Mitomycin-induced Chromatid Breaks in HeLa Cells: A Consequence of Incomplete DNA Replication

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ABSTRACT

The formation of chromosome aberrations induced by alkylating agents such as mitomycin C has been shown to require the passage of the treated cell through S phase. However, the exact mechanisms by which mitomycin C-induced DNA lesions are translated into chromosome aberrations during S phase are not known. The purpose of these studies was to better understand the molecular basis of chromosome aberration formation after mitomycin C treatment. The morphology of metaphases of cells treated in G1 phase with mitomycin C resembled that of prematurely condensed chromosomes of S-phase cells. Consequently we postulated that chromosome aberrations resulted from cells reaching mitosis without completing DNA replication. This was tested by treating HeLa cells in G1 phase with mitomycin C and then analyzing these cells at mitosis for residual DNA damage and DNA content. Utilizing the DNA alkaline elution assay for DNA damage, we showed that HeLa cells progress through S phase into mitosis with intact DNA-DNA interstrand cross-links. These cross-links, originally induced into parental DNA, were associated equally with parental and newly replicated DNA at the time the cells reached mitosis. This suggests that recombination events had taken place during the DNA replication process. Cells that were treated in G1 phase and allowed to proceed to mitosis in the presence of bromodeoxyuridine to density label newly replicated DNA were analyzed with cesium chloride density sedimentation. Unreplicated DNA was present in the mitotic cells of the treated populations but not in the untreated control cells. Further, flow cytometric measurements, made under hypotonic conditions in order to reduce chromatin condensation effects, demonstrated that the mitotic cells from the mitomycin C-treated populations contained 10–20% less DNA than untreated mitotic controls. These results indicate that chromatid breaks induced by mitomycin C are the result of cells reaching mitosis without having fully completed DNA replication.

INTRODUCTION

Many chemical and physical agents used in the treatment of malignancy are known to produce DNA lesions that ultimately result in chromosome aberrations (1). While the existence of chromosome aberrations has been recognized since Muller's first description in 1927 (2), little is known about how specific types of DNA lesions are translated into aberrations at the chromosomal level (for reviews, see Refs. 3 to 5). The purpose of the studies reported here was to gain a better understanding of how DNA damage induced by MMC might cause the formation of one type of chromosome aberration, e.g., the chromatid break.

Mitomycin C, a therapeutic drug used in the treatment of malignancy, covalently binds to the DNA of cells and produces both monoadducts and DNA-DNA and DNA-protein cross-links, most likely involving the O6 position of guanine (6). MMC-induced damage results in a reduction in the rate of DNA replication and a dose-dependent delay in cell cycle progression (7, 8). At the chromosome level, MMC is thought to produce chromosome aberrations by means of an S-phase dependent mechanism; i.e., chromosome aberrations are not observed unless the treated cell has undergone replicative DNA synthesis following the induction of DNA damage (9). When the treated cells eventually reach mitosis, chromatid-type breaks and exchanges are observed (10).

The chromatid breaks after MMC treatment are noteworthy in that the deleted regions can be quite lengthy in appearance, suggesting that a portion of the genome might be missing. Similarly, highly damaged metaphase spreads closely resemble S-phase cells which have been induced into premature chromosome condensation. These observations led to the hypothesis that cells containing MMC-induced damage can eventually attain mitosis without having fully replicated their DNA. Some chromatid breaks would therefore be the result of unreplicated chromosome regions.

If this hypothesis holds, the cells that reach mitosis after MMC treatment should exhibit several distinct characteristics. (a) The damaged mitotic cells should still contain MMC-induced DNA lesions. (b) These cells should contain a portion of their DNA in an unreplicated form. (c) The treated cells should exhibit a diminished DNA content upon reaching mitosis. In this paper we have tested and confirmed these predictions using a variety of techniques including DNA alkaline elution (to detect residual DNA-DNA and DNA-protein cross-links), CsCl density sedimentation (to detect unreplicated DNA), and flow cytometry (to detect mitotic cells with diminished DNA content). Thus, it is likely that MMC-induced chromatid breaks are a consequence of incomplete DNA replication caused by the presence of residual MMC-induced DNA lesions.

MATERIALS AND METHODS

Cell Culture, Cell Synchronization, and Drug Dilution. HeLa cells were grown as monolayer cultures on Lux 150-mm dishes in Hsu's modified McCoy's Medium 5A (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (K C Biological, Lenexa, KS), 3 mM l-glutamine (GIBCO), penicillin (100 µg/ml) (GIBCO), streptomycin (100 µg/ml) (GIBCO), and 0.1 mM CaCl2 (M CB, Norwood, OH). Cultures were maintained at 37°C in a humidified 5% CO2 incubator. Cells were passaged when they reached confluence, and were subcultured with 0.125% trypsin (GIBCO). The procedures for obtaining HeLa mitotic cells were described previously (11). Briefly, exponentially growing cells were treated with 2.5 mM dThd for 22–24 h, released from the dThd block, and then accumulated in mitosis in the presence of 90 psi nitrous oxide. After selective detachment of mitotic cells, this procedure routinely yielded populations with a mitotic index of at least 95%. Upon release from nitrous oxide (N2O), the cells completed mitosis and entered G1 phase in a synchronous wave. MMC (Mutamycin, Bristol Laboratories, Syracuse, NY) was dissolved just before use in sterile distilled water to a 100-µg/ml stock solution and then diluted in McCoy's Medium 5A with 1% fetal calf serum to a final concentration of 1 µg/ml.

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4 The abbreviations used are: MMC, mitomycin C; BrdUrd, bromodeoxyuridine; dThd, thymidine; HH, heavy-heavy; HL, heavy-light; LL, light-light; PCC, prematurely condensed chromosomes.

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Experimental Protocol. The general experimental protocol was identical for every type of study described in this paper. HeLa cells were synchronized to mitosis and selectively detached, and 4 x 10^6 cells were plated per 150-mm Petri dish. The cells were allowed to divide, attach to the dishes, and progress into G1 phase. Five hours after plating, the G1 cells (labeling index, ≤1%) were treated with MMC (1 μg/ml) for 1 h, rinsed 3 times with medium, and reincubated in fresh McCoy's medium with 10% fetal calf serum. After 18 h, the old medium containing floating cells was removed, and fresh medium was added. Cells were then accumulated in mitosis in the presence of N2O, and mitotic cells were selectively detached for analysis after appropriate time intervals.

For the alkaline elution and CsCl analyses, the cells were radioactively labeled with [3H]dThd (0.02 μCi/ml) (40-60 Ci/mm) for two full cell cycles prior to the first synchronization. For the studies concerning damage to newly replicated DNA, [3H]dThd was added to the cultures after MMC treatment. The procedure for alkaline elution has been previously described (12). For the CsCl assays of unreplicated DNA, BrdUrd (20 μg/ml) was added after MMC treatment.

Neutral Cesium Chloride Density Centrifugation. After drug treatment and cell cycle progression, mitotic cells were accumulated in N2O, selectively detached, centrifuged, and resuspended in Tris buffer (0.15 M NaCl, 0.01 M EDTA, 0.1 M Tris, pH 8.1) to a concentration of 10^7/ml. Cells were lysed by addition of Sarkosyl to 0.1%, incubated for 30 min at 60°C with RNase A (80 μg/ml) (Millipore Corporation, Freehold, NJ), and then incubated for 1 h at 60°C with Pronase C (0.5 mg/ml) (Calbiochem-Behring Company). The cell lysate was mixed with an equal volume of Sevag chloroform/isomyl alcohol (24:1, v/v) at 4°C. After centrifugation, the aqueous phase was removed, and the Sevag procedure was repeated until the interface between the sample and Sevag was clear. The DNA was then precipitated with 2 volumes of 95% ethanol at 0°C and collected by a 20-min centrifugation at 4°C and 10,000 rpm. The DNA was resuspended in 0.15 M NaCl, 0.01 M Na2HPO4, (1:100) and used in the preparation of the neutral CsCl density gradients as previously described (13). Individual gradient fractions were mixed with 1 ml of water and 10 ml of Scintiverse II (Fisher Scientific, Houston, TX), and radioactivity was measured with a Packard Model 2650 liquid scintillation spectrometer.

Flow Cytometry Analysis. Samples of mitotic cells designated for flow cytometric DNA content analysis were washed in physiological saline and then exposed to either hypotonic 0.075 M KCl or physiological saline for 25 min in the presence of Colcemid (0.05 μg/ml). Following centrifugation (3 min at 150 g), the cell pellets were resuspended in 1 ml of 0.5% Pepsin (Accurate Chemicals, Hicksville, NY) and held for 4 min at 37°C. After centrifugation, the pellet was resuspended in 1 ml of 0.5% trypsin (Worthington Biochemical Corporation, Freehold, NJ) containing 0.05% EDTA (pH 8.0) and 0.8 mg/ml N-ethylmaleimide. After digestion, the cell pellet was treated with 0.1 M HCl (pH 1.0) for 10 min, followed by 0.5 M NaCl, 0.01 M Na2HPO4, (pH 7.4) to neutralize the HCl. After centrifugation, the DNA was resuspended in 0.25 ml of 1x standard saline citrate (pH 6.0), fixed in suspension with 70% ethanol (4°C), and held at 4°C until analysis.

For DNA fluorochrome staining, 2 x 10^6 cells were resuspended in Dulbecco's phosphate-buffered saline containing 1 mm MgCl2 and centrifuged, 1 ml of 0.5% pepsin (Accurate Chemicals, Hicksville, NY) was added, and the solution was held for 4 min at room temperature. Digestion was stopped by the addition of 2.5 ml of cold Tris buffer (0.1 M Tris base:0.1 M NaCl:0.84 M HCl, pH 7.4). After centrifugation, the cell suspension was resuspended in 1.5 ml of ethidium bromide (25 μg/ml) (Sigma Chemical Co., St. Louis, MO) for 10 min, followed by the addition of 1.5 ml of mithramycin (50 μg/ml) (Worthington Biochemical Corporation, Freehold, NJ) containing 30 mM MgCl2. Immediately before analysis each sample received 3 drops of 1% RNase (Worthington) and was filtered through a 65-μm nylon mesh. The integral DNA fluorescence emission per cell was measured with an ICP-11 Phywe instrument interfaced to a Nuclear Data 256 channel pulse height analyzer equipped with an HP-26 48A terminal and tape storage device (14, 15).

Cell Fusion. The procedure for cell fusion has previously been described in detail (16). Briefly, mitotic HeLa cells (mitotic index ≥95%) were fused with an equal number of MMC-treated HeLa cells using UV-induced fusion. After fusion, the cells were exposed to hypotonic, fixed in methanol/glacial acetic acid (3:1, v/v) dropped on clean, wet slides, and stained with Giemsa.

RESULTS

Visualization of Chromosome Damage after MMC Treatment. When HeLa cells were synchronized in G1 phase, treated for 1 h with MMC, and then allowed to progress to mitosis, chromosome breaks, gaps, and exchanges were observed in metaphase preparations that were not seen in the untreated controls (Fig. 1). In addition, some MMC-treated cells exhibited unusually long chromatid deletions. In Fig. 1B, 2 and 3, the MMC-treated cells prepared with an equal number of MMC-treated HeLa cells using UV-induced fusion. After fusion, the cells were exposed to hypotonic, fixed in methanol/glacial acetic acid (3:1, v/v) dropped on clean, wet slides, and stained with Giemsa.
Fig. 1. Chromosome morphology of mitomycin C-treated cells. Synchronized HeLa cells were treated in G₁ phase with or without MMC for 1 h, washed free of drug, and reincubated in drug-free medium to allow cell cycle progression. A, mitotic figure of untreated control; B, mitotic figure of MMC-treated cell demonstrating chromosome damage with many apparent large chromatid deletions (arrows); C, S-phase PCC of an untreated control cell; D, S-phase PCC of MMC-treated cell blocked in S phase demonstrating continuity of single and double chromatid regions (arrows). × 1500.

(7 and 10% for the control and MMC treatment, respectively) suggests that most of the cross-links induced by MMC are DNA-DNA rather than DNA-protein cross-links.

To determine whether cells treated in G₁ phase could enter mitosis despite residual DNA damage, prelabeled HeLa cells were treated in G₁ phase with MMC and allowed to progress to mitosis, and then mitotic cells were selectively detached and analyzed by alkaline elution. As shown in Fig. 3A, the MMC-treated cells exhibited a slight decrease in DNA retention compared to the controls (○ versus ●, respectively). This suggested the presence of DNA strand breaks or alkaline-labile sites in the MMC-treated cells. When the two populations were irradiated on ice just prior to elution to allow the detection of residual cross-links, the parental DNA of the MMC-treated cells still exhibited increased DNA retention on the filters compared to the controls, suggesting the presence of residual cross-links in the MMC-treated cells that attained mitosis (● versus ○, respectively). Proteinase K digestion prior to elution indicated that the cross-links remaining in the treated cells at mitosis were DNA-DNA interstrand type.

It is not understood how the treated cell might replicate past cross-linked DNA, but it is feasible that the DNA synthesized by the cells after MMC might contain breaks in the regions of residual cross-links in the parental DNA. To test this, the parental DNA was prelabeled prior to MMC treatment in G₁ phase with [14C]dThd, and then the cells were incubated in [3H]dThd during the S phase after MMC treatment to label the newly replicated DNA. As before, cells that accumulated at mitosis were selectively detached and analyzed by DNA alkaline elution. Surprisingly, the elution rate of 3H-labeled DNA synthesized after the MMC treatment was equally retarded as that of the parental DNA (Fig. 3B). This suggests that DNA synthesized after MMC treatment also contained DNA-DNA cross-links.

That DNA-DNA interstrand cross-links that were present in equivalent amounts in the parental and newly replicated DNA after MMC treatment might be a consequence of one of three mechanisms. (a) The conditions used for liquid scintillation counting or double radioactive labeling might have produced an artifact. (b) The formation of MMC-induced cross-links might occur by a two-step mechanism over a period of hours similar to that described for cis-platinum (20), nitrosoureas (21), and melphalan (22). (c) A recombinational mechanism might be involved during replication past cross-links (see “Discussion”).

The first mechanism was tested by not prelabeling the paren-
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tal DNA but labeling only the newly replicated DNA after MMC treatment. The elution profiles of these cells after proteinase K digestion were very similar to those seen in the double label experiments (data not shown). This result indicates that the double radioisotope labeling procedure was not interfering with the assay.

The second possible explanation involves a time-dependent mechanism for cross-link production. If interstrand cross-links were formed in two discrete steps over a period of time, the maximum cross-link levels produced would not be observed immediately after MMC treatment but at some later time (i.e., 6–17 h after drug treatment). Thus if the first step of cross-link formation occurred in Gₐ phase while the second step occurred after some DNA replication had occurred, it would be possible for newly replicated DNA to be involved in cross-links. To determine whether MMC forms cross-links by a similar mechanism, the parental DNA was prelabeled with [³⁵C]dTdh, and the cells were treated with MMC as before in Gₐ phase. Treated and control populations were harvested for alkaline elution analysis at 0, 6, and 15 h after MMC treatment. To allow detection of cross-links, the cells were irradiated on ice with 400 rads of γ-rays just prior to elution. As shown in Fig. 4A, fewer cross-links were present in the MMC-treated cells 6 h after treatment compared to that observed immediately after treatment as evidenced by a decreased DNA retention (∇ versus Δ, respectively). The decrease in retention for the MMC-treated cells was not due to the initiation of DNA synthesis because increased elution kinetics was not observed in the unirradiated control samples (data not shown), and the number of nicks produced by the irradiation is large compared to the number of nicks associated with DNA replication. Thus the drop in filter retention reflected repair of DNA-DNA cross-links. Similarly, the degree of cross-linking evident in the parental DNA was also decreased at 15 h posttreatment when compared to that observed immediately after treatment (Fig. 4B). Since the maximum cross-link levels were observed immediately after MMC treatment, these results suggest that a delayed two-step mechanism for cross-linked formation could not explain the finding that cross-links become associated with DNA synthesized after MMC treatment.

Demonstration of Unreplicated DNA in Mitotic Cells after MMC Treatment. The cytological observations described earlier suggested that some MMC-treated cells might enter mitosis without completing DNA replication. If so, the cells reaching mitosis would be expected to contain some unreplicated DNA. This was tested by prelabeling cells with [³H]dThd for two cell generations, synchronizing to Gₐ phase, and treating with MMC as before. After drug treatment, the cells were reincubated in medium containing BrdUrd to density-label the newly replicated DNA. After appropriate periods of time, the cells attaining mitosis were accumulated in N₂O, selectively detached (mitotic index, >95%), and the DNA was isolated and subjected to neutral CsCl density gradient analysis. In this assay, BrdUrd-
Fig. 4. Alkaline elution analysis of cell populations at various times after MMC treatment in G1 phase compared to that found immediately after MMC treatment. A, 0 and 6 h post-MMC treatment; B, 0 and 15 h post-MMC treatment. •, untreated controls immediately after sham MMC treatment; O, untreated controls at 6 or 15 h after sham treatment; A, MMC-treated cells immediately after treatment; D, MMC-treated cells at 6 or 15 h after treatment. All populations were irradiated on ice just prior to DNA elution.

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containing replicated DNA can be separated from unreplicated DNA on the basis of density.

As shown in Fig. 5A, the untreated control mitotic preparations yielded one major DNA peak present at a density of 1.75 g/cm³, typical of DNA replicated once in the presence of BrdUrd. By weight, the LL region of the control comprised 3.8% of the gradient compared to 85.8% in the HL region. In contrast, DNA from the MMC-treated populations selected at mitosis contained two distinct peaks, with 18.5% of the gradient in the LL peak (1.71 g/cm³) and 67.8% in the HL peak (1.75 g/cm³) (Fig. 5B). In a repeat experiment, approximately 15% of the gradient of the MMC-treated sample was found in the LL region, typical of unreplicated DNA. Since the mitotic indices of the analyzed populations were all greater than 95%, these results suggest that the MMC-treated populations are reaching mitosis with some unreplicated DNA.

Detection of Decreased DNA Content in Mitotic Cells after MMC Treatment. The preceding experiments showed that cells that enter mitosis after MMC treatment still contain detectable DNA-DNA interstrand cross-links and some unreplicated DNA. If chromosome breaks were a consequence of the cell’s inability to complete DNA replication past the residual DNA damage, one would also expect these drug-treated cells to have a deficiency in DNA content at mitosis. To test this, the DNA contents of control and MMC-treated populations selected at mitosis were determined by flow cytometry using ethidium bromide and mithramycin as the fluorescent dyes (23, 24).

Since some drugs have been shown to interfere with mithramycin staining (25), and since the presence of interstrand cross-links could conceivably diminish the binding of the intercalating dye ethidium bromide, it was first necessary to ensure that MMC treatment alone would not affect the DNA content measurements. To test this, HeLa mitotic cells were treated with up to 100 µg of MMC per ml for 1 h in the presence of Colcemid, and then the cells were analyzed for DNA content. No difference in the measured DNA content due to MMC treatment was observed (data not shown). Similar results were also obtained in MMC-treated mitotic cells exposed to a hypotonic solution (0.075 M KCl) for 25 min prior to fixation. Thus, the presence of even high levels of DNA-DNA cross-links does not interfere with the accurate determination of DNA content.

Fig. 5. Cesium chloride analysis of mitotic populations of cells treated in G1, with or without MMC, washed free of drug, reincubated in medium containing BrdUrd to density label newly replicated daughter DNA, and selectively detached at mitosis. A, untreated controls; B, MMC-treated population. Note the presence of a significant light-light peak in the MMC-treated population demonstrating the presence of unreplicated and/or recombined DNA.
To determine whether cells treated in G₁ could come to mitosis with a deficiency in DNA content, HeLa cells were treated in G₁ with MMC, selectively detached at mitosis as described above. The cells were then analyzed by flow cytometry using human peripheral blood lymphocytes as an internal DNA content marker. The DNA content measurements for the control (mitotic index, 95%) and MMC-treated (mitotic index, 96%) cell populations are shown in Fig. 6A. While it was expected that the drug-treated cells would exhibit less fluorescence than the controls due to a diminished DNA content, just the opposite was observed (modal peak channel numbers of 52 and 50 for the MMC-treated and control populations, respectively). These results suggested that either the MMC-treated cells reached mitosis with an increased DNA content or that the chromatin of MMC-treated cells was more open, allowing increased dye binding. The latter notion is possible because it has previously been shown that mitotic cells blocked with Colcemid for prolonged time periods have their peak channels diminished by as much as 20% (26). Also, PCC studies have shown that MMC treatment induces chromatin elongation during the repair process (27). To minimize the effects of chromatin condensation differences on DNA content measurements, the MMC-treated and control populations were exposed to hypotonic solution (0.075 M KCl) prior to fixation and staining with mithramycin and ethidium bromide. Preliminary studies showed that the greatest dye binding was observed in cells swollen with hypotonic solution for 25 min (data not shown). As shown in Fig. 6B, when the MMC-treated and control populations were analyzed by flow cytometry after hypotonic exposure, the control cells now showed increased DNA-bound dye when compared to the MMC-treated cells (modal peak channel numbers of 57 and 52, respectively). Thus the MMC-treated cells appear to reach mitosis with a decreased DNA content.

Interestingly, with hypotonic treatment, the control population increased in peak channel number from 50 to 57 (a net 14% increase), while the MMC-treated cells remained at channel 52. The result with the control population is consistent with the observation of others that hypotonic treatment increases dye binding (28, 29). The contrasting result with the MMC-treated cells suggests that the chromatin of these cells is already in a conformation that allows maximum dye binding. Assuming a HeLa cell in G₁ phase contains 17 pg of DNA while a normal human lymphocyte contains 6.5 pg of DNA, the MMC-treated cells appear to be lacking 9–10% of their expected DNA when they reach mitosis. In a repeat experiment, a 20% difference in DNA content was observed between the MMC and control mitotic populations. These figures are on the same order of magnitude as those obtained by cesium chloride measurements of the amount of unreplicated DNA in the MMC-treated populations.

**DISCUSSION**

The purpose of this study was to determine how MMC-induced DNA lesions present during S phase induce chromosome aberrations. The preceding experiments demonstrate that MMC-treated cells reach mitosis without completing either DNA damage repair or DNA replication and with a decreased DNA content. This suggests that chromosome breaks result from incomplete DNA replication prior to mitosis. A similar idea has been previously postulated to explain the formation of breaks after fluorodeoxyuridine treatment (30).

DNA alkaline elution analysis of the cells reaching mitosis demonstrated measurable residual DNA-DNA cross-links in the mitotic populations of cells that had been treated in G₁ with MMC. The fact that cross-links can persist in cells for extended periods of time is consistent with results from several other studies. Palitti et al. (31) showed that some MMC-induced damage remains in Chinese hamster ovary cells until G₂ phase, since their repair could be inhibited with hydroxyurea, aphidicolin, or caffeine. Murnane and Byfield (32) reported that a portion of the cross-links induced in C3H10T½ cells by nitrogen mustard, phosphoramide mustard, or melphalan was not repairable even though the cells were cross-link repair proficient if challenged with a second drug dose. Similarly, Sognier and Hittelman (33) reported that, although some initial repair was observed during the first 48 h after MMC treatment of density-inhibited normal human fibroblasts, measurable cross-linking remained in the cells for at least 96 h after a 1-h treatment with...
MMC (3 µg/ml). Slow repair kinetics of MMC-induced cross-links was also reported by Dorr et al. (34). These studies all suggest that a fraction on the cross-links induced in cellular DNA might be irreparable. Indeed, some evidence indicates that there might be regions of the genome that are resistant to repair functions (35).

Since DNA damage in these studies was measured with alkaline elution, only the presence or absence of breaks and cross-links was measured. However, MMC induces monoadducts at 5 times the frequency of cross-links (36). It is therefore not possible to exclude a contributory role for monoadducts on the effects observed. However, it is unlikely that monoadducts are primarily responsible for these results for several reasons. (a) Monofunctional alkylating agents are less efficient inducers of chromosome aberrations than polyfunctional alkylating agents (37); (b) O6-alkyl guanine adducts are poor inducers of damage induced by bifunctional MMC is potentiated by hydroxyurea in G2 while damage induced by monofunctional MMC is not (31).

It is not known how cells replicate past lesions such as intact DNA-DNA cross-links. Cleaver has suggested that the presence of two inhibitory DNA lesions in a replicon might prevent synthesis of the DNA between the lesions (41). Interestingly, however, our DNA alkaline elution data suggest that the DNA-DNA cross-links become associated with DNA synthesized after drug treatment. Since control experiments showed that this result was not a consequence of a delayed two-step mechanism of cross-link formation nor an artifact of the radioisotope labeling and counting conditions, this finding suggests that some form of recombinogenic process takes place when the cell replicates past these DNA lesions. One possible mechanism for this process is illustrated in Fig. 7 where two cross-links within a replication unit prevent the completion of DNA synthesis between the cross-links. If a recombinogenic process occurred at or near the cross-links, the resultant structure would satisfy our observations; i.e., (a) cross-links would be present and would be distributed in both parental and daughter DNA; (b) both parental and daughter DNA would also have single strand breaks; and (c) a portion of the genome would remain unreplicated.

A number of investigators have attempted to demonstrate the existence of recombination in mammalian cells by looking for the presence of HH DNA in CsCl density gradients. However, only very small amounts of HH DNA (i.e., 0.1–0.2%) were ever detected in these reported studies (42, 43), and it was believed that recombination probably did not occur in mammalian cells. On the other hand, recent evidence has suggested that recombination can occur in mammalian cells after damaging treatment. Using UV endonuclease to probe the location of thymidine dimers in 3T3 cellular DNA after UV treatment, Meneghini et al. (44) showed that dimers originally induced into parental DNA were transferred to newly synthesized DNA. Earlier studies had failed to show this effect, since it mainly occurs soon after damage induction and declines thereafter. Subsequently, Fournace (45) confirmed Meneghini’s results using DNA alkaline elution. Interestingly, a mammalian enzyme similar to the RecA protein in E. coli, known to be involved in recombinational events, has recently been isolated (46).

We have shown in this paper that cells reaching mitosis after treatment in G1 phase with MMC contain unreplicated DNA and exhibit a decreased DNA content when compared to untreated control populations. In previous reports, other investigators reached a different conclusion when they measured DNA content after either UV irradiation and caffeine (47) or X-ray treatment (48). Although these studies involved different damaging agents, it is possible that diminished DNA content was actually present in the treated populations but was not detectable using standard flow cytometry methods. The results presented here suggest that an altered chromatin configuration might be present in the damaged cells that allows increased fluorescent dye uptake compared to the control cells. Indeed, it has recently been demonstrated that agents such as UV irradiation and alkylating agents induce a chromatin decondensation process that can be visualized in the interphase PCC (27, 49–51) as well as in mitotic figures (52). However, hypotonic treatment of the cell populations prior to flow cytometry analysis decreases the influence of chromatin condensation differences between the two populations and allows a more accurate estimation of DNA content.

The results of this study raise the question of how a cell can proceed to mitosis with unrepaired and unreplicated DNA. The transition to mitosis in treated cells can be thought of as a product of two counterbalancing processes. First, as the cell proceeds through the cell cycle, chromosome condensation/mitotic factors gradually accumulate to prepare the cell for mitosis (53, 54). On the other hand, cell fusion studies have shown that both S-phase cells and damaged quiescent cells can block G2 cells from progressing to mitosis (55), and extracts from damaged cells have been shown to contain factors that bind to and inactivate mitotic factor activity (56, 57). Thus, when a cell has unrepaired or extensive damage, it often becomes blocked in S or G2 phase, and only the least damaged cells reach mitosis (58, 59). Eventually, however, the decondensation process may subside, and the accumulated mitotic condensation factors finally force the cell to enter mitosis even though neither DNA repair nor DNA replication is complete. A similar situation has been observed in cells that exhibit a temperature-sensitive mutation for S-phase functions (60). After periods of time at nonpermissive temperature, these cells still undergo chromosome condensation without completion of DNA synthesis and exhibit an S-phase PCC morphology. Further, these cells can induce PCC in other cells, suggesting that even though they cannot complete DNA replication, they still continue to produce chromosome condensation factors (61). Taken together, the results of this study suggest the following model for chromosome break production. Due to the presence of residual DNA lesions, the treated cells are unable to complete DNA replication. Nevertheless, some cells continue to prepare for and eventually enter mitosis. The unreplicated portion of
the chromatin would then appear as a chromatic deletion at mitosis.

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