Relationship between Morphological Transformation and [3H]Thymidine Incorporation Stimulated by a Chemical Carcinogen in Postconfluent Cultures of Hamster Embryo Cells

Stefan G. D. Mironescu, Sheldon M. Epstein, and Joseph A. DiPaolo

ABSTRACT

Postconfluent cultures of Syrian hamster embryo cell strains were exposed to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or to combinations of MNNG, hydroxyurea (HU), caffeine (CF), and/or theophylline. At various intervals, [3H]thymidine incorporation into DNA was determined by autoradiography and liquid scintillation spectrometry. In parallel cultures, drug- and/or carcinogen-elicited cytotoxicity and carcinogen-induced morphological transformation were determined. The results indicate that: (a) MNNG induces, in a relatively small proportion of cells, a round of HU- and CF-suppressible [3H]thymidine incorporation superimposed on, or immediately followed by, a wave of inhibitor-resistant incorporation; (b) the transformation frequency varies with the detectable [3H]thymidine in the acid-insoluble fractions and the relative size of cell subpopulations undergoing HU- and CF-suppressible DNA replication; (c) the cells exposed to 1 mM CF or theophylline during the treatment with MNNG (0.5 μg/ml), as well as for the first 24 hr after being plated for focus formation, develop statistically significant fewer (p < 0.001) transformed foci, without measurable cytotoxic effects; and (d) at the doses used, HU inhibited [3H]thymidine incorporation of carcinogen-stimulated cells. It is concluded that MNNG-stimulated [3H]thymidine incorporation into DNA that is suppressible by both HU and CF may be associated with the initiation event(s) of morphological transformation; however, the nature of suppression by HU and CF appears to be different.

INTRODUCTION

Experiments with postconfluent, mitotically quiescent cell culture systems may contribute to the elucidation of the mechanism(s) of chemically induced transformation. DDIR cultures (18) are viable and metabolically active (5). When appropriately stimulated, such cells reenter the mitotic cycle without requiring the removal of drug-induced metabolic blocks. Furthermore, DDIR cultures with rare or very few cells synthesizing DNA are remarkably resistant to the cytotoxic effect of MNNG (14), but do develop, if replated shortly (e.g., 15 to 25 hr) following treatment (8, 14), transformed foci.

In previous studies (13, 14), 2 structurally unrelated chemical carcinogens, benz(a)pyrene and MNNG, elicited nuclear incorporation of [3H]thymidine in a relatively small proportion of DDIR HEC. In both dose- and time-dependent experiments, the number of morphological transformants, quantitated either by colony (13) or focus formation (14) assay, correlated with the percentage of cells of the carcinogen-responsive population that incorporated [3H]thymidine.

In the current study, autoradiographic and scintillation spectrometry techniques were used in conjunction with quantitative assays for morphological transformation and single-cell survival of DDIR HEC cultures. The latter were exposed to MNNG, or to various combinations of MNNG, HU, CF, and/or TP, to determine (a) the sensitivity of carcinogen-stimulated [3H]thymidine incorporation to the above inhibitors and (b) which of the DNA synthesis inhibitors used is able to suppress the morphological transformation in the absence of a cytotoxic response.

MATERIALS AND METHODS

Primary cultures initiated from 12- to 14-day-old Syrian hamster (Mesocricetus auratus) embryos (LVG-LAK; Lakeview Hamster Colony, Newfield, N. J.) were prepared and propagated in Dulbecco’s modified Eagle’s minimal essential medium with Earle’s salts and high glucose concentration (4.5 g/liter) as previously described (13, 14). The medium was routinely supplemented with nonessential amino acids (1%, v/v), vitamins (1%, v/v), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (North American Biologicals, Inc., Miami, Fla.). All cultures were negative for Mycoplasma contamination (Diagnostic Laboratory of Microbiological Associates; Contract K9440).

 Autoradiography. Cluster 35-mm-diameter plastic Linbro wells (9.6-sq cm growth area; Belco Glass, Inc., Vineland, N. J.) containing 22-mm-diameter glass coverslips were seeded with 1.3 × 10⁴ cells/sq cm and incubated in a humidified atmosphere flushed with 10% CO₂ at 37°C. Four to 6 days later, confluency was achieved, and only rare, if any, mitotic figures were observed by phase microscopy. At saturation density (12.0 × 10⁴ cells/sq cm), the number of HEC per oil immersion field was 23 ± 2.7 (S.D.). DNA synthesis, as indicated by the percentage of labeled nuclei, had essentially ceased. MNNG- or DMSO-treated cultures were pulsed (1 hr) or continuously labeled with [3H]thymidine (1 μCi/ml; specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, Mass.). The autoradiographic technique has been described in detail (13, 14). The results were expressed as percentage of labeling index.
Measurement of Radioactivity in Acid-insoluble Fractions. Similar confluent cultures plated in 25-sq cm plastic flasks (Costar) 5 to 6 days before being used at an initial density of 2.4 x 10^5 cells/sq cm were also pulse-labeled or continuously labeled with [3H]thymidine after MNNG (0.5 μg/ml of medium) administration. Incorporation of labeled precursor was stopped by rinsing the monolayers twice with cold (2°C) Hanks’ balanced salt solution and in situ freezing of the cells in a bath of dry ice methanol. The frozen cell sheets were stored at -86°C until analyzed. Immediately after thawing, the unincorporated nucleoside was extracted with 0.4 M perchoric acid, and the extracted cells were dissolved with 0.1 N NaOH to isolate the acid-insoluble fractions. A 0.5-ml aliquot was mixed with 15 ml Aquasol (New England Nuclear) and counted in a Packard liquid scintillation spectrometer at an efficiency of 35% for 3H in Aquasol. The results were expressed as dpm/culture. To minimize the influence of variations in cell number on dpm/culture, each control and experimental series was duplicated by confluent monolayers which were trypsinized at the end of the labeling period, and the monodispersed cells were counted on a Model B Coulter counter (Coulter Electronics, Hialeah, Fla.); dpm were corrected when differences greater than 5% occurred between the number of cells in control and experimental samples. Triplicate cultures were used for each scintillation and autoradiographic count.

Survival Studies. At different intervals after the confluent monolayers were treated with MNNG, or after the cultures were seeded for transformation assay (see below), the cells were dispersed with trypsin-Versene mixture and diluted with complete medium. Aliquots of 5 x 10^5 cells in 0.5 ml were inoculated in 25 sq cm plastic flasks containing 5 ml of a prewarmed mixture of conditioned/fresh medium (1:2; v/v). After a 10- to 14-day incubation period in a humidified incubator with 10% CO₂, the colonies were fixed, stained, and counted (13, 14). Colonies containing 64 or more cells were considered viable. The surviving fraction was calculated as a fraction of the number of untreated colonies. For each data point a minimum of 10 flasks were used.

Focus Transformation. Confluent cultures of HEC were treated with MNNG or with MNNG plus HU, CF, and/or TP for different intervals. The monolayers were trypsinized, and aliquots of 5 x 10^4 cells in 0.75 ml were seeded in 25-sq cm plastic bottles containing 4.25 ml of medium. Medium was changed twice weekly. After 3 to 6 weeks, the medium was removed, and the cultures were fixed with absolute methanol and stained with diluted (1:9, v/v) Giemsa solution. Transformed foci were identified with the aid of a Stereo Zoom microscope (Bausch & Lomb, Inc., Rochester, N. Y.) at x5 to x125. The average number of transformed foci in a minimum of 10 flasks was determined.

Chemicals. MNNG and HU were purchased from Schwarz/Mann (Orangeburg, N. Y.), TP was obtained from Sigma Chemical Co. (St. Louis, Mo.), CF was from Calbiochem (La Jolla, Calif.), and DMSO was from Crown Zellerbach (Camas, Wash.). All chemicals, except for MNNG, were dissolved directly in Hanks’ balanced salt solution to prepare 100-fold concentrated stock solution from which appropriate amounts were used. MNNG was solubilized in DMSO and used at pH 6.8 to 6.9.

Statistical Analysis. The dose-response curves of [3H]thymidine incorporation in MNNG-treated cultures were computer analyzed using a quantal bioassay program written in FOR-TRAN to fit the model using maximum likelihood estimation. The model utilizes a natural response rate for the background labeling plus an exponential component for the relationship due to dose, according to the method described by Finney (6). The other results, indicated in Tables 2 and 3, were analyzed using Student's t test.

RESULTS

Analysis of MNNG-stimulated [3H]Thymidine Incorporation

Dose-Response Studies. MNNG, at concentrations ranging between 0.1 and 0.5 μg/ml, is known to trigger DNA replication in DDIR cultures of HEC (14); this replication is related to the dose of carcinogen in a linear manner. However, we considered that a dose-response curve derived from a broader range of MNNG concentrations would be more informative with respect to the nature of carcinogen-induced DNA synthesis. Thus, experiments were done with HEC treated with MNNG doses varying between 10⁻⁷ and 10⁻² μg/ml (Chart 1). No detectable changes in labeling index occur until 0.1 μg MNNG per ml of medium. The ascending portion of the curve between 0.1 and 0.5 μg MNNG per ml corresponds, according to the quantal bioassay computerized program, to a model fit by the equation:

\[
\hat{P} = \left( b + \left( 1 - e^{-d \cdot c} \right) \right) / \left( d \cdot c \right)
\]

in which \( \hat{P} \) represents the estimated probability that a cell will...
become labeled at the dose $d$, $b$ and $\lambda$ indicate the background labeling and scale parameter, respectively, and $c$ equals the value of the threshold dose.

The fit and derived parameters of Equation A are presented in Table 1. These estimates substantiate the adequacy of the model for the labeling data obtained with 0.1 to 0.5 $\mu$g MNNG per ml. Moreover, by simultaneously considering all doses within this range, it becomes evident that the changes of $\hat{P}$, being in the interval

$$\hat{P} = 5.93 \pm 0.0653 \pm 0.1172$$

are within a confidence limit of 95%.

The straight ascending line in Chart 1 is suggestive of a one-hit mechanism of induction. However, one hit/one target theory would also predict a linear relationship between the log of fraction of unlabelled cells and the dose. Such a plot is shown in the inset of Chart 1. The linearity with which the fraction of unlabelled cells decreases with each increment of the doses may be accepted as additional evidence for a one-hit mechanism of MNNG-induced $[^3H]$thymidine incorporation in DDH cultures of HEC.

The size of subpopulation of HEC stimulated to incorporate $[^3H]$thymidine is about 30% at the optimal dose of MNNG (0.5 $\mu$g/ml). Interestingly, the MNNG dose which maximally stimulates $[^3H]$thymidine incorporation in DNA is also most efficient in inducing morphological (1, 14) and neoplastic (2) transformation.

Kinetic Studies. A typical experiment in which the time course of $[^3H]$thymidine incorporation elicited in the acid-insoluble fractions of DDH HEC by a single application of 0.5 $\mu$g MNNG per ml was investigated with pulse (Chart 2A) and continuous (Chart 2B) labeling regimens. In both cases, cell labeling commences 4 to 5 hr posttreatment and continues until the 10th hr, when in pulsed monolayers the specific activity decreases. In these cultures, the maximum incorporation coincides with the plateau of $[^3H]$thymidine uptake observed in continuously labeled monolayers. These 2 different results indicate that during the 10th hr of the experimental period the rate of $[^3H]$thymidine incorporation elicited by MNNG decreases considerably. This decrease is probably due to the termination of carcinogen-stimulated DNA synthesis and does not result from the exhaustion of $[^3H]$thymidine from the incubation medium (Table 2).

Effects of HU and CF on Carcinogen-stimulated $[^3H]$Thymidine Uptake. Although CF did not affect DNA synthesis in DMSO-treated cultures (not shown), it did inhibit MNNG-elicited $[^3H]$thymidine incorporation by approximately 50%. The inhibitory action of CF occurs in pulse-labeled (Chart 2A) and continuously labeled (Chart 2B) cultures. HU (10 $\mu$m) inhibited precursor uptake 80 to 85% when administered to MNNG-treated DDH HEC; synergistic effects occurred when CF and HU were given simultaneously. After a relatively longer lag period, the observed HU-resistant uptake of $[^3H]$thymidine increased and reached a peak 8 to 10 hr following that of HU- and CF-suppressible incorporation (Chart 2, A and B).

Cytotoxicity Studies

Survival of DDH HEC treated with MNNG (Chart 3) demonstrates the lack of measurable toxicity of medium containing 0.5 $\mu$g MNNG per ml medium. This contrasts with the extreme lethality of an MNNG pulse on logarithmically growing cell cultures of hamster (1, 11) or mouse origin (2) assayed by colony formation. HU (10 $\mu$m) caused a progressive decrease in colony-forming ability if treatment of DDH HEC (Chart 3) extends beyond 12 hr. The cell killing in cultures treated with both HU and MNNG (Chart 3) is not significantly different from that observed with HU only. CF (1 $\mu$m), either alone (Chart 3) or associated with MNNG (Chart 3), was not toxic for confluent cultures of hamster embryo cells.

The cytotoxic effects of CF (1 $\mu$m) and HU (0.1 and 10 $\mu$m) on HEC seeded for focus formation following treatment for 25 hr with 0.5 $\mu$g MNNG per ml were also investigated (Chart 4). This information is essential for determining whether the influence of HU and CF upon MNNG-induced morphological transformation is mediated by a nonspecific cell killing of HU and CF exerted on carcinogen-treated cells. Neither CF nor HU at a dose of 1 and 0.1 $\mu$m, respectively, are cytotoxic for the first 36 hr after plating for focus formation, but a time-dependent inhibition of single-cell survival occurs after relatively short incubation periods with 10 $\mu$m HU (Chart 4). Furthermore, treatment for 25 hr at confluency of HEC with MNNG or DMSO resulted in similar survival rates when the cells seeded for focus formation were tested for colony-forming ability during the first 36 hr after plating (Chart 4).

Transformation Studies

In agreement with earlier findings (14), the average number of transformed foci of HEC per flask versus the time interval that the cells are maintained as DDH monolayers subsequent to MNNG treatment forms a single-peaked curve (Chart 5). Few

Table 1

<table>
<thead>
<tr>
<th>MNNG dose ($\mu$g/ml)</th>
<th>No. of cells counted</th>
<th>No. of cells labeled</th>
<th>Proportion labeled</th>
<th>Exponential fit</th>
<th>Equation parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>10,099</td>
<td>855</td>
<td>0.08471 ± 0.0390</td>
<td>0.08610</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>9,763</td>
<td>1,244</td>
<td>0.12743 ± 0.0431</td>
<td>0.12953</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>9,954</td>
<td>1,727</td>
<td>0.17352 ± 0.1008</td>
<td>0.17237</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>10,090</td>
<td>2,426</td>
<td>0.24039 ± 0.1172</td>
<td>0.24143</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>9,995</td>
<td>3,199</td>
<td>0.32002 ± 0.0863</td>
<td>0.31076</td>
<td></td>
</tr>
</tbody>
</table>

* In Chart 1, 0.1 ≤ d, ≤ 0.5 represents the exponential portion of the curve illustrated. Each dose level has been investigated in triplicate in 2 separate experiments.

** a: background labeling; b: curve threshold; c: scale parameter (see also the text).

The fit and derived parameters of Equation A are presented in Table 1. These estimates substantiate the adequacy of the model for the labeling data obtained with 0.1 to 0.5 $\mu$g MNNG per ml. Moreover, by simultaneously considering all doses within this range, it becomes evident that the changes of $\hat{P}$, being in the interval

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Chart 2. Incorporation of [3H]thymidine into the acid-insoluble fraction of 6-day-old DDIR cultures of tertiary HEC exposed for increasing intervals to medium containing 0.5 μg MNNG/ml (C). The effects exerted by 10 mM HU (△), 1 mM CF (□), or both (●) on MNNG-elicited [3H]thymidine uptake are also indicated. The experiments summarized were conducted with both pulse (A) and continuous (B) labeling conditions. Incorporation of [3H]thymidine in DMSO-treated controls (▲) is also presented. The amount of inhibition induced by CF and HU was determined from the dpm values accumulated over a 20 hr-exposure period to MNNG-containing medium (B).

Table 2
Lack of [3H]thymidine exhaustion in medium of DDIR cultures of HEC exposed to MNNG

<table>
<thead>
<tr>
<th></th>
<th>5-day-old DDIR monolayersa</th>
<th>MNNG (0.5 μg/ml)</th>
<th>[3H] dpm × 10^{-1} / culture</th>
<th>Significance (Student’s t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinogen stimulated</td>
<td>48</td>
<td>46.57 ± 0.03b</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Serum stimulated</td>
<td>1</td>
<td>96.0 ± 2.57</td>
<td>5.0 0.01</td>
<td></td>
</tr>
</tbody>
</table>

a Five-day-old density-inhibited monolayers of secondary HEC in 25-sq cm Falcon plastic bottles received 1μCi of [3H]thymidine of medium plus the indicated dose of MNNG (see below) and were kept for an additional 2 days in a humidified atmosphere of 10% CO2 in air. They were designated carcinogen-stimulated cultures. At the end of this interval, the radioactive medium of carcinogen-stimulated cultures was collected, and the acid-insoluble radioactivity of the cells was measured as indicated under “Materials and Methods.” The collected radioactive medium was centrifuged for 20 min at 3000 rpm, filtered through a 0.45-nm assembly membrane (Natigel), and stored at -20°C. Twenty-four hr later, fresh medium was aspirated and replaced by an equal amount of radioactive medium thawed and prewarmed at 37°C just prior to use. The serum-stimulated cultures were incubated in radioactive medium for 1 hr, and at the end of this interval the radioactivity incorporated into acid-insoluble fractions was determined as before in a Model 3390 Packard scintillation counter. b Mean ± S.D. of 3 different cultures.

transformed foci occur when 10 hr or less elapse between the exposure to MNNG and the plating for transformation assay. When the cultures were plated to ascertain morphological transformation between 10 and 25 hr following the MNNG pulse, the frequency of transformed foci steadily increases to a maximum. As the time interval before plating is extended beyond 30 hr, the number of transformed foci decreases again.

The effects upon morphological transformation of concomitant and postcarcinogenic administration of HU, CF, and TP are summarized in Table 3. Both CF and TP, as well as HU, inhibited the development of transformed foci with HEC cultures. No differences between the inhibitory effects of CF and TP were noted. Finally, no morphological transformation is induced in cultures treated with CF, TP, or HU.
occurs immediately after treatment, whereas the DDIR cultures of HEC. This incorporation is fundamentally different from \[^{3}H\]thymidine incorporation into MNNG-stimulated DDIR cultures of HEC. With unscheduled DNA synthesis, maximum incorporation rate occurs immediately after treatment, whereas the DDIR cultures present a unique situation in which the HU-resistant incorporation is delayed for more than 10 hr after MNNG treatment (Chart 2). Concomitant with the inhibitor-induced reduction in rate of \[^{3}H\]thymidine incorporation, there is a reduction in the number of transformed foci. Evidence for a causal relationship between the number of transformed foci and \[^{3}H\]thymidine incorporation was shown previously using a 5'-bromo-2'-deoxyuridine-photolysis protocol (14). In those experiments, morphological transformed foci induced by MNNG in the DDIR cultures of HEC was related to the subpopulation of cells responsible for \[^{3}H\]thymidine incorporation. The inactivation of this subpopulation by 5'-bromo-2'-deoxyuridine and fluorescence light suppressed the transformation due to MNNG.

The response of the DDIR cultures to concentrations of CF and HU used differ on the basis of the relative amount of inhibition, as well as in terms of the maximum amount of inhibition obtained. The induced incorporation of \[^{3}H\]thymidine in hamster DDIR cultures is ordered MNNG > MNNG plus CF > MNNG plus HU > MNNG plus HU plus CF. The addition of HU to MNNG cultures or the combination of CF and HU to MNNG cultures caused an additional delay in reaching the maximum amount of \[^{3}H\]thymidine incorporation relative to that obtained with MNNG alone or the combination of MNNG and CF. However, whether the delay in attaining maximum incorporation rate reflects the course of the wave of \[^{3}H\]thymidine uptake that is resistant to HU and/or CF could not be evaluated with certainty from the results.

The survival data obtained in cultures treated at confluence with the various combinations suggest that different DNA pathways had been inhibited. The inhibition of \[^{3}H\]thymidine incorporation into responsive cells inhibited by CF was not accompanied by a difference in survival relative to cultures treated with MNNG alone (Chart 3). On the other hand, HU-treated

**DISCUSSION**

The addition of inhibitors of DNA synthesis reduced the \[^{3}H\]thymidine incorporation into MNNG-stimulated DDIR cultures of HEC. This incorporation is fundamentally different from what is referred to as unscheduled DNA synthesis or excision repair, because only a fraction of cells incorporate the \[^{3}H\]thymidine and because the kinetics of incorporation is different. With unscheduled DNA synthesis, maximum incorporation rate occurs immediately after treatment, whereas the DDIR cultures present a unique situation in which the HU-resistant incorporation is delayed for more than 10 hr after MNNG treatment (Chart 2). Concomitant with the inhibitor-induced reduction in rate of \[^{3}H\]thymidine incorporation, there is a reduction in the number of transformed foci. Evidence for a causal relationship between the number of transformed foci and \[^{3}H\]thymidine incorporation was shown previously using a 5'-bromo-2'-deoxyuridine-photolysis protocol (14). In those experiments, morphological transformed foci induced by MNNG in the DDIR cultures of HEC was related to the subpopulation of cells responsible for \[^{3}H\]thymidine incorporation. The inactivation of this subpopulation by 5'-bromo-2'-deoxyuridine and fluorescent light suppressed the transformation due to MNNG.

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cultures demonstrated a significant loss in survival and a greater degree of inhibition of \(^{3}H\)thymidine incorporation. Cells of DDIR cultures exposed to MNNG and HU, plated for focus formation and exposed to the inhibitor for an additional 24 to 36 hr of incubation, had a decreased survival relative to that obtained when HU treatment was limited to confluent cultures for 20 hr (Chart 4). Therefore, the loss of \(^{3}H\)thymidine uptake as a result of exposure to a combination containing HU may be due to reduced cell survival.

Conceivably, the lack of an incorporation response at MNNG doses less than 0.1 \(\mu\)g/ml of medium is due to the relative insensitivity of the techniques used. Nevertheless, low levels of damage are expected because the amount of —SH groups, which influence the alkylation of DNA by MNNG (10, 11), is less in DDIR cultures than in sparse monolayers (11). An equally plausible interpretation is that the absence of a response represents very little DNA damage that is not removed and, therefore, not repaired. Recently, it has been documented that sister chromatid exchanges are a very sensitive index for carcinogen-induced unexcised DNA damage (16). Application of this chromosomal analysis with doses of MNNG that did not increase \(^{3}H\)thymidine incorporation resulted in an increased frequency of sister chromatid exchanges (17, 19).

Although the effects of HU appear mediated by its marked cytotoxicity (Charts 3 and 4), the inhibition of carcinogen-stimulated \(^{3}H\)thymidine incorporation by nontoxic doses of CF may be instrumental in inhibiting the development of morphologically altered foci. CF has been reported to enhance as well as to inhibit chemically induced transformation in vivo and in vitro (4, 7, 9, 15). In previous experiments (4), transformation of HEC by MNNG could be enhanced when CF was added while cells were in their early multiplication stage. It seems, therefore, that the inhibitory (9, 15) or enhancing (4, 7) effects of CF on morphological cell transformation depends, among other factors, on the state of cell proliferation at the time of drug administration. Differences between transformation assays and procedures used, CF concentration, and the sequence of carcinogen and CF treatment may account for the observation in which the inhibition of in vitro transformation was reported (4).

Recently, it has been demonstrated that CF does not inhibit DNA synthesis at sublethal doses even in UV-treated cells (3). However, incorporation of \(^{3}H\)thymidine in carcinogen-responsive cells is sensitive to CF at sublethal doses, suggesting future studies of postreplication repair under these conditions.

The possible explanation(s) for the shape of the transformation curve (Chart 5) have been discussed (14). The present experiments are consistent with the concept that the reduced transformation frequency observed after 30 to 35 hr following MNNG treatment is controlled by the intervention of repair processes, which can occur when carcinogen-treated cells are maintained in a nondividing state (8).

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REFERENCES

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