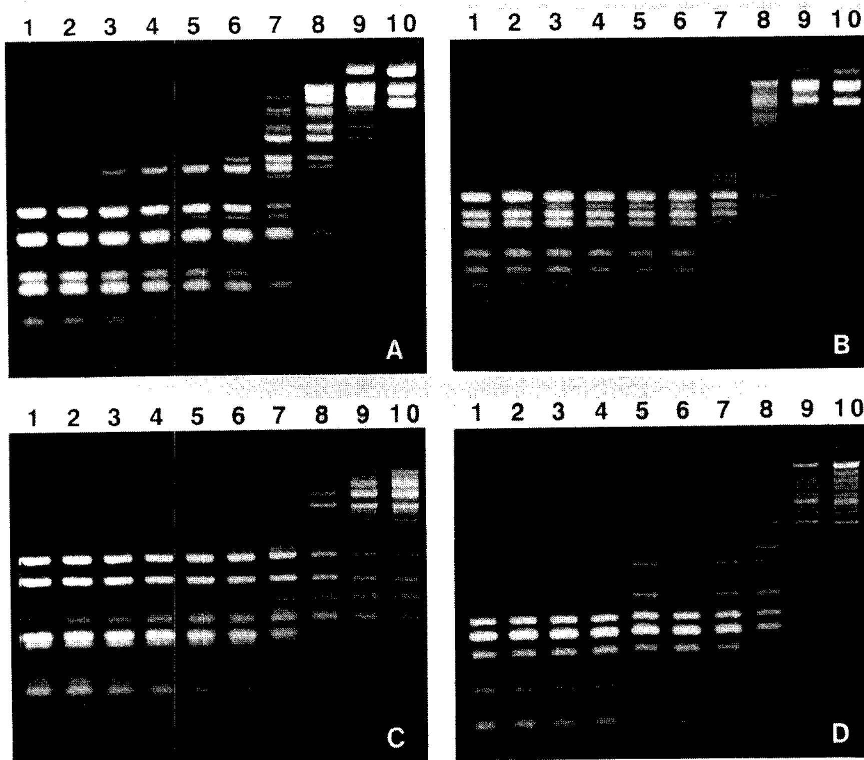

Fig. 3 Electrophoresis in agarose gels following digestion of platinated pUC18 DNA with various restriction enzymes. The reaction mixture (20 μl in final volume) for cisplatin treatment contained 1 μg pUC18 DNA, cisplatin at the indicated concentrations, 10 mM Tris-HCl and 1 mM EDTA. After having been incubated at 37 °C for 16 h in dark, 10 μl (0.5 μg DNA) of the mixture was taken out and mixed with 1 unit of a restriction enzyme and 2 μl of 10-fold-concentrated, appropriate ionic strength buffer for the enzyme (12). The final volume was adjusted to 20 μl with TE (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The mixture for restriction enzyme digestion was incubated at 37 °C for 2 h, and then mixed with 4 μl of 6-fold-concentrated gel loading buffer (12). Twelve μl (0.25 μg DNA) per lane of the solution was loaded into a 2 % agarose gel containing 0.5 μg/ml EtdBr, and electrophoresed in the presence of 0.5 μg/ml EtdBr at 50 volts for 100 min. Channels (1-10) correspond to DNA samples incubated in the molar ratio of cisplatin and nucleotide of pUC18 DNA at 0, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.025, 0.05, 0.075 and 0.1. Digested with *Hae*III (A), *Hpa*II (B), *Alu*I (C) and *Hha*I (D).

otherwise indicated. The detection limit of the cisplatin-induced conformational change in the present system was the molar ratio of 0.025 (Fig. 1). The diminished mobility of the cisplatin-damaged superhelical DNA was not observed when DNA was electrophoresed in the presence of ethidium bromide at 0.5 μg/ml.

Analysis of cisplatin-damaged DNA by restriction endonucleases. Cisplatin forms adducts regioselectively with DNA (1). The platinated sites are known to be resistant to the restriction enzymes which recognize and cleave the initial, non-platinated sequence (1, 9, 18). Platination kinetics can be analyzed by using pUC18 DNA, whose restriction sites are known, and appropriate restriction enzymes. We used several restriction enzymes to detect and analyze the platinated sites. In the present experiment, the gel electrophoresis of restriction enzyme-digested, platinated DNA was conducted in the presence of ethidium bromide at 0.5 μg/ml to avoid confusion due to the coexistence of cisplatin-mediated, conformationally changed molecules and restriction fragments. To analyze the site-dependent (regioselective) platination efficiency, we used first single cut-restriction endonucleases for pUC18 DNA such as *Sma*I (recognition sequence, CCC-GGG) and *Eco*RI (GAATTC). About a half molecules of pUC18 DNA became *Sma*I resistant when it was treated by cisplatin at the 0.01 molar ratio per DNA nucleotide, and the DNA became completely resistant to the enzyme when it was treated at the 0.05 molar ratio (data not shown). Cisplatin-treated DNA was more resistant to *Sma*I than to *Eco*RI, as indicating that the*Sma*I site was platinated more easily than the *Eco*RI site.

Further studies were conducted using restriction enzymes having multiple cutting sites on pUC18 DNA. They were *Hae*III (recognition sequence, GGCC; 10 cut), *Hpa*II (CCGG; 12 cut), *Alu*I (AGCT; 15 cut) and *Hha*I (GCGC; 17 cut). Digestablility by these enzymes of cisplatin-treated pUC18 DNA decreased cisplatin-dose-dependently (Fig. 3). The digestability decrease was detected by the appearance of partially digested fragments and concomitantly decrease of the restriction fragments formed by digesting completely the undamaged pUC18 DNA with the same enzyme. The digestability

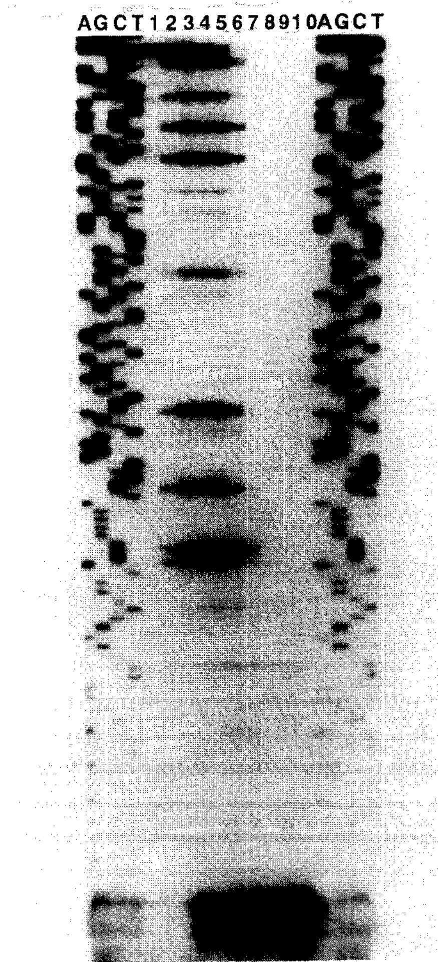

Fig. 4 Sequencing gel analysis of reaction products by Sequenase with platinated pUC18 DNA template. pUC18 DNA was treated with cisplatin as described in Materials and Methods and legend to Fig. 1. The molar ratio of cisplatin and pUC18 DNA nucleotide was 0 in lane 1, 0.001 in lane 2, 0.0025 in lane 3, 0.005 in lane 4, 0.0075 in lane 5, 0.01 in lane 6, 0.025 in lane 7, 0.05 in lane 8, 0.075 in lane 9 and 0.1 in 10. The primer used was P8 described in Materials and Methods. AGCT lanes in left and right sides are sequence ladders determined by Sanger's dideoxy-termination method (16) using non-platinated pUC18 DNA and primer P8.

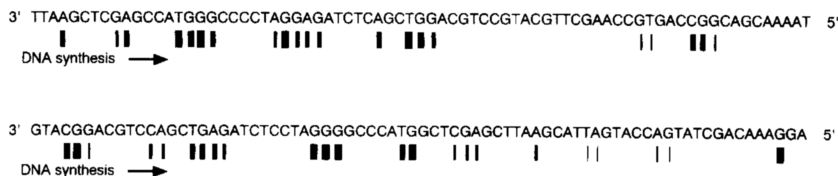

Fig. 5 Schematic diagram showing the arrest sites of Sequenase on platinated pUC18 DNA strand. Experiments were conducted as described in Materials and Methods. A part of the results used for this diagramatic representation is shown in Fig. 7. The result obtained using the primer M4 is also shown schematically. The width of the bar represents the intensity of each band, which is roughly estimated.

decrese was detected at the 0.001 molar ratio of cisplatin per DNA nucleotide by *Hae*III, 0.0025 molar ratio by *Hpa*II and *Alu*I, and 0.0075 molar ratio by *Hha*I (Fig. 3A, B, C and D). pUC18 DNA treated with cisplatin at the 0.1 molar ratio per DNA nucleotide was highly resistant to *Hae*II and *Hpa*II, and cleaved only at a single site. Whereas, *Alu*I and *Hha*I digested the DNA treated by cisplatin at the 0.1 molar ratio into multiple fragments. These results indicate the platinated DNA is resistant to *Hae*III, *Hpa*II, *Alu*I and *Hha*I in the order.

Considering the recognition sequences and cutting sites of these restriction enzymes, the sequences of GGCC, CCGG, AGCT and GCGC are thought to be attacked readily in the order by cisplatin and become resistant to the corresponding restriction enzyme.

Sequencing gel analysis of cisplatin-mediated DNA damage. Sequence-dependent termination of the DNA synthesis by cisplatin was monitored on DNA sequencing gels using the large fragment of DNA polymerase I (10). We conducted a similar experiment using pUC18 DNA platinated at the present condition and Sequenase as a DNA polymerase. When the drug concentration was increased from the 0.001 to 0.1 molar ratio of cisplatin per DNA nucleotide, DNA chain elongation was decreased markedly (Fig. 4). The sequence-dependent termination of *in vitro* DNA synthesis by cisplatin is shown schematically in Fig. 5. The chain termination occurred most frequently at oligo (dG) regions of the platinated template strand, and also occurred at 3'-d(ApG)-5' and less frequently at d(GpNpG). Although all stop bands appeared in correlation with guanine base on the template strand, actual stop bands were variable depending on the regional sequence of the template strand

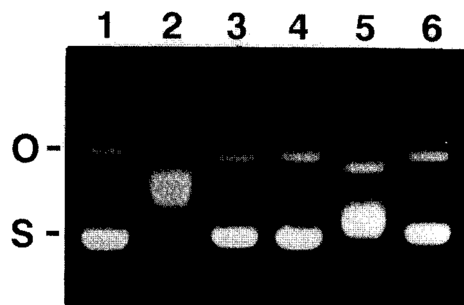

Fig. 6 Effects of some salts on platination of pUC18 DNA measured by the conformatinal change. The reaction mixture (20 μl in final volume) for cisplatin treatment contained 1 μg (150 μM pUC18 DNA, a salt at the concentration indicated below, 15 μM cisplatin, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. After having been incubated at 37°C for 16 h in dark, 5 μl (0.25 μg DNA) of the mixture was taken out and mixed with 1 μl of 6-fold-concentrated gel loading buffer. The sample was loaded into a slot of a 1% agarose gel and electrophoresed at 50 volts for 100 min. The gel was stained with EtdBr (0.5 μg/ml) for 60 min. Lane 1, the original preparation of pUC18 DNA; lane 2, platinated pUC18 DNA; lane 3, pUC18 DNA treated with cisplatin in the presence of 100 mM NaCl; lane 4, in the presence of 100 mM KCl; lane 5, in the presence of 2 mM $CaCl_2$; lane 6, in the presence of 5 mM $MgCl_2$.

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(Fig. 4 and 5). For example, the major stop band(s) was the complementary base(s) to the thymine on a 3'-d(CpTpGpGp)-5' sequence, to the cytosine and first guanine on 3'-d(CpGpG)-5'sequence, and to adenine on 3'-d(ApG)-5' sequence of the template strand. These sequence-dependent major stop bands were for Sequenase, but DNA polymerase used is shown to affect the sequence-dependent termination (10, 11). Sequencing gel analysis of cisplatin-mediated DNA damage was sensitive method for detecting DNA platinated as low as the 0.001 mole of cisplatin per DNA nucleotide (Fig. 4).

Application of the present electrophoretic analysis. Effects of some salts and reducing agents on cisplatin-mediated DNA damage were anlyzed using the present method. Sodium chloride (100 mM), potassium chloride (100 mM), magnesium chloride (5 mM) and calcium chloride (2 mM) at their nearly physiological concentrations, all inhibited cisplatin-mediated DNA damage (Fig. 6). Inhibitory effects of reducing agents such as 2-mercaptoethnanol (10 mM) and thiourea (10 mM) on cisplatin-mediated DNA damage were also demonstrated by the present system (Fig. 7). The effect of ascorbic acid on platination of DNA was not clear, because ascorbic acid itself induced double-strand breaks of pUC18 DNA (Fig. 7).

We are trying to find a repair enzyme for cisplatin-damaged DNA using the present detection methods of cisplatin-mediated DNA damage and the activity blotting technique for detecting DNA repair enzymes in cell extracts (19. 20). The study may be important for understanding practically the mechanisms of the development of cisplatin resistance in tumors and nephrotoxicity of the drug but also the basic cellular mechanism of DNA repair.

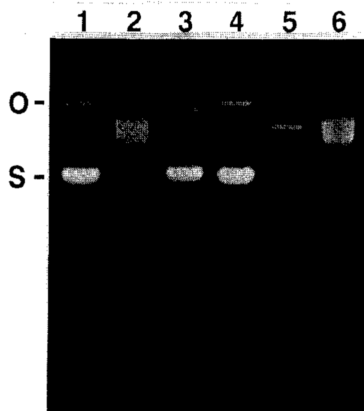

Fig. 7 Effects of some reducing agents on platination of pUC18 DNA measured by conformatinal change. The reaction mixture (20 μl in final volume) for cisplatin treatment contained 1 μg (150 μM) pUC18 DNA, a reducing agent at the concentration indicated below, 15 μM cisplatin, 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. After having been incubated at 37°C for 16 h in dark, 5 μl (0.25 μg DNA) of the mixture was taken out and mixed with 1 μl of 6-fold-concentrated gel loading buffer. Agarose gel electrophoresis and gel staining with EtdBr were perfomed as described in legend to Fig. 10. Lane 1, the original preparation of pUC18 DNA; lane 2, platinated pUC18 DNA; lane 3, pUC18 DNA treated with cisplatin in the presence of 10 mM thiourea; lane 4, in the presence of 10 mM 2-mercaptoethanol; lane 5, in the presence of 10 mM sodium ascorbate; lane 6, in the presence of 10 mM glucose.

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