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Inhibition of Deoxyribonucleic Acid Synthesis and Replicon Elongation in Mammalian Cells Exposed to Methyl Methanesulfonate

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SUMMARY

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DNA synthesis in Chinese hamster V-79 cells exposed to the alkylating agent methyl methanesulfonate (MMS) was investigated by radioactively labeled thymidine incorporation, followed by Geiger counting, liquid scintillation counting, cell radioautography, and DNA fiber radioautography. Exposure of randomly dividing cells to MMS results in a rapid, dose-dependent decline in the rate of cellular DNA synthesis, followed later by an apparent recovery in the rate of DNA synthesis. The inhibition of DNA synthesis cannot be explained by effects on cell progression or thymidine transport, or by any apparent effects on thymidine metabolism. Analysis of the lengths of labeled segments in DNA fiber radioautograms indicated that MMS significantly decreases either the rate or extent of DNA chain elongation. This inhibition is only temporary, however, since by 6 or 10 hr following treatment with 1 or 3 mM MMS, respectively, the lengths of replication segments return to control values. The degree of inhibition of cellular DNA synthesis following exposure to MMS parallels the inhibition of DNA chain elongation but is quantitatively greater over the entire time course of the experiment. This suggests that exposure to MMS results in constant but protracted inhibition of replicon initiation events. These findings are discussed in terms of the lesions that MMS is known to produce in DNA and their possible relationship to two known mechanisms of inhibition of DNA synthesis.

INTRODUCTION

For the purpose of replication, the DNA in a eukaryotic chromosome is divided into tandemly arranged segments called replicons. Operationally, a replicon can be considered to be a region of duplex DNA, somewhere between 15 and 100 μm in length, in which is centered an origin of replication and which is flanked by somewhat loosely defined termini. Upon initiation of DNA synthesis, a replication "bubble" is postulated to form by localized denaturation of the DNA at the origin of replication. Replication forks then diverge bidirectionally from the origin, elongating the growing DNA chain opposite both parental strands, thus creating two semiconservatively replicated DNA duplexes. Growing points converging from opposite directions from adjacent replicons probably meet near their boundary, and in some

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manner fuse, ensuring linear continuity of the chromosome (1–5).

The rate of DNA synthesis in a cell is controlled by two parameters: the number of replicons that initiate DNA synthesis per unit of time and the average rate of DNA chain elongation within individual replicons. Both these parameters can be artificially modified. For example, X-irradiation (6–8) and photolytic strand breakage of BUdR[1]-substituted DNA (9) have been shown to inhibit initiation of DNA synthesis in replicons in various mammalian cells. Furthermore, photoproducts induced by ultraviolet radiation act to block DNA chain elongation in HeLa S-3 cells (10). To a first approximation, it appears that agents which inhibit replicon initiation (X-irradiation, BUdR photolysis) produce DNA strand breaks, while agents that inhibit DNA chain elongation because of their effect on the template properties of DNA (ultraviolet radiation, 4-nitroquinoline oxide) produce covalent modification of certain DNA bases.

Methyl methanesulfonate is a chemical mutagen that has been widely studied in terms of its mutagenic and cytotoxic properties in many cell systems (11–14). MMS can produce both covalent modification of nucleotide bases (alkylated bases) and strand breakage of DNA via spontaneous or enzymatic pathways involving an apurinic intermediate.

MMS has been demonstrated to inhibit DNA synthesis in mammalian cells. However, the mechanism of this inhibition at the subchromosomal level remains controversial. For example, Buhl and Regan (15), utilizing the technique of alkaline sucrose velocity sedimentation, determined that the average molecular weight of nascent DNA synthesized during a 30-min pulse period was diminished in various human cell lines at 2.5 hr (but not at 0.5 or 4.0 hr) following a 1-hr exposure to 1 mM MMS. These authors suggested that such a result might occur in one of two ways: MMS-induced lesions could act as blocks to the replicase enzymes (DNA chain elongation effect), or MMS could act to inhibit the initiation of adjacent replicons and provoke degradation of nascent DNA (replicon initiation effect).

Scudiero and Strauss (16) and Kato and Strauss (17) determined that when DNA synthesis is inhibited in HEp.2 cells by exposure to 2.5 mM MMS for 1 hr there is an accumulation of intermediate molecules that possess the characteristics of the replication fork. At 8 hr after exposure to MMS, however, no such accumulation occurs. This and other evidence presented by both groups was interpreted in the following way: MMS produces a lesion(s) in DNA that freezes the growing point at a site near the lesion(s). Repair synthesis near the growing points later removes the lesion(s) and allows the replication fork to continue.

In contrast to the results above, Makino and Okada (18), employing alkaline sucrose velocity sedimentation, found that a 20 min exposure of mouse L5178Y cells to a concentration of 200 μg of MMS per milliliter results in only a slight retardation of the rate of DNA chain elongation immediately after exposure.

Finally, Painter (19), also utilizing alkaline sucrose velocity sedimentation, has shown that if HeLa cells are exposed to 1 mM MMS for 15 min, only low molecular weight nascent DNA is underrepresented in gradient profiles immediately after exposure. However, by 2 hr after exposure, both low and high molecular weight nascent DNA is decreased. The interpretation offered by Painter was that MMS acts immediately to depress replicon initiation but that over longer intervals MMS also acts to slow or block DNA chain elongation.

The discrepancies among the results noted above may be due to the differences in cell lines used, concentration or duration of exposure to MMS, methodology employed by the various groups, or the dual nature of the lesions that MMS produces in DNA.

The purpose of the present study was to investigate the inhibition of DNA synthesis in mammalian cells after exposure to

[1] The abbreviations used are: BUdR, 5-bromo-2'-deoxyuridine; MMS, methyl methanesulfonate; PCA, perchloric acid.

MMS. Special emphasis has been placed on trying to answer two questions: What is the mechanism of inhibition? What are the long-term effects on the ability of cells to synthesize DNA after exposure to MMS?

METHODS

Cells and culture conditions. Chinese hamster V-79 cells, obtained from Dr. L. Tolmach, Washington University, were routinely grown in 75-cm^2 tissue culture flasks (Falcon) in Ham's F-10 nutrient medium supplemented with 10% calf and 10% fetal calf serum as well as antibiotics. The cultures were maintained in an incubator at 37° in a water-saturated atmosphere of 5% CO_2 and 95% air.

Approximately 45 hr before the beginning of each experiment, nearly confluent monolayers of cells were treated with 0.025% trypsin and identical cell inocula (approximately 18,000 cells) were plated onto 35-mm plastic Petri dishes (Falcon).

MMS treatment. To fresh, previously warmed medium, the appropriate amounts of vacuum-redistilled MMS (Eastman Kodak) were added to prepare solutions that were 3 times the treatment concentration. Then 1 ml of this solution was rapidly pipetted (less than 3 min) into each Petri dish of cells, which already contained 2 ml of medium. After a 30-min exposure, the medium containing MMS was removed and the Petri dishes were rinsed once with warmed Hanks' balanced salt solution and once with regular warmed medium, and reincubated with fresh medium. The addition and removal of MMS were performed at room temperature.

Determination of rate of DNA synthesis. DNA synthesis was measured by [^{14}C]-thymidine incorporation followed by Geiger counting, or by [^{3}H]thymidine incorporation followed by liquid scintillation counting or radioautography. The concentration and specific activities of the radioactive precursors are given in the respective table and figure legends.

The procedures used for measuring [^{14}C]thymidine incorporation were similar to those described previously by Griffiths and Tolmach (20). Briefly, replicate cultures growing in 35-mm plastic Petri dishes (whose inside diameter had been ringed with a wax pencil to confine cells to the center of the dish) were pulse-labeled with [2-^{14}C]thymidine (Amersham/Searle) for 30 min. At the end of the pulse period, the radioactive medium was removed and the Petri dishes were washed twice with cold (4°) 0.9% NaCl, then incubated overnight in 2% PCA at 4°. The PCA was removed, and the Petri dishes were washed twice with distilled water and once with 70% ethanol and dried. The bottoms of the Petri dishes were then punched out and counted in a low-background gas-flow Geiger counter with a probable counting error of less than 5%.

For experiments utilizing [^{3}H]thymidine, cells were cultured as usual, incubated in [^{3}H]thymidine (Amersham/Searle) for 7.5 or 10 min, washed twice with cold (4°) 0.9% NaCl, then incubated overnight in 2% PCA. The PCA was removed, the cells were washed twice with cold 0.9% NaCl and scraped off the Petri dishes with a rubber policeman, and the PCA-precipitable material was collected by vacuum filtration onto glass fiber filters. The filters were washed with 95% ethanol, dried, and immersed in toluene-based scintillator, and the radioactivity was determined by liquid scintillation counting (Beckman).

DNA fiber radioautography. DNA fiber radioautograms were prepared according to Edenberg (10). Briefly, cells were exposed to a 30-min pulse of [^{3}H]thymidine of high specific activity (100 μCi/ml, 50 Ci/mmole). After removal of the radioactive medium, the attached cells were rinsed three times with ice-cold buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 25 mM Tris, and 10 mM unlabeled thymidine, pH 7.4). The cells were then scraped off the Petri dishes with a rubber policeman and resuspended in ice-cold buffer at a concentration of around 5 $\times$ 10^4 cells/ml. Then 20 μl of this suspension were allowed to mix with an equal volume of lysing solution (2% sodium dodecyl sulfate in 10 mM EDTA adjusted to pH 8.1 with Trizma base) for 5–15 min on gelatin-subbed microscope slides. The drops were then spread across the slide in

the same manner in which a blood smear is made. The slides were dried, then rinsed for successive 1-min periods in 5% trichloracetic acid (twice), 1 N HCl, and 95% ethanol. The slides were dried again, dipped in NTB-3 photographic emulsion that was diluted 1:1 with double-distilled water, allowed to dry again, and stored in light-tight boxes containing desiccant at 4° for 2½ to 3½ months. Standard procedures were followed to develop and fix the emulsion. All photographic supplies were obtained from Eastman Kodak.

In order to prevent bias in scoring the radioautograms, the code number that was marked on each slide to identify the treatment regimen was masked with a paper tag. Each slide was scanned at 400 × magnification to locate areas where the DNA fibers were well spread. Within such regions, internally located replication segments in separated tandem arrays were measured at 1000 × magnification by use of a calibrated ocular micrometer. Only internally located segments were scored, to minimize the chance of including fragmented replication segments from the ends of long DNA fibers.

Conventional radioautography. Conventional radioautography was performed *in situ* on cells growing in Petri dishes according to Hopwood and Tolmach (21). The cells were incubated with [^{3}H]thymidine (2 μCi/ml, 5 Ci/mmole) for 30 min. Pulse termination and PCA fixation were performed as described above. After removal of the PCA, the cells were rinsed with 0.9% NaCl at room temperature. The cells were then overlaid with NTB-3 photographic emulsion (mixed 1:1 with double-distilled water) and stored at 4° in light-tight boxes for 4–7 days. Development and fixation were carried out by standard procedures. Giemsa staining was then employed to stain the cells lightly. After coding to ensure unbiased scoring, the percentage of labeled cells was determined by microscopic observation at 400 ×.

Cell counting. Attached cells were scraped from Petri dishes with a rubber policeman and suspended in 10 ml of 0.9% NaCl. The cell suspension was forcibly drawn through a 10-ml pipette to break up clumps of cells. The number of cells per Petri dish was then determined by counting with an electronic particle counter (Coulter counter, model B).

RESULTS AND DISCUSSION

The rate of DNA synthesis, as measured by pulsed incorporation of [^{14}C]thymidine as a function of time after a 30-min treatment with graded doses of MMS, is shown in Fig. 1. The data in Fig. 1 are presented as ratios of incorporation by MMS-treated cultures compared with control cultures. Exposure to MMS results in a rapid decrease in the rate of DNA synthesis. Examination of Fig. 1, for which 30-min pulse times were utilized, shows that the initial decline was so rapid that the dose response of the deceleration of synthesis could not be determined. However, when 7.5-min pulses of [^{3}H]thymidine were used to measure the rate of DNA synthesis during the 30-min exposure to MMS, it became evident that the initial deceleration in the rate of DNA synthesis is actually dose-dependent (Fig. 2). The half-times for the initial decline in rate of DNA synthesis in cells exposed to 1, 3, and 8 mM MMS are

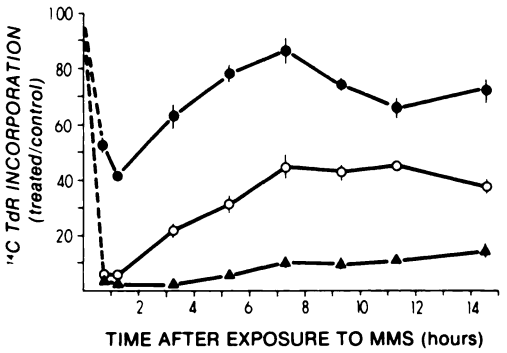

FIG. 1. *Time course of thymidine incorporation into V-79 cells following a 30-min exposure to 1* (●), *3* (○), *or 8 mM MMS* (▲)

Cells were pulse-labeled for 30 min with [2-^{14}C]thymidine (0.2 μCi/ml, 50 Ci/mole) at the times shown. Data are presented as ratios of radioactivity incorporated by MMS-treated cultures compared with control, sham-treated cultures. Each point is centered at the midpoint of the pulse interval and represents the mean ± standard error of duplicate plates from three separate experiments.

approximately 56, 13.8, and 6.2 min, respectively. These half-times were determined by regression analysis of the data points shown in Fig. 2 plus additional experiments utilizing a 10-min pulse interval. The relative extent of the minima of the DNA synthesis rate in MMS-treated cells is also dose-dependent (Fig. 1).

After an initial delay of about 1.5 hr following the termination of MMS exposure, the rate of DNA synthesis starts to recover. The relative rates of recovery are similar in cells exposed to 1 or 3 mM MMS, but not in cells exposed to 8 mM MMS. The recovery phase lasts approximately 6 hr and is followed by a plateau or even a second decline in the rate of DNA synthesis. The extent of the recovery is dose-dependent. Complete recovery of MMS-treated cells was not observed over the dose range and time course of these experiments.

Drawing conclusions about the long-term inhibition of biosynthetic processes as measured by precursor incorporation in cultures of attached cells can be hazardous because of possible effects on cell progression, cell lysis, redistribution of cells into different cell-cycle phases, and so on (22). In order to determine whether any of these factors plays a role in the results reported in this study, the percentage of S-phase cells and the relative number of cells per Petri dish were measured at various times following exposure to MMS.

At various times following treatment with 3 mM MMS, the number of cells per dish in treated cultures is reduced slightly relative to control cultures, but is not reduced absolutely (Table 1). This effect could be due either to an MMS-induced retardation of cell progression, as has been reported by others (23), or to limited MMS-induced cell lysis or both. Note, however, that MMS does not produce a depression in the percentage of S-phase cells.

Even after considering the corresponding differences in the number of cells per Petri dish in controls and treated cells, it is obvious that a true depression in the rate of DNA synthesis at the cellular level occurred. For example, attached cells treated with 3 mM MMS for 30 min exhibit

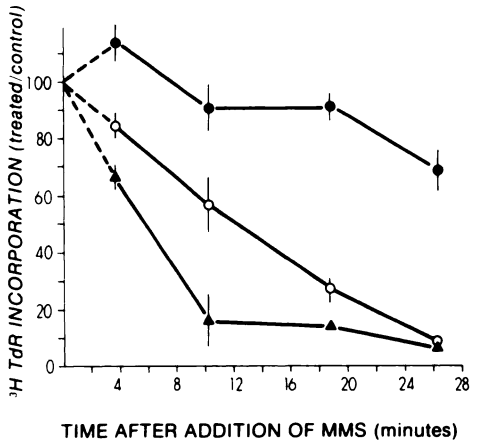

FIG. 2. *Initial decline in rate of thymidine incorporation during a 30-min exposure to 1* (●), *3* (○), *or 8 mM MMS* (▲)

Cells were pulse-labeled with [³H]thymidine (2 μCi/ml, 5 Ci/mmole) for 7.5 min. Data are presented as the ratios of radioactivity incorporated by MMS-treated cultures compared with control cultures. The data points are centered at the midpoint of the pulse interval and represent the mean ± range of duplicate plates.

a 62% reduction in the rate of thymidine incorporation per Petri dish 14.5 hr after exposure. The relative number of cells per Petri dish, however, is reduced by only 24% compared with control cultures. Therefore the true depression in the rate of DNA synthesis per cell in the MMS-treated cells is about 50%, or $[1-(1-0.62)/(1-0.24)] \times 100$.

Since MMS has been shown to induce repair synthesis (16, 17, 24), another concern was that our measurements of DNA synthesis might reflect a considerable component of repair synthesis. Cleaver (25) has shown that semiconservative DNA synthesis, but not repair synthesis, is selectively abolished in the presence of hydroxyurea. Therefore the amount of [¹⁴C]thymidine incorporated into acid-precipitable material was determined in the presence and absence of 10 mM hydroxyurea. When hydroxyurea was added just before each pulse, at least 95% of the [¹⁴C]thymidine incorporation into DNA of control or MMS-treated cells (for up to 14 hr after exposure) was hydroxyurea-sensitive and was therefore presumed to be

TABLE 1

Percentage of labeled cells and relative cell number at various times after termination of exposure to MMS

Time after MMS exposure	Labeled cells[a]		Relative cell number[b]	
	−MMS	+3 mM MMS	−MMS	+3 mM MMS
hr	%	%		
0			1.0	1.14 ± 0.06
0.5	52.6	48.6		
1	56.0			
3	54.9	52.5		
5	52.6	50.2	1.23 ± 0.07	1.11 ± 0.09
7	51.8	51.2		
9			1.47 ± 0.04	1.12 ± 0.05
14.2	51.2	55.4	1.80 ± 0.04	1.37 ± 0.05

[a] Averages of triplicate plates from two separate experiments. The [^{3}H]thymidine pulse duration was 30 min.

[b] Averages of duplicate plates from two separate experiments.

semiconservative in nature (data not shown).

Another possibility that we considered was that our measurements of depressed DNA synthesis in MMS/treated cells might be an artifact arising from an alteration in the rate of transport of labeled thymidine across the cell membrane, or from MMS-induced perturbation of the metabolic conversion of labeled thymidine to labeled dTTP. Two methods were utilized to investigate whether thymidine transport was affected. In the first, the amount of total radioactivity retained in NaCl-washed cells minus the radioactivity incorporated into PCA-precipitable form was measured in control and MMS-treated cells (Fig. 3). The difference between the two measurements reflects the amount of thymidine uptake across the cell membrane into the acid-soluble pool (22). In the second method, the PCA-soluble radioactivity extracted after overnight fixation of NaCl-washed cells was measured directly by liquid scintillation counting (Fig. 4). It can be seen that there is no difference in thymidine transport in MMS-treated or control cells.

Since thymidine must be metabolically converted along a three-step biosynthetic pathway to dTTP before it is incorporated into DNA, and because dTTP can be produced by the pathway *de novo* as well as the salvage pathway (22), we were concerned that MMS might in some manner cause an expansion of the endogenous

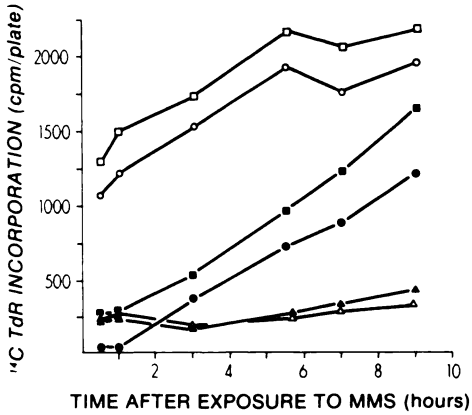

FIG. 3. *Total cellular uptake of thymidine (squares), uptake of thymidine into acid-precipitable material (circles), and uptake into the acid-soluble pool (triangles) in control cells (open symbols) and cells treated with 3 mM MMS (solid symbols)*

At the times shown, cells were pulse-labeled for 30 min with [2-^{14}C]thymidine (0.2 μCi/ml, 50 Ci/mole), and radioactivity was measured before or after a 3-hr PCA fixation. The difference between total uptake and incorporation into acid-precipitable material is a measure of pool uptake. Data points refer to the means of duplicate plates from two experiments.

dTTP pool (possibly by provoking DNA degradation and reutilization of the unlabeled nucleotides resulting from this breakdown). Such an effect would result in a decline in the specific activity of the dTTP pool and lead to a decrease in incorporation of label into DNA even in the absence of a true depression of the rate of DNA synthesis. In order to investigate

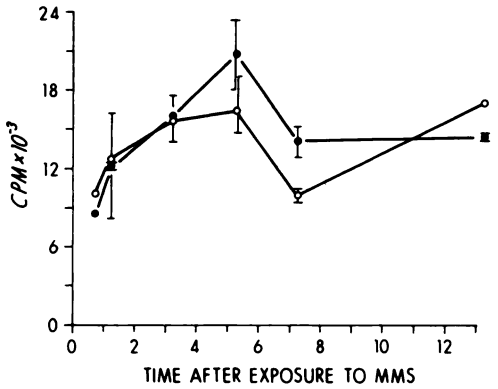

FIG. 4. *PCA-soluble radioactivity extracted from sham-treated (●) or MMS-treated (○) cells at various times after exposure*

At various times following exposure to 3 mM MMS, cells were pulse-labeled for 30 min with [³H]thymidine of high specific activity (25 μCi/ml, 50 Ci/mmole) for 30 min. The cells were then rinsed twice with cold 0.9% NaCl and incubated overnight in 1 ml of PCA. The PCA fraction was removed, and the radioactivity (corrected for quenching) in a 0.1-ml aliquot was determined by liquid scintillation counting.

this possibility, DNA fiber radioautograms were prepared. By use of this technique, we were able to measure the specific activity of incorporation along the DNA chain and, in addition, to determine the effects of MMS on DNA synthesis at the level of individual replicating units (replicons). Exposure to MMS probably results in no major effect on precursor utilization, since the specific activities per unit length are similar for control and MMS-treated cells at all times after exposure (Table 2). This technique has been utilized previously by Watanabe (8) to show that X-irradiation does not perturb the thymidine pool.

The rapid, dose-dependent nature of the decrease in the rate of DNA synthesis in MMS-treated cells strongly suggests that at least some of the inhibition is due to an effect on DNA chain elongation, since even total, immediate inhibition of replicon initiation cannot produce a deceleration in the rate of DNA synthesis as rapid as that seen in cells exposed to 8 mM and probably 3 mM MMS.

Kinetic data, however, cannot be used to determine the long-term effects of MMS on replicon elongation. In order to obtain such long-term data, DNA fiber radioautography was employed. Blumenthal, Kreigstein and Hogness (2) and Edenberg (10) have shown that the average lengths of replication segments synthesized during a single pulse of [³H]thymidine provide a valid measure of the extent of unidirectional DNA chain growth during the pulse interval, provided that (a) the duration of the pulse is less than the time necessary to complete synthesis of an average-sized replicon and (b) no drastic and rapid effects on replicon initiation occur. A drastic effect on initiation with little or no effect on elongation or termination would by itself greatly alter the lengths of labeled segments observed in DNA fiber radioautograms at certain times after insult, since one would be scoring primarily the termination of replicons that had initiated prior to insult. To monitor long-term DNA chain elongation effects when initiation effects are expected, the first pulse of [³H]-thymidine should be delayed until all replicons that had initiated prior to the insult have completed synthesis. In the cell line used for these studies, we obtained data from hot pulse–warm pulse labeling experiments which indicate that at least 98% of the replicons would require more than 37 min but less than 56 min to complete synthesis. Thus, in the cell line used in this study, fiber data based on pulses of a single duration cannot be used to estimate the effects on elongation within the first 56 min following MMS treatment. The first data point in Fig. 5 represents

TABLE 2

Specific activity in cells at various times after termination of treatment with MMS

Cells were pulsed for 30 min with [³H]thymidine of high specific activity (100 μCi/ml, 50 Ci/mmole). Values are means and standard errors.

Time after MMS exposure	Specific activity	
	−MMS	+3 mM MMS
hr	*grains/μm DNA*	
1	1.13 ± 0.10	1.38 ± 0.07
5	1.05 ± 0.05	1.27 ± 0.04
9	1.13 ± 0.08	1.22 ± 0.04
14.2	1.19 ± 0.10	1.11 ± 0.04

the average lengths of labeled fibers from cells pulsed from 60 to 90 min following termination of MMS treatment. The data indicate that exposure to MMS results in a transient, dose-dependent decrease in either the rate or extent of DNA chain elongation along individual replicons during the period from 1 to 14 hr following exposure. Since pulses of only a single duration were used in this experiment, it is impossible to ascertain whether MMS causes a generalized decrease in the rate of DNA chain growth or whether MMS-induced lesions act as discrete blocks to continued fork progression until they are removed. However, as noted in the INTRODUCTION, Scudiero and Strauss (16) and Kato and Strauss (17) have obtained data that are consistent with the latter possibility. Examples of the replication segments that can be visualized in DNA fiber radioautograms prepared from control and MMS-treated cells are shown in Fig. 6.

Qualitatively, the pattern of inhibition and recovery of the rate or extent of DNA chain elongation is similar to that exhibited in the inhibition and recovery of total cellular DNA synthesis. However, the two recovery phenomena differ quantitatively. With a few exceptions, at all doses and at all times after exposure, the degree of inhibition of the rate or extent of DNA chain elongation is less than the degree of inhibition of the rate of total cellular DNA synthesis. This is true even when corrections for differences in the number of cells per Petri dish in MMS-treated and control cultures are taken into account. Furthermore, nearly complete recovery of DNA chain elongation occurs after exposure to 1 or 3 mM MMS, as contrasted with the limited extent of recovery that occurs in cellular DNA synthesis. The finding that the effect of MMS on DNA chain elongation can only partially account for the depression of cellular DNA synthesis indicates that replicon initiation may also be inhibited in MMS-treated cells. Furthermore, from analysis of the data in Figs. 1 and 4 and Table 1, the degree of inhibition of cellular DNA synthesis that cannot be attributed to effects on the rate or extent of chain elongation

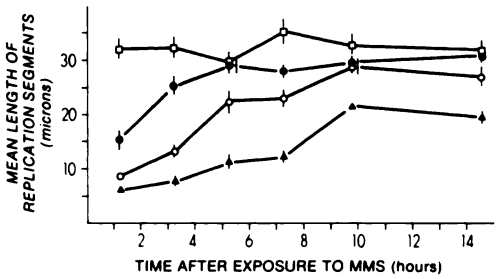

FIG. 5. *Time course of length of replication segments synthesized following a 30-min exposure to 0* (□), *1* (●), *3* (○), *or 8 mM MMS* (▲)

At various times following MMS treatment, cells were pulse-labeled for 30 min with [^{3}H]thymidine of high specific activity (100 μCi/ml, 50 Ci/mmole), the DNA was spread for fiber radioautography, and replication segments were visualized and measured as explained in METHODS. Each data point is centered at the midpoint of the pulse interval and represents the mean ± standard error of at least 20 replication segments.

is fairly constant at each time point and persists over the entire time course of these experiments.

These findings are consistent with the interpretation that exposure to MMS results in two types of damage to the DNA-synthesizing capabilities of the cell. One type of damage causes the rate or extent of DNA chain elongation to decline. Although it is difficult to use our kinetic or single-pulse fiber data to estimate the initial effects of MMS on replicon elongation, it is clear that by 1 hr following termination of MMS treatment, a marked decrease in the rate or extent of chain elongation occurs. Cells appear to recover from this type of damage within 6–10 hr. The second type of damage appears to cause a decline in the number of replicons that are actively engaged in DNA synthesis. The inhibition resulting from this type of damage is manifested uniformly over a protracted period of time.

MMS is known to produce four general types of damage in DNA: base alkylation (mainly 3- and 7-methylpurines plus other minor products such as O^6-methylguanine), phosphotriesters, apurinic sites, and DNA strand breakage (26). Most of these lesions can be characterized as to their stability, their structural effect on

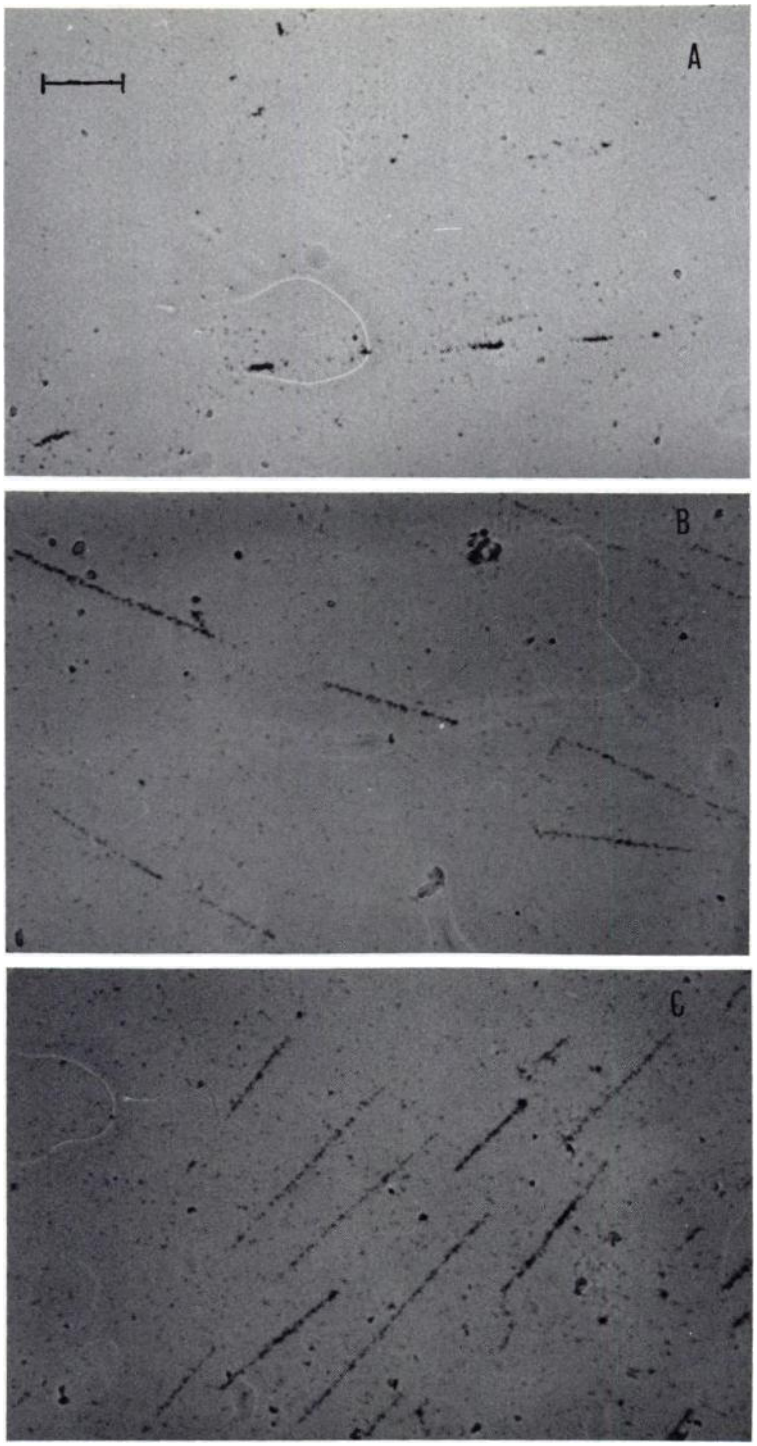

FIG. 6. *Photomicrographs of replication segments visualized in DNA fiber radioautograms prepared from sham-treated or MMS-treated cells at various times following treatment*

A. 3 mM MMS-treated; midpoint of pulse interval, 1.25 hr following treatment. B. 3 mM MMS-treated; midpoint of pulse interval, 14.5 hr following exposure. C. Sham-treated; midpoint of pulse interval, 14.5 hr following exposure. Bar = 20 μm.

the DNA helix, and their breakdown products. A detailed discussion of each lesion would be unwieldy. Therefore, for clarity and brevity, we have reviewed the literature concerning the characteristics of the major types of damage induced by MMS in both bacterial and mammalian systems. Table 3 shows a compilation of the data we are aware of. Although none of the information of Table 3 was derived from experiments involving cultured V-79 cells, the majority of the data were obtained from experiments involving mammalian cells *in vivo* and *in vitro*.

Two additional observations are pertinent for this discussion. Recently several investigators have reported that X-rays or 313 nm photolysis of BUdR-substituted DNA depresses the DNA synthetic rate by transiently inhibiting replicon initiation in various mammalian cell lines (7–9, 27). Only at doses that render more than 95% of the cells nonviable can either agent inhibit DNA chain elongation. Since the majority of lesions produced in DNA by either X-rays or BUdR photolysis are strand breaks, and since the technique of BUdR photolysis results almost exclusively in direct damage to DNA, it has been hypothesized that strand breaks are directly responsible for inhibiting replicon initiation (9).

Exposure of mammalian cells to ultraviolet radiation, however, causes a dramatic inhibition of DNA chain elongation without apparently affecting replicon initiation (10). By comparing the lengths of replication segments synthesized after various exposures to ultraviolet light with the expected distribution of pyrimidine dimers in DNA from cultured HeLa S-3 cells, Edenberg (10) concluded that pyrimidine dimers (and possibly other minor ultraviolet-induced photoproducts) act as blocks to the replicase enzymes, preventing DNA chain elongation past the site of the lesion for up to 90 min. Pyrimidine dimers have been classified by Cerutti as lesions that cause helix distortion (29). Thus they cause structural damage to the DNA helix that may be similar to the damage produced by the 3-methylpurines or O^6-methylguanine.

Therefore, because of the characteristics of damage to the DNA synthetic machinery that we have observed in MMS-treated V-79 cells, because of the properties exhibited by the various types of lesions produced in DNA by exposure to MMS (as categorized in Table 3), and because of the recent studies concerning the effects of X-ray-induced lesions and ultraviolet-induced photoproducts on semiconservative DNA replication, we propose that certain MMS-induced lesions affect either the rate or extent of chain elongation, while other lesions may inhibit replicon initiation. Since O^6-methylguanine and the 3-methylpurines are lesions that might structurally restrict the template character of DNA in a manner analogous to the effects of ultraviolet photoproducts, it is likely that these lesions are responsible for the observed effects of MMS on DNA chain

TABLE 3

Characteristics of various lesions produced by exposure of cells to MMS

Lesion	Persistence in DNA	Degree of distortion of DNA helix	Spontaneously produced secondary lesions	References
7-Methylpurines	$t_{\frac{1}{2}}$[a] ≅ 24 hr	None	Apurinic sites[b] Single strand breaks	26, 28–33
3-Methylpurines	$t_{\frac{1}{2}}$ ≅ 1–3 hr[c]	Major	Apurinic sites[b] Single strand breaks	26, 28–34
O^6-Methylguanine	$t_{\frac{1}{2}}$ ≅ 3 hr[d]	Major	Unknown	26, 28–34
Phosphotriesters	Unknown	Unknown	Unknown	31, 35, 36

[a] $t_{\frac{1}{2}}$ = biological half-time.

[b] The majority of apurinic sites are formed from spontaneous breakdown of 7-methylpurines; however, unrepaired 3-methylpurines also yield apurinic sites (26).

[c] Enzymatically removed.

[d] Possibly enzymatically removed.

elongation. Furthermore, since these lesions are removed from DNA within a few hours, there should be only a transient effect on DNA chain elongation.

We also hypothesize that strand breaks are the MMS-induced lesions responsible for inhibition of replicon initiation. Strand breaks are produced at a relatively constant rate in the DNA of MMS-treated cells for long periods of time, owing mainly to the breakdown of 7-methylguanine to apurinic sites, which are then converted to strand breaks (16, 26). The kinetics of breakdown of 7-methylguanine could account for the relatively constant but persistent inhibition of replicon initiation that we believe occurs in MMS-treated cells. Since very little is known about the biological characteristics of phosphotriesters, it is difficult to assess their importance in relation to MMS-induced depression of the DNA synthetic rate. However, since they are produced in relatively high yields, their possible importance should not be ignored.

We believe that the classification proposed here provides a comprehensive picture of the manner in which MMS acts to inhibit eukaryotic DNA synthesis. In particular, the proposal accounts for the findings of Kato and Strauss (17) as well as Scudiero and Strauss (16) that some MMS-induced lesion(s) acts to block DNA chain elongation until it (they) is removed by repair replication. In addition, it accounts for Painter's finding (19) that MMS inhibits replicon initiation. Although Painter did observe repair of the MMS-induced lesions responsible for the inhibition of replicon initiation, the amount of repair was minimal, so that by 2.75 hr following exposure to MMS, effects on replicon initiation were still observed.

The proposal we have made must be regarded as tentative. Our interpretations are based only on a qualitative assessment of our data and on the observations of many other groups. Only through careful quantitative studies that relate the intragenomic distribution of different types of MMS-induced lesions to the amount and type of inhibition at the replicon level can this hypothesis be rigorously tested.

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