MOLECULAR AND CELLULAR BIOLOGY, Apr. 1991, p. 2075–2080
0270-7306/91/042075-06$02.00/0
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Vol. 11, No. 4

Cross-Resistance to UV Radiation of a Cisplatin-Resistant Human Cell Line: Overexpression of Cellular Factors That Recognize UV-Modified DNA

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Received 24 October 1990/Accepted 16 January 1991

A human cell line selected for cisplatin resistance (CPR) was irradiated with UV light and showed cross-resistance to UV light. Applying a modified chloramphenicol acetyltransferase assay, we observed that CPR cells acquired enhanced host cell reactivation of a transfected plasmid carrying UV damage. Gel mobility shift analysis indicated that two nuclear factors that recognize UV-modified DNA were overexpressed in CPR cells. In addition, factors that bind UV-modified DNA were independent from the factors that bind cisplatin-modified DNA. The significance of the identified binding factors, possibly DNA repair enzymes, is discussed.

cis-Diamminedichloroplatinum(II) or cisplatin is a widely used chemotherapeutic agent in clinical trials for a variety of cancers. Although it is a potent drug, the resistance of cancer cells often hinders the treatment (25, 33). An understanding of the mechanism of cisplatin resistance (CPR) is necessary for the development of a powerful treatment regimen, and it therefore becomes an urgent matter. An effective, although not perfect, way to attain this goal is through studies done in a cell culture model, partly because established cell lines or cancer cells are readily demonstrated to be resistant to drugs through the mechanism of gene amplification (13, 37–40) or other types of mutation (16). Substantial lines of indirect evidence have shown that CPR in human cell cultures is associated with enhanced DNA repair (1, 12, 19, 21, 23, 26, 31, 41, 42). This idea is supported by studies of host cell reactivation of transfected plasmids (5, 7, 36). However, there is nothing in the literature that documents thus far evidence in any depth for the mechanism of resistance.

It has been reported that cells acquiring CPR also developed resistance to alkylating agents and other types of DNA-damaging chemicals (e.g., see references 14 and 34). It is therefore reasonable to speculate that CPR cells have also developed resistance to physical types of damaging agents, such as UV light, whose lesion involves a bulky repair mechanism (15). High enzymatic activity for DNA repair in these cells is expected. This can result from the mutation of repair enzymes with persistent high activity or from overexpression of the repair enzymes. The latter situation can be monitored by the nature of DNA-protein binding because the interaction of the repair enzymes with the damaged DNA target is a prerequisite for effective DNA repair. In fact, nuclear extracts from human and yeast repair mutants showed reduced ability to interact with damaged DNA (8, 30).

In this study, we demonstrated the cross-resistance of CPR cells to UV light. Enhanced DNA repair was detected in CPR cells by a modified chloramphenicol acetyltransferase (CAT) analysis. Using a gel mobility shift assay, we identified two cellular factors that recognize UV-modified DNA (UVMD). Overexpression of the UVMD binding activity was observed in CPR cells. In addition, factors that interact with cisplatin-modified DNA (CMD) were independent of UVMD binding activity.

MATERIALS AND METHODS

Cell lines and determination of UV resistance. CPR cells were isolated by stepwise exposure of HeLa cells to cisplatin (Platiamine; Farmitalia Carloerba Ltd.) by gradually increasing the concentration of the drug up to a maximum of 8 μM (5). CPR and parental cells were maintained in monolayer culture in Dulbecco modified Eagle medium (GIBCO Laboratories, Gaithersburg, Md.) containing 10% (vol/vol) fetal bovine serum, 100 μg of streptomycin per ml and 100 units of penicillin per ml and incubated at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2 in air. Control cell lines VA13 (WI-13 subline; American Type Culture Collection) and XP (xeroderma pigmentosum complementation group A; American Type Culture Collection) were maintained according to the manufacturer's instructions.

The acquired resistance of cells to UV light is defined as the ratio of F_{37} (UV fluence at 37% survival) of CPR cells to that of parental cells. Cytotoxicity was assayed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric method (28). Using this assay, CPR cells showed 15- to 20-fold resistance to cisplatin.

UV irradiation and platination of plasmid DNA. pRSVcat and f130 DNAs (see below) at a concentration of 100 μg/ml were irradiated in the dark with UV germicidal lamps as described previously (6). DNA was irradiated with a fluence rate 25 J/m^2/s from a VL-100C UV irradiation unit (Vilbert Lourmat, France). The fluence rate was measured by a VLX-254 radiometer (Vilbert Lourmat). The f103 DNA (see below) was treated in the dark with cisplatin in 3 mM NaCl–1 mM Na phosphate (pH 7.4) at 37°C for 18 to 24 h. This treatment, according to Ushay et al. (39), generated an estimated molar ratio of free cisplatin to nucleotide phosphate of $r_f = 0.008$.

DNA-mediated gene transfer. A modification of the technique of Chu et al. (10) was used to introduce pRSVcat DNA

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into the cells. Cells were seeded at 3×10^6 cells per 100-mm plate 1 day before electroporation. Cell suspensions of 1 ml in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer were added to a sterile cuvette containing 20 μg of pRSVcat plasmid, gently mixed, and subjected to electroporation by a GenePulser (Bio-Rad). Conditions of 1,000 μF and 250 V were typically used. After resting for 10 min at 25°C after transfection, cells were returned to the incubator. The following day, the medium was replaced with fresh medium and incubated for another 48 h to allow expression. Cells were then harvested into 1 ml of phosphate-buffered saline and centrifuged. The cell pellet was then either stored at −80°C or processed for CAT assay (see below).

Modified CAT assay. The CAT assay (17, 18) was adapted to study the host cell reactivation of transfected plasmid. pRSVcat contains the bacterial *cat* gene inserted between the 3' long terminal repeat of Rous sarcoma virus and simian virus 40 polyadenylation sequences to make a gene transcription unit. The expression of the *cat* gene in transfected cells depends on the intactness of the gene (7, 22, 24, 32). The transfected cell pellet was resuspended in 0.1 ml of 0.25 M Tris-HCl (pH 7.5), subjected to sonication at 4°C (120 W, 2 min), and centrifuged in a microcentrifuge at 4°C for 10 min. The supernatant containing the CAT activity was extracted twice with 150 μl of ethyl acetate, evaporated to dryness under vacuum, and subsequently suspended in 20 μl of ethyl acetate. The assay for CAT enzymatic activity contained, in a total volume of 150 μl, 116.5 μl of H_2O, 25 μl of 1 M Tris-HCl (pH 7.5), 2 μl (0.05 μCi) of [^{14}C]chloramphenicol (54 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), 1.5 μl of 40 mM acetyl coenzyme A, and 5 μl of cell extract. The reaction mixture was incubated at 37°C for 1 h and then developed on a silica thin-layer chromatography plate (Merck, Darmstadt, Federal Republic of Germany). After autoradiography, material on the thin-layer chromatography plate corresponding to the modified chloramphenicol was cut out, and its radioactivity was counted in a toluene-PPO (2,5-diphenyloxazole) scintillant. Activity is calculated as percentage of [^{14}C]chloramphenicol converted into acetylated derivatives.

DNA probes and gel mobility shift assay. The DNA fragment f130 was the 130-bp *Sph*I-*Bgl*I fragment from pSVT (2), ligated to *Sph*I-*Sma*I-opened pBS(+) (Stratagene), with the 5' recessed *Bgl*I site filled in with Klenow fragment, and ligated. The *Hin*dIII-*Eco*RI f130 fragment containing a 17-bp AT-rich region is a potential target for UV modification. The DNA fragment f103 was the 103-bp *Stu*I-*Ava*II fragment from pcD-α-globin (29), filled and attached to *Eco*RI and *Xba*I linkers, respectively, and cloned into pBS(+). The f103 fragment containing the 14-bp dG · dC-rich region is a potential target for cisplatin modification. *Hin*dIII-*Eco*RI-generated f103 and f130 fragments were ^{32}P labeled (3×10^4 cpm/ng of DNA) and purified in spin columns by standard methods (35). The labeled DNA fragments were irradiated with 1,000 J of UV light per m^2 and used as a probe for a gel mobility shift assay.

Nuclear extracts were prepared by the method of Dignam et al. (11). The protein concentration was measured by the method of Bradford (3). The gel mobility shift assay was performed as previously described (4, 20). Briefly, the UVMD probe was incubated with nuclear extracts at 30°C for 30 min. The reaction mixtures were then subjected to 4% polyacrylamide gel electrophoresis under low ionic strength at 25°C and 15 mA constant current. Resolved gels were directly exposed to Kodak XAR-5 X-ray film without further processing. Relative expression of DNA binding factors was determined by scanning desitometry of the X-ray film.

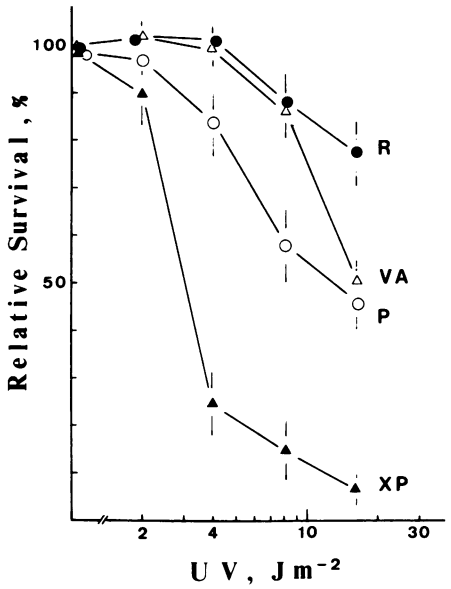

FIG. 1. Cytotoxicity dose-response curves of parental HeLa (P), CPR (R), and VA and XP control cells determined by colorimetric MTT assay (see Materials and Methods for details). Points with error bars (standard deviation) show the mean of five separate experiments.

RESULTS

Cross-resistance of CPR cells to UV light. Sensitivity of CPR and parental HeLa cells to UV light was analyzed by colorimetric MTT assays (see Materials and Methods for details). Known repair-competent VA13 cells (VA) and repair-deficient XP complementation group A cells (15) (XP) were included as controls. The data were quantitated and are shown in Fig. 1. F_{37} (UV fluence at 37% cell survival) was approximately 7 and 20 J/m^2 for parental and CPR cells, respectively. A threefold resistance was acquired by the CPR cells. The difference in the survival pattern between CPR and parental cells was evident at high UV fluence, i.e., greater than 15 J/m^2. This is somewhat different from the survival pattern observed in XP and VA cells, in which an apparent difference in sensitivity appeared at a much lower fluence, i.e., 4 J/m^2.

Enhanced host cell reactivation of transfected plasmids. A modified CAT assay was applied to monitor cellular ability to repair DNA. pRSVcat was treated with increasing amounts of cisplatin before transfection into cells for transient expression analysis (see Materials and Methods for details). Relative CAT activity was quantitated from silica thin-layer chromatography autoradiographs and is presented in Fig. 2. For comparison, the quantitated relative CAT activity of VA and XP cells is also indicated in Fig. 2. For this control experiment, a difference in CAT activity became evident at low UV fluence. This is in contrast to the apparent divergence in the pattern at relatively high UV fluences observed for CPR and parental HeLa cells, as is also observed in cytotoxicity studies.

Overexpression of UVMD binding activity in CPR cells. Since accessibility of DNA repair enzymes to the damaged DNA is necessary for effective repair, the interaction be-

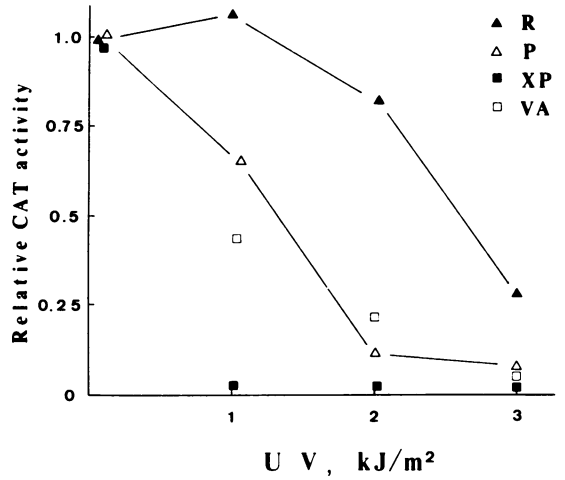

FIG. 2. Host cell reactivation of UV-irradiated pRSVcat plasmid in CPR (R), parental HeLa (P), and XP and VA cells. Relative CAT activity for the above cells is shown as a function of UV fluence. The estimated error due to uncertainties in transfection and the CAT assay was about 20% as ascertained from three experiments.

tween nuclear factors and DNA was measured by gel mobility shift assays. Optimal conditions for the assays were determined after titration of the amounts of nuclear extracts. When 2 μg of poly(dI-dC) and UVMD probes were included in the binding reactions, the 2-μg extracts gave a consistent specific binding band (Fig. 3, b) in both CPR and parental cell extracts. These optimized conditions were used for the following dose-response and competition studies. In Fig. 3, binding activity for CMD is indicated with a B. When UVMD was used as a probe, two binding activities from the nuclear extracts were identified (Fig. 4, b1 and b2) that increased with the levels of UV damage, suggesting that the binding activity is UVMD specific. b2 is equivalent to b in Fig. 3 (also see the legend to Fig. 4). The CPR nuclear extracts showed a three- to fourfold-stronger UVMD binding activity than parental activity (Fig. 4, compare R and P). A significant UVMD binding activity was also observed in the

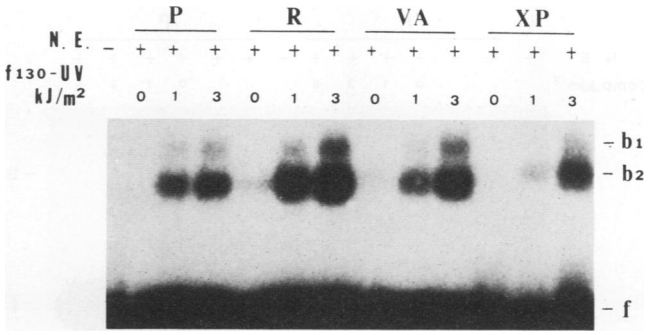

FIG. 4. Binding activity specific for UVM DNA in parental HeLa (P), CPR (R), and VA and XP control cells. Nuclear extracts (N.E.) of 2 μg were used for each binding reaction, with the UVMD probe carrying various fluences of UV light as indicated. b1 and b2 (equivalent to b in Fig. 3), Bound DNA; f, unbound DNA probe. The minor DNA binding activity, b1, was seen after prolonged autoradiography.

control VA and XP cells. This was confirmed by competition assays which showed that increasing the concentration of UVMD as a competitor effectively competed for UVMD binding activity in both the CPR and parental HeLa cell extracts (Fig. 5). This conclusion was supported further by an alternative competition in which a fixed amount of unlabeled f130 DNA was used as the competitor. With increasing levels of UV damage, the competition became apparent (Fig. 6). Therefore, the overexpression of UVMD binding activity in CPR cells is specific.

Competition of UVMD binding activity by CMD. To verify that the UVMD binding activity is independent from CMD binding activity, we performed a combination competition assay. For this purpose, CMD competitor was used for UVMD binding studies (Fig. 7, left panel). UVMD competitor was used for CMD binding studies (Fig. 7, right panel). It is interesting that 30 ng of CMD (i.e., 100-fold molarity) was able to compete slightly for UVMD binding activity. Similarly, 30 ng of UVMD competed for CMD binding.

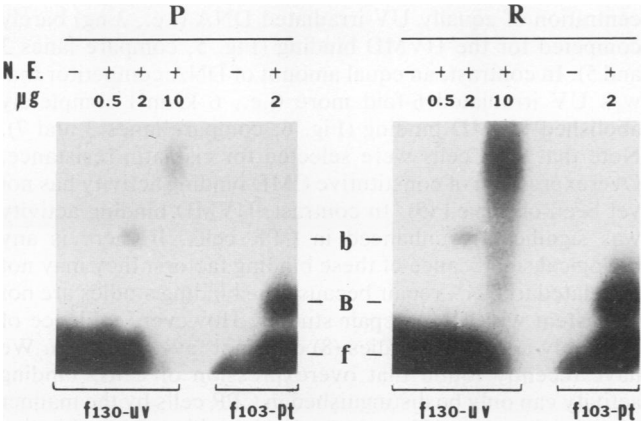

FIG. 3. Identification of binding activity specific for UVMD. Nuclear extracts (N.E.) were incubated with the following DNA probes: f130-UV, 1-kJ/m² UV-modified f130 fragment; f103-Pt, cisplatin-modified f103 fragment (as described in Materials and Methods). b, Bound UVMD; B, bound CMD; f, unbound DNA probe; P, parental HeLa cells; R, CPR cells.

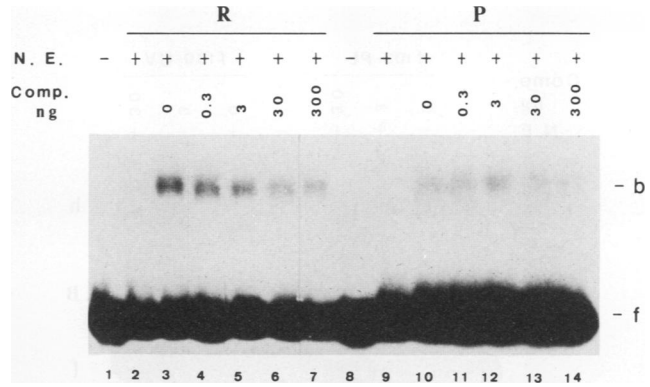

FIG. 5. Competition of UVMD binding activity with increasing amounts of UVMD in CPR (R) and parental HeLa (P) cells. A 0.3-ng sample of UVMD probe was incubated for 30 min before competitor (Comp.) was added in the indicated amounts (lanes 3 to 7 and 10 to 14). An additional 30 min of incubation followed for competition. Control lanes: 1 and 8, without nuclear extract (N.E.); 2 and 9, unmodified f130 probe. b, Bound DNA; f, unbound DNA.

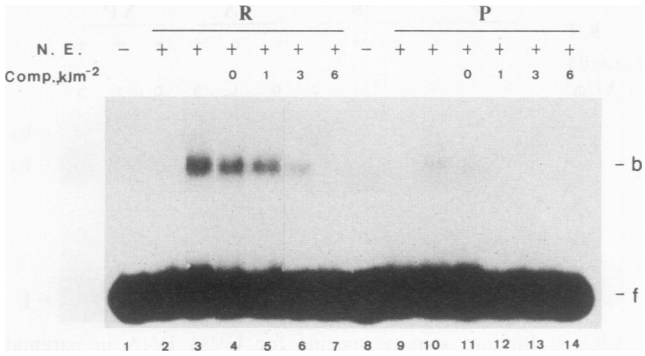

FIG. 6. Competition of UVMD binding activity with equal molarity of unlabeled f130 DNA carrying increasing UV damage in CPR (R) and parental HeLa (P) cells. Competitors (Comp.) with the indicated UV damage were added 30 min after the incubation of nuclear extract (N.E.) with the probe (lanes 4 to 7 and 11 to 14). Control lanes: 1 and 8, without nuclear extract; 2 and 9, unmodified f130 probe; 3 and 10, without competitor. b, Bound DNA; f, unbound DNA.

DISCUSSION

We identified at least two UVMD binding activities in human cells. This binding activity was overexpressed in a cell line selected for CPR. Since CPR HeLa cells acquired enhanced host cell reactivation of transfected plasmid (representing DNA repair), it is reasonable to speculate that the identified UVMD binding activity is associated with DNA repair functions and probably represents repair enzymes. The basis for this speculation follows. First, the levels of acquired DNA repair in CPR cells are similar to the overexpressed UVMD binding activity. Second, repair-defective XP cells showed less UVMD binding activity than repair-competent VA cells. However, one should also recognize that when comparing these two different experiments in which a small fragment is the target for UV light (in plasmid reactivation) compared with the large genomic DNA (in the cytotoxicity assay), there is an enormous difference in target size.

According to genetic complementation assays, human DNA repair may depend on at least nine *trans*-acting factors (15). It is reasonable to speculate that at least one binding factor specific for DNA damage can be monitored by a gel mobility shift assay. In fact, we identified at least two UVMD binding activities. The others among the nine repair factors are either not directly involved in DNA binding or they do exist but at an amount below the sensitivity of detection. The detection of factors that bind modified DNA in cell extracts provides a powerful tool for studies on DNA-damaging agents. First, a labeled DNA probe that has been damaged can be used as a probe to clone a gene(s) for a binding factor(s) and possibly a repair enzyme(s) by screening an expression library. Second, a DNA column can be established for the isolation of binding factors depending on the nature of the DNA used. Third, a similar well-designed DNA probe modified with other DNA-damaging agents can also be used to identify corresponding binding factors. In addition, this methodology may become an effective tool to screen mutagens and carcinogens. Therefore, in general, the significance of our findings should be crucial.

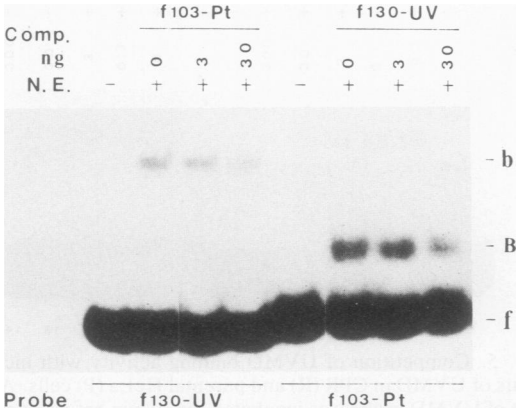

FIG. 7. Competition of UVMD binding activity with CMD. Probes are indicated at the bottom. Amount of competitors (Comp.) is indicated at the top. b, Bound UVMD; B, bound CMD; f, unbound DNA probe.

However, several immediate questions arise. For example, how are UVMD and CMD able to compete with each other? UVMD and CMD may share a common binding domain for the binding factor(s). This is considered because both the UV- and cisplatin-induced DNA damages are repaired through a bulky repair pathway (15). Alternatively, both UVMD and CMD binding may involve multiple domains containing a site for unmodified DNA. The observed competition therefore may not be specific for the lesion of the DNA molecule; instead, a domain of the competitor binds to an unmodified region of the treated DNA molecule. If the unmodified bound region is near the modified DNA region, this nonspecific binding can affect the specific binding. One should be aware that a DNA molecule treated with either UV light or cisplatin is not 100% modified along the entire molecule; large proportions of the DNA molecule remain intact. We hypothesize that at least one domain of the UVMD or CMD binding factor fits the unmodified DNA and may be more flexible in the domain interaction between molecules than the domain specific for the damaged DNA region. This is supported by the observations that unmodified DNA could also compete for UVMD and CMD binding (4b, 8). It is also confirmed by differently designed competition studies. For example, increasing to 10-fold the concentration of equally UV-irradiated DNA (i.e., 3 ng) barely competed for the UVMD binding (Fig. 5, compare lanes 3 and 5). In contrast, an equal amount of DNA competitor that was UV irradiated 6-fold more (i.e., 6 kJ/m^2) completely abolished UVMD binding (Fig. 6, compare lanes 3 and 7). Note that CPR cells were selected for cisplatin resistance. Overexpression of constitutive CMD binding activity has not yet been observed (9). In contrast, UVMD binding activity was significantly enhanced in CPR cells. If there is any biological significance of these binding factors, they may not be related to DNA repair because the binding studies are not consistent with DNA repair studies. However, evidence of our study and other studies (8) does not favor this idea. We have recently found that overexpression of CMD binding activity can only be distinguished in CPR cells by the manner of induction (4a). Therefore, the identified DNA binding activity may still be relevant to DNA repair. A further understanding of CPR or UV resistance can be facilitated by the molecular cloning of binding factors identified in this study. Efforts aiming toward this goal are currently in progress.

ACKNOWLEDGMENTS

We express our appreciation to Delon Wu and Chau-Hsiung Chang for encouragement and Mary Helen Lu Young for a critical reading of the manuscript.

This research was supported by grants from Chang Gung Medical College (CMRP256) and the National Science Council, Republic of China (NSC79-0412-B182-50) to C.C.-K.C. and by a visiting specialist fellowship from the National Science Council, Republic of China, to S.L.-C.

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