Cell cycle-specific mutagenesis at the hypoxanthine phosphoribosyltransferase locus in adult rat liver epithelial cells

cell synchronization/chemical mutagens/6-thioguanine resistance/S-phase sensitivity/mammalian cells

CHARLES TONG, MARYELLEN FAZIO, AND GARY M. WILLIAMS

Division of Pathology and Toxicology, Naylor Dana Institute for Disease Prevention, American Health Foundation, 1 Dana Road, Valhalla, New York 10595

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ABSTRACT The cell cycle specificity of chemical mutagenesis was studied by use of two cell synchronization techniques, one a nontoxic technique involving serum deprivation and the other a double thymidine block, to obtain rat liver epithelial cells in different phases of the cell cycle to be exposed to chemical mutagens. For both methyl methanesulfonate and N-methyl-N'-nitro-N-nitrosoguanidine, there was a cell cycle specificity of chemical mutagenesis, with the most sensitive phase being the period of DNA synthesis.

During replication, the susceptibility of mammalian cells to malignant conversion by chemicals in vitro is increased (1–4). Similarly, in vitro neoplastic transformation is enhanced in cells entering or in the S phase (5, 6). Despite the suggested relationship between carcinogenesis and mutagenesis, studies in mammalian cells by Orkin and Littlefield (7) using baby hamster kidney cells, by Goth-Goldstein and Burki (8) using Chinese hamster ovary cells, and by Vergara and Terzi (9) using Chinese hamster lung (V79) cells have not revealed a cell cycle specificity for mutagenesis by chemicals. This is conceptually in contrast to findings in bacterial or yeast mutagenesis (10, 11).

To reexamine the question of whether mammalian cells have differing susceptibilities to the mutagenic effects of chemicals during the cell cycle, we have studied the response to chemical mutagens/carcinogens of proliferating and non-proliferating cultured rat liver epithelial cells, which in vitro are more susceptible to carcinogenesis during replication (1–4). In preliminary studies, we demonstrated that replicating liver cells were more sensitive to chemical mutagenesis by methyl methanesulfonate (MeMes) than were nonreplicating cells (12, 13). In the present studies, we have used two distinct synchronization procedures and two chemical mutagens/carcinogens to identify the most sensitive phase in the cell cycle. The results presented consistently demonstrate a cell cycle sensitivity to chemical mutagenesis in mammalian cells, with the phase of DNA synthesis being the most sensitive.

MATERIALS AND METHODS

Cell Lines. Adult rat liver epithelial cell line 6 (ARL6) (14) was initiated from a primary hepatocyte culture prepared from cells dissociated from the liver of an adult male Wistar rat after continuous perfusion by collagenase (15). It was maintained in Williams' medium E (Flow Laboratories, Rockville, MD) supplemented with 10% (vol/vol) fetal bovine serum.

Cells frozen in liquid nitrogen at around the 150th passage were used in all experiments. They were revived a few days before the start of any experiment and discarded at the completion of the experiment.

Synchronization by Serum Deprivation. The procedure for arresting the growth of ARL cells by depletion of serum growth factors (16–18) has been described (13). The epithelial cells were plated at 1 × 10^5/cm² in Williams' medium E supplemented with 10% fetal bovine serum. Twenty-four hours later, these cultures were washed and refed with medium supplemented with only 1% fetal bovine serum. These cultures were kept for 10 days with one change of medium. In this condition, growth quiescence occurred in the subconfluent stage (13) with less than 10% of the cells in S phase. Cells were released from quiescence by being fed with growth medium containing 10% fetal bovine serum.

Synchronization by Double Thymidine Block. The procedure of Kuroki and Sato (19) was modified. Confluent cultures of ARL6 cells were plated at 1 × 10^5/cm² in medium E/10% fetal bovine serum containing 2 mM thymidine. Eighteen hours later, these cultures were washed and kept for 8 hr in medium E/10% fetal bovine serum. Then they were subjected to a second thymidine block for 18 hr. Cells were released from the block by washing the cultures and refeeding them with medium E/10% fetal bovine serum.

Exposure to Mutagen. For exposure to mutagen, cells were washed twice with Williams' medium E and then exposed to MeMes or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) for 30 min at 37°C in medium E. Controls were given medium E alone. After exposure, the cells were washed twice with medium E and refed with medium E/10% fetal bovine serum. The concentration of the two mutagens was chosen from dose-response studies so that the survival at 24 hr after exposure to mutagen of the S-phase populations, which are most sensitive to the cytotoxic effects (13), would be 40–60% of the control S-phase population. This survival range is optimal in inducing 6-thioguanine-resistant (TG') mutants in ARL cell lines.

Recovery of Mutants Resistant to the Purine Analog. After exposure to the mutagen, the exposed cells and the controls were maintained with intermittent replating for a minimum of 14 days for optimal mutant expression (20, 21) before they were replated for TG' mutant selection. During the mutant expression period the cells were maintained in Falcon or Corning T-75 cell culture flasks. The cultures were replated whenever they reached 75% confluency. For the control population at a seeding density of 2 × 10^4 cells per cm², this would be about every 4 days.

For selection of mutants, cells were seeded at 10⁴ cells per cm² in Williams' medium E/10% fetal bovine serum in 25-cm² cell culture flasks. Twenty-four hours later, medium E/10% fetal bovine serum containing 10 μg of 6-thioguanine per ml (Sigma) was added, and the cells were refed every 4 days thereafter. Fetal bovine serum was added to the medium con-

Abbreviations: ARL6, adult rat liver epithelial cell line 6; MeMes, methyl methanesulfonate; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; TG', 6-thioguanine resistance.
taining 6-thioguanine only on the day of feeding. After 14 days, colonies were fixed with formalin and stained with Giemsa for counting. The TG\(^+\) mutants that were isolated were phenotypically stable, contained residual amounts of hypoxanthine phosphoribosyltransferase, and were sensitive to aminopterin (20–22).

The colony-forming efficiency of these cultures in nonselective medium was determined by seeding 20 cells per cm\(^2\) in Williams\' medium E/10% fetal bovine serum in 25-cm\(^2\) cell culture flasks. At the end of 7–9 days, colonies were fixed and stained as above.

**Determination of Labeling Index.** For pulse labeling, 1 \(\mu\)Ci of \(^{3}H\)dThd per ml (40–60 Ci/mmol; 1 Ci = 3.7 \(\times\) \(10^{10}\) becquerels) was added to the medium for 30 min. Emulsion autoradiography was performed (23), and the percentage of labeled cells was determined.

**Determination of Mitotic Index.** Cells were fixed with formalin and stained with eosin and hematoxylin. More than 1000 cells were scored per slide, and a minimum of two slides was used per experimental time point.

**RESULTS**

To monitor the cell cycle kinetics of synchronized cultures, we measured the labeling and mitotic indices. Pulse-labeling autoradiography studies of liver epithelial cell cultures brought to growth arrest by serum deprivation indicated that under this condition, about 10% of the cells were in S phase (Fig. 1). Upon stimulation with the complete growth medium, the number of cells entering S phase began to increase slightly during the next 10 hr and then increased markedly, reaching a peak at 14 hr. Mitotic index analysis of these cultures revealed the appearance of two small peaks of mitosis prior to the peak of DNA synthesis and a major mitotic peak approximately 10 hr after the peak of DNA synthesis. The mitoses that occurred immediately after stimulation must be a result of progression of some cells arrested in late G\(_2\) by serum deprivation. The second peak 5 hr later must be due to cells that were arrested in late S or early G\(_2\). Thus, because quiescent cultures contain at least some, and possibly a significant population of G\(_2\) cells, we have designated the cell population at the end of quiescence as G\(_2\) cells. At 8 hr after stimulation, most cells were past mitosis but not yet in S phase. Therefore, we have assigned the cell population at this point to the G\(_1\) phase. At 14 hr after stimulation, the peak of DNA synthesis was reached; thus, this population can be considered to be in the S phase. Exposure of these cell populations to 3 mM MeMes resulted in the highest incidence of TG\(^+\) mutants being produced in exposed S-phase cells (Table 1). With a second mutagen, MNNG, at 50 \(\mu\)M, and the same synchronization procedure, the highest incidences of mutants were again observed when S-phase populations were exposed (Table 1). Also, the incidences of mutants of S-phase cells exposed to a lower concentration of the potent mutagen MNNG were higher than those exposed to higher, but equitoxic, concentrations of MeMes. Cultures representing G\(_1\) and G\(_2\) cells were also sensitive to the two chemical mutagens, with the G\(_2\) population being perhaps slightly more sensitive. This could be due to the presence of late S-phase cells.

To ensure that these observations were not caused by an artifact of the synchronization procedure, we extended the studies by using the more commonly employed double thymidine block technique for synchronization. In our modified procedure, the epithelial cells were synchronized with less than 5% of the cells in S phase at the end of the second thymidine block (Fig. 2). Upon release from the blockage, the arrested population immediately entered S phase and reached a peak of DNA synthesis at around 4 hr after release. The only peak of mitosis under these conditions occurred approximately 8 hr after DNA synthesis (Fig. 2). Thus, synchronization of AR6L cells by the double thymidine block technique permitted study of arrested cells at the G\(_1\)/S interphase of the cell cycle, cells in S phase at 4 hr after release, cells in G\(_2\) at 8 hr after release, and cells in mitosis at 12 hr after release (Fig. 2). Exposure of these populations to MNNG at different phases of the cell cycle again revealed that cells in S phase were the most sensitive to the chemical mutagen (Table 2). In these studies, during which a significant population of cells in M phase was also exposed, the M-phase cells in two of three experiments were equally sensitive to mutagenesis by MNNG as cells in G\(_1\)/S or G\(_2\) phase.

![Graph](https://example.com/graph.png)

**Table 1. Incidence of TG\(^+\) mutants per 10\(^6\) colony-forming cells**

<table>
<thead>
<tr>
<th>Mutagen</th>
<th>Exp.</th>
<th>G(_1)/S</th>
<th>S</th>
<th>G(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeMes</td>
<td>I</td>
<td>60 ± 14</td>
<td>26 ± 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>490 ± 21</td>
<td>39 ± 17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>164 ± 31</td>
<td>21 ± 8</td>
<td></td>
</tr>
<tr>
<td>MNNG</td>
<td>I</td>
<td>1015 ± 83</td>
<td>305 ± 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>632 ± 219</td>
<td>56 ± 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>695 ± 186</td>
<td>305 ± 62</td>
<td></td>
</tr>
</tbody>
</table>

The cells were exposed to 3 mM MeMes or 50 \(\mu\)M MNNG for 30 min in Williams\' medium E. The average mutant incidence for the control populations was 6 ± 8 (SD) (range, 0–10). ND, not done. The mutant incidence per time point is the average from six selection flasks with a total of 1.5 \(\times\) 10\(^6\) cells plated for selection.

![Graph](https://example.com/2graph.png)

**Table 2. Incidence of TG\(^+\) mutants per 10\(^6\) colony-forming cells upon exposure to MNNG**

<table>
<thead>
<tr>
<th>Exp.</th>
<th>G(_1)/S</th>
<th>S</th>
<th>G(_2)</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>275 ± 25</td>
<td>754 ± 120</td>
<td>104 ± 39</td>
<td>626 ± 39</td>
</tr>
<tr>
<td>II</td>
<td>22 ± 14</td>
<td>470 ± 82</td>
<td>274 ± 56</td>
<td>240 ± 8</td>
</tr>
<tr>
<td>III</td>
<td>214 ± 40</td>
<td>355 ± 54</td>
<td>205 ± 50</td>
<td>164 ± 33</td>
</tr>
</tbody>
</table>

The cells were exposed to 50 \(\mu\)M MNNG for 30 min in Williams\' medium E. The average mutant incidence for the control populations was 5 ± 5 (SD) (range, 0–13). The mutant incidence per time point is the average from six selection flasks with a total of 1.5 \(\times\) 10\(^6\) cells plated for selection.
FIG. 2. Percentage of cells in S phase (●) and in mitosis (O) in ARL6 cell populations upon release from a double thymidine block synchronization procedure. Each time point is the average from three slides, with a minimum of 1000 cells counted per slide.

DISCUSSION

The findings in this study document that mammalian cells in the S phase are more susceptible to chemical mutagens than during other phases of the cell cycle. Mutagenesis in S-phase mammalian cells has been reported for base analogs that are incorporated during DNA synthesis (24–26) and for UV mutagenesis (27, 28); in the present study, we reveal this phenomenon for chemical mutagens. A possible reason for the absence of sensitivity of S-phase cells in the studies reported by other groups is that all studies involved hamster cells, which have been shown to be deficient in the removal of the highly promutagenic O6-alkylguanine produced by alkylating agents (29).

The present finding of sensitivity of S-phase cells to chemical mutagenesis provides a correlation between mutagenesis and neoplastic conversion. Susceptibility to chemical carcinogenesis is enhanced by cell replication (1–4). Marquardt (6) reported that induction of malignant transformation in cell cultures by MNNG was significantly greater in the S phase than in the G1 or M phase. Enhancement of chemical mutagenesis in mammalian cells during the S phase, therefore, correlates with these observations on transformation and supports the concept that the two phenomena may be caused by similar mechanisms.

The molecular mechanism(s) of sensitivity of S-phase cells remains to be established. The sensitivity could be due to a lack of opportunity for the cell to repair DNA damage before the damaged regions are used as templates for the synthesis of new, and thereby faulty, DNA. Support for this concept is provided by the studies of Maher et al. (30), which have demonstrated that cells from excision repair-deficient xeroderma pigmentosum patients are more susceptible to chemical mutagenesis than cells from normal subjects. Moreover, a recent study by Smith et al. (31) in 10T1/2 cells has indicated that excision of O6-methylguanine and N7-methylguanine is decreased or absent in S-phase cells compared with pre-S-phase cells. Alternatively, the sensitivity of S-phase cells could be due to the physical state of the DNA during synthesis, with critical sites being exposed for damage by the mutagen. The delineation of an S-phase sensitivity to mutagenesis in cultured epithelial cells provides a system in which these two possibilities can be studied. Understanding the nature of events occurring during the most sensitive phase of the cell cycle may help to identify a lesion that is involved in the induction of mutation and neoplastic conversion.

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