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Differential cell cycle phase specificity for neoplastic transformation and mutation to ouabain resistance induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in synchronized C3H10T½ Cl8 cells

(chemical carcinogens/mutagenesis/methylating agent/toxicity)

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ABSTRACT **The transformable mouse embryo fibroblast cell line C3H10T½ Cl8 has been employed to study the induction by the carcinogen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) of morphological transformation and mutation to ouabain resistance throughout the cell cycle. Cells were synchronized by means of isoleucine deprivation for 24 hr and initiated DNA synthesis with a high degree of synchrony 7.5 hr after release of the isoleucine block. At various intervals throughout the cell cycle cultures were treated with MNNG at 1.0 μg/ml and the induction of cytotoxicity, morphological transformation, and ouabain-resistant colonies was determined. All three phenomena exhibited marked cell-cycle phase dependency. Maximal induction of transformation occurred in cultures treated 7.5 hr after release from isoleucine deprivation, when the cells were at the G_1/S boundary. In contrast, induction of ouabain-resistant colonies was at a minimum at the time of maximal induction of transformation, and peak induction of ouabain resistance did not occur until 16–18 hr after release from the isoleucine block, when cells were in late S phase. A close correlation was observed between the induction of cytotoxicity and of ouabain-resistant mutants. The results suggest that differences exist in the production or cellular processing of the various early lesions.**

Many studies have shown an excellent correlation between carcinogenicity and mutagenicity for a wide range of chemicals (1, 2). The development of *in vitro* mammalian cell systems capable of simultaneously detecting transformation and mutation in the same cell system has made it possible to more closely examine the relationship between these two events (3–7). While somatic mutation has been closely studied at the genomic level (8), neoplastic transformation is increasingly viewed as a complex series of events requiring the interplay of both genetic and epigenetic factors (9).

Both the induction of neoplastic transformation and the induction of mutation have been shown to vary quantitatively throughout the cell cycle; however, analysis of these two events within the same experiment has not been previously reported. The C3H10T½ Cl8 cell line was selected for these studies because it has been used extensively for quantitative studies of chemical carcinogenesis (10, 11) and, more recently, for the mutational induction of ouabain resistance (12). Previous studies of the cell-cycle specificity of transformation in 10T½ cells, using a variety of carcinogens and methods of synchronization, have implicated cells in late G_1 (13) and early S phase (14–16) as displaying the greatest sensitivity to transformation. In this paper we report temporal differences in the cell-cycle phase-specific induction of neoplastic transformation and mutation to ouabain resistance in synchronized C3H10T½ cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG).

MATERIALS AND METHODS

Cells. The C3H10T½ Cl8 mouse embryo fibroblast line described by Reznikoff *et al.* (17) was used throughout these studies. Stock cells between passages 8 and 14 were passaged weekly in antibiotic-free Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO). At confluence, cells were re-fed with complete medium plus gentamicin (25 μg/ml) and held for 2 days prior to synchronization.

Synchronization. Cells were synchronized by means of isoleucine deprivation. Confluent cultures were gently treated with 0.1% trypsin and plated at the desired concentration in isoleucine-free medium supplemented with 10% dialyzed heat-inactivated fetal calf serum. Cultures were held for 24 or 36 hr in isoleucine-free medium and released with complete Eagle's basal medium supplemented with 15% undialyzed heat-inactivated fetal calf serum and isoleucine at 100 μg/ml. Cells were released in two groups. Those cells to be treated at 0–12 hr were released at 0900 and treated throughout the day, whereas cells to be treated at 14–24 hr were released at 2100 and treated throughout the following day. The increased holding time did not noticeably affect the degree or timing of synchrony in these two groups.

Monitoring of Synchrony. DNA synthesis in cells plated at 10^5 cells per 60-mm dish and synchronized as described above was followed by addition of [^{3}H]thymidine (1 μCi/ml, 50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) for 1 hr to groups of four previously untreated dishes at intervals of 2 hr. The degree of synchrony was determined by autoradiography and trichloroacetic acid precipitation as described (13). The cells in an additional two dishes were counted electronically at each time period to determine the rate of cell doubling.

Cytotoxicity. Triplicate cultures seeded at 400 cells per 60-mm dish were synchronized and treated with MNNG (Sigma) in acetone, or acetone (0.5%) as control, at 4-hr intervals after release. After 7 days the cells were fixed in MeOH and stained by the Giemsa procedure, and the plating efficiency was determined as described (13). Control cultures synchronized in isoleucine-deficient medium as described above had a plating efficiency of 10–15%.

Transformation. Cells were plated into 60-mm dishes in isoleucine-free medium and synchronized as described above. To compensate for the higher level of cytotoxicity in S phase as

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Abbreviation: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

opposed to G_1, plating densities during these periods were adjusted to provide a surviving fraction of at least 100–200 cells per dish as previously determined from cytotoxicity studies. In a typical experiment, dishes treated with MNNG at 1.0 μg/ml at 0–8 hr were seeded with 2,000 cells per dish, and dishes treated at 8–24 hr were seeded with 7,000 cells per dish. Replicate cultures were treated with MNNG at 1.0 μg/ml at four hourly intervals after release; typically 10 dishes were treated with MNNG and 2 dishes were treated with acetone. An additional 24 cultures seeded at 2,000 cells per dish received treatment with acetone as control 14 hr after release. Five days after release from isoleucine deprivation and at weekly intervals thereafter, cells were re-fed with complete medium plus 5% heat-inactivated fetal calf serum and gentamicin at 25 μg/ml (18), and the numbers of type II and III morphologically transformed foci were determined after a 5- to 6-week interval (11).

Mutation. Cells were seeded at 1×10^5 per 100-mm dish in isoleucine-free medium and synchronized as described above. After release, four dishes were treated per time point with MNNG at 1.0 μg/ml or acetone as a control. Selection for ouabain-resistant mutants was carried out as described by Thompson (19). Three, six, or nine days after treatment with MNNG the cells were trypsinized and subcultured 1:2 into 100-mm dishes in medium containing 2 mM ouabain (Sigma). The cells were then re-fed weekly with this medium. After 2 weeks surviving cells were fixed in MeOH and stained by the Giemsa procedure, and the number of ouabain-resistant colonies was determined. For characterization some colonies were first identified microscopically then cloned by using a glass ring.

Statistical Analysis. Cytotoxicity data were analyzed by Student's *t* test without assumption of equal variances and mutagenicity data were analyzed by means of the *t* test and by the Z test statistic for proportions. Analysis of transformation frequency was complicated by the low frequency of transformation in treated dishes. Pooled data from four individual experiments, involving a total of 100 dishes per time point, were analyzed for significance by using the Z test statistic. Interpretation of the data was simplified by our failure to detect spontaneous mutation or transformation in the extensive series of cultures examined. For transformation, all dishes scored contained no more than one transformed focus per dish. All statistical tests were conducted at a level of significance of 0.05.

RESULTS

Synchrony. Attempts to obtain synchrony by using our previously published protocols (13) gave inadequate levels of synchrony. Modifications of the protocol by plating confluent cells in 10% dialyzed heat-inactivated fetal calf serum, holding them in deficient medium for only 24 hr, and releasing them from cell-cycle blockade with 15% heat-inactivated fetal calf serum supplemented with L-isoleucine at 100 μg/ml was found to give the high level of synchrony shown in Fig. 1. After a delay of about 8 hr DNA synthesis rapidly increased, as measured by incorporation of [^{3}H]thymidine. Autoradiography indicated a cohesive entry of cells into S phase at this time. The labeling index increased from 8% at 6 hr after release to approximately 90% 14 hr after release, the time of peak S phase. Thereafter the number of labeled cells decreased rapidly. Between 17 and 20 hr after release about 90% of the cells divided as indicated by cell counting. Thus, within the first cell cycle after release 87.6 ± 1.9% of the cells became heavily labeled with a 1-hr pulse of [^{3}H]thymidine and 97.5 ± 3.0% of the cells subsequently divided. Therefore, the 90% of attached cells that incorporated [^{3}H]thymidine during mid-S phase must all have continued on through the cycle and divided synchronously 3–6 hr later. Cells held in deficient medium for 1–4 days failed to incorporate [^{3}H]thymidine and did not divide.

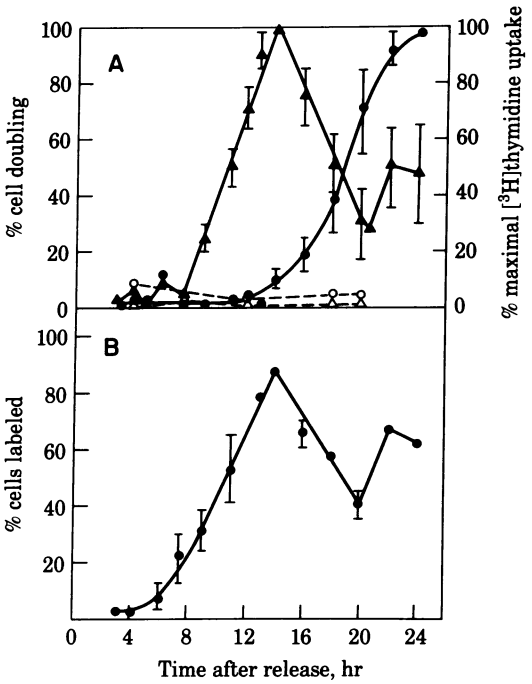

FIG. 1. Induction of synchrony established by release from 24-hr isoleucine deprivation. (*A*) ▲, Incorporation of [^{3}H]thymidine expressed as a percentage of maximal uptake. Due to variation of peak uptake of [^{3}H]thymidine in each experiment, values of [^{3}H]thymidine incorporation at each time point are expressed as a percentage of the maximal incorporation for each experiment. ●, Percentage cell doubling. ○ and △ represent controls held in a state of isoleucine deprivation. Means ± SEM of six experiments are shown. (*B*) Labeling index obtained by autoradiography after a 1-hr pulse of [^{3}H]thymidine. Cells with light labeling were excluded from the data. Means ± SEM of three experiments are shown.

Cytotoxicity. As demonstrated previously (13), the cytotoxicity of MNNG increased as cells approached and progressed through S phase. Preliminary experiments with MNNG at 2.0 μg/ml resulted in greater than 95% cell killing throughout the cell cycle. MNNG doses of 1.0 and 0.5 μg/ml resulted in proportionately less cell killing (Fig. 2*A*). Because of the absence of transformation detected with 0.5 μg/ml, 1.0 μg/ml was chosen for all subsequent studies. This concentration resulted in survivals of 14–48% of control with statistically significant differences in survival within the cell cycle (Table 1). Replacement with fresh medium 1 hr after treatment with MNNG resulted in no substantial reduction in cytotoxicity over those cultures treated simultaneously with MNNG that did not receive a change of medium.

Transformation. C3H10T½ cells synchronized by isoleucine deprivation as described above were treated with MNNG at 1.0 μg/ml at various intervals throughout the cell cycle after release. The results shown in Fig. 2*B* and Table 1 indicate that transformation was significantly increased 7.5 hr after release, close to the G_1/S boundary, 6 hr prior to peak DNA synthesis. Little transformation was seen during periods of peak toxicity. A smaller, second peak of transformation occurred 20 hr after release and is believed to represent transformation of cells in their second cycle after release. No transformation was found in acetone-treated control cells or cells treated with MNNG at 0.5 μg/ml throughout the cell cycle.

Mutation. In the synchronous population of cells used for the studies of transformation, the frequency of mutation to ouabain

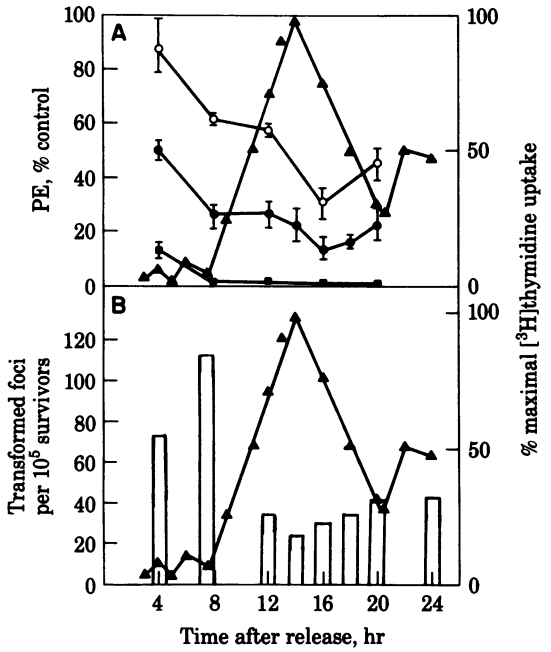

FIG. 2. Cell-cycle phase specificity for the induction of cytotoxicity and neoplastic transformation in synchronized 10T½ cells. (*A*) Cytotoxicity is expressed as plating efficiency (PE) as percentage of control for cells treated with MNNG at 0.5 (○), 1.0 (●), or 2.0 (■) μg/ml, as cells progressed through the cycle. Means ± SEM of three experiments are shown. (*B*) Bars represent transformation frequency expressed as the numbers of transformed foci per 10^5 survivors for cultures treated with MNNG at 1.0 μg/ml. Cumulative data from four experiments (see Table 1) are shown. For *A* and *B*, ▲ indicates percentage maximal uptake of [^{3}H]thymidine (see Fig. 1 legend).

resistance was also determined. A significant increase in the yield of ouabain resistance was observed 16–18 hr after release from isoleucine deprivation, when the majority of cells were in late S phase or were entering G_2–M (Table 1). In cells treated during G_1 or at the time of peak induction of transformation (7.5 hr after release) significantly lower yields of mutants were obtained. The ratio of transformants to ouabain-resistant mutants was 5:1 in the latter cells, whereas at the time of peak induction of mutation the ratio was 0.2:1. No spontaneous mutants were seen in acetone-treated controls subcultured into 2 mM ouabain.

Table 1. Comparison of transformation and mutation to ouabain resistance in synchronized C3H10T½ cells*

Time, hr	Tox,† %	No. foci/ no. survivors × 10^{-3}	Transf‡	No. Oua^r colonies/ no. survivors × 10^{-5}	Mut§	Transf/ Mut¶
4	45.5	9/12.3	74.6	49/3.8	12.9	5.8
7.5	27.6	23/23.1	112.5	62/2.5	24.4	4.6
12	29.6	9/24.8	34.4	138/2.3	59.9	0.6
14	23.9	4/20.0	23.2	138/1.9	72.9	0.3
16	13.5	5/16.2	29.5	220/1.4	157.1	0.2
18	16.4	6/18.7	32.2	268/1.9	141.8	0.2
20	23.6	7/19.8	37.6	87/1.6	54.0	0.7
24	48.3	10/26.1	43.3	174/3.2	53.3	0.8

* The data for transformation and mutation represent cumulative results of four separate experiments involving 100 60-mm dishes and 32 100-mm dishes per time point, respectively.

† Toxicity as a percentage of control; for SEMs see Fig. 2*A*. For MNNG at 1.0 μg/ml 4 and 24 hr are significantly different from all other points; 7.5 and 12 hr are signficantly different from 16 or 18 hr; 20 hr is significantly different from 16 hr.

‡ Transformed foci per 10^5 survivors. The 7.5-hr value is significantly different from all other data except the 4-hr value and is significantly different from the mean transformation frequency for all points combined (20).

§ Ouabain-resistant (Oua^r) mutants per 10^5 survivors. The 16-hr datum is significantly different from all other data but 18 hr.

¶ Transf/Mut, ratio of transformation frequency to mutation frequency.

Studies of the effect of expression time on the recovery of ouabain-resistant mutants are presented in Fig. 3. No marked differences in the recovery of ouabain-resistant mutants were seen after expression periods of 3, 6, or 9 days, and the levels of induced mutation frequencies were highest at 16–18 hr after release regardless of the expression period allowed. As demonstrated in Fig. 4, the induction of mutation bore a linear relationship to the logarithm of cytotoxicity throughout the cell cycle.

Survival curves of 10 ouabain-resistant colonies cloned at random at various times within the cell cycle are presented in Fig. 5. All clones demonstrated a log-linear relationship of survival with increasing drug concentration, with only a 30% reduction in plating efficiency at a concentration of 1 mM ouabain, which resulted in greater than 99% killing of wild-type cells. Clones grown in the absence of ouabain for 4 weeks demonstrated no loss of resistance. Although their saturation densities were slightly increased, all clones isolated grew as stable contact-inhibited monolayers characteristic of control 10T½ cells, demonstrating that ouabain-resistant mutants were not also transformed.

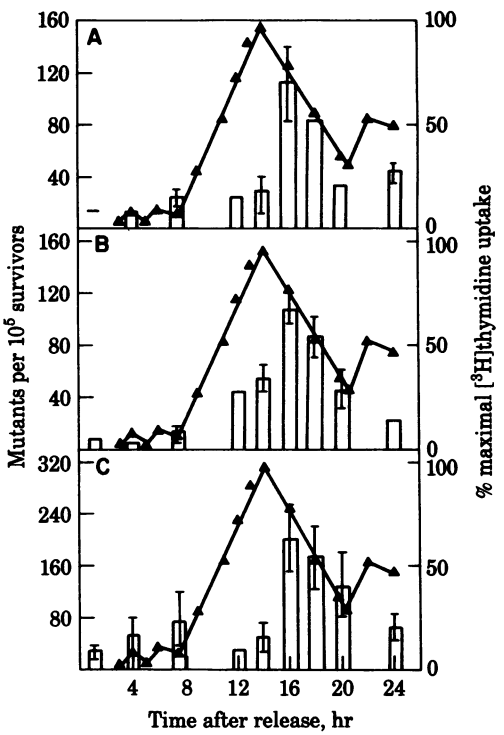

FIG. 3. Cell-cycle phase specificity for the induction of mutation to ouabain resistance in cultures synchronized by isoleucine deprivation and treatment with MNNG at 1.0 μg/ml as a function of time after release. Mutation frequencies, expressed as number of mutants per 10^5 survivors (open bars) are shown for expression periods of 3 (*A*), 6 (*B*), or 9 (*C*) days. Each panel represents the mean ± SEM for three separate experiments. In all panels, ▲ indicates [^{3}H]thymidine uptake (see Fig. 1 legend).

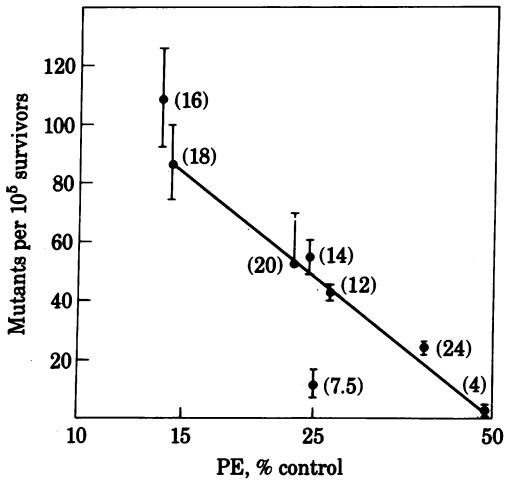

FIG. 4. The relationship between cytotoxicity and mutation frequency in synchronized 10T½ cells treated with MNNG at 1.0 μg/ml at various times after release from isoleucine deprivation. For the data shown in Fig. 3B, mutation frequency (±SEM) is presented as a function of cytotoxicity, which is plotted on a logarithmic scale. PE, plating efficiency. Numbers in parentheses indicate hr after release at time of treatment with MNNG. Correlation coefficient = 0.906.

DISCUSSION

The results of this study demonstrate that synchronized cultures of C3H10T½ cells exhibit marked cycle phase specificity for the induction by MNNG of neoplastic transformation, mutation to ouabain resistance, and cytotoxicity. Whereas the induction of transformation is greatest in cells treated at the G_1/S border 7.5 hr after release from isoleucine deprivation, peak mutation to ouabain resistance was found 16–18 hr after release when cells were exiting S phase and entering G_2–M. Cytotoxicity varied throughout the cell cycle, increasing progressively through G_1 and reaching a maximum in late S phase.

The timing of peak mutation to ouabain resistance was constant regardless of the expression time allowed (Fig. 3), corresponded closely to periods of peak cytotoxicity (Fig. 2A), and exhibited a log-linear relationship to the cytotoxicity throughout the cell cycle (Fig. 4). Such a relationship has also been reported

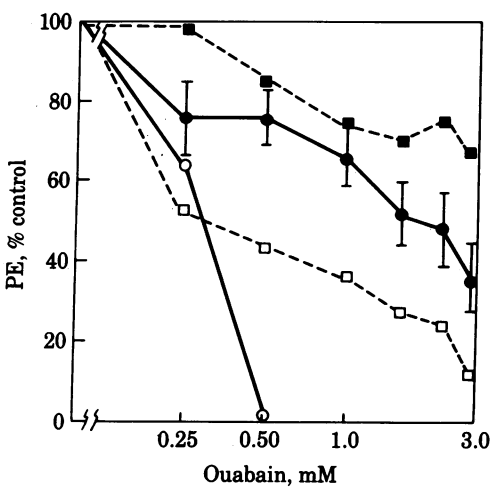

FIG. 5. Cytotoxicity of ouabain to wild-type and ouabain-resistant 10T½ cells. Ten ouabain-resistant colonies were randomly isolated during experiments described in Table 1, plated at 40 cells per ml, and exposed to the stated concentrations of ouabain 1 day after seeding. PE, plating efficiency; ○, wild-type; ■, maximally resistant clone; □, minimally resistant clone; ●, means ± SEM for all 10 clones.

for the induction of 8-azaguanine resistance in synchronous Chinese hamster ovary cells (21). Maximal induction of mutation during periods of peak cytotoxicity has also been noted in other studies (22–24). Several possible explanations for the observed cell-cycle phase specificity for mutation may be advanced. One hypothesis, increased alkylation of DNA during specific periods of the cell cycle, seems unlikely because gross methylation of cellular macromolecules by MNNG has been shown to be independent of cell-cycle position (25). In addition, Bowden and Boutwell (26) have demonstrated a lack of preferential binding of [^{3}H]MNNG to replicating and nonreplicating mouse skin epidermal DNA. A second hypothesis, enhanced mutagenesis of the DNA growing fork, follows from work done with bacterial (8) and yeast (27) systems and Chinese hamster cells (24, 28). Other studies have failed to demonstrate cell-cycle phase-specific mutagenesis (29, 30). Although the growing fork hypothesis is attractive mechanistically, it fails to explain the close correlation seen throughout the cell cycle between cytotoxicity and mutagenicity because it is unlikely that the lesions involving the ouabain locus are the sole lesions involved in cytotoxicity and mutation. The results of this study suggest that a third hypothesis, enhanced fixation of the mutagenic lesion, may account for the strong correlation between toxicity and mutation seen in this study and that by Jostes *et al.* (21). Maher *et al.* (31) have shown that excision repair (during early G_1) of damage induced with *N*-acetoxy-4-acetylaminostilbene results in decreased frequencies of mutation to 6-thioguanine resistance in human fibroblasts, whereas cells treated later in G_1 yielded greater numbers of mutants. Thus the increasing induction of mutagenesis and cytotoxicity seen in 10T½ cells treated progressively closer to late S phase suggests that fixation is maximal at this time period.

The predominance of transformation at the G_1/S border (Fig. 2B) is in close agreement with our earlier study, in which peak transformation was found in late G_1 6 hr prior to mid-S phase (13). Unfortunately, excess cytotoxicity due to the high concentrations of MNNG employed in this earlier study complicated interpretation of the data. The introduction of lower serum concentrations to enhance the expression of transformation (32) enabled us to reexamine this question at more acceptable levels of cytotoxicity. Not unexpectedly, the reduced levels of cytotoxicity achieved in this study were accompanied by a reduction in the overall yield of transformants at each time point (Table 1). To compensate for this, 100 dishes per time point were seeded with 1.2–4.2 × 10^5 cells per 60-mm dish. In spite of the low overall yield of transformants, which could be enhanced only by unacceptable increases in number of dishes, seeding density (33), or drug dosage, statistical analysis in confirmation of our earlier study (13) has shown that significant variations do exist in susceptibility to transformation within the cell cycle.

While our studies have indicated peak incidence of transformation in late G_1/early S phase, other workers have shown S phase specificity for transformation with 5-fluoro-2′-deoxyuridine (14), 1-β-D-arabinofuranosylcytosine (15), combined 5-bromodeoxyuridine and near UV irradiation (34), and MNNG (16). It is not surprising that these compounds are selectively transforming during S phase of the cell cycle, because all masquerade as normal substrates for DNA synthesis. It is perhaps reassuring that this is the case, because it strengthens the argument for the direct role of DNA in the carcinogenic process (35). However, the studies by Grisham *et al.* (16) and Marquardt (36) both implicated S phase as being most susceptible to transformation, and both studies utilized MNNG, which is not similarly biased toward S phase. Marquardt's study in all probability did not examine G_1 as stated because 2 mM thymidine was utilized to synchronize cells, a procedure that does not

block cells in G_1, but in early S phase (37). Studies by Grisham *et al.* (16) demonstrating a mid-S phase sensitivity to transformation allowed adequate analysis of G_1 events, yet did not demonstrate efficient transformation in cells treated in G_1. It is difficult to reconcile these two results with those obtained in the present study. The only notable differences between the experiment of Grisham *et al.* and ours are the methods of synchrony employed and timing of peak periods of cytotoxicity. Under the conditions employed by Grisham *et al.* the duration of S phase was 17 hr and peak levels of cytotoxicity were found in early S. In contrast, cells synchronized in our studies by isoleucine deprivation (Fig. 1) or postconfluence release (13) required 12 hr to complete S phase and maximum cytotoxicity was found during late S phase. Clarification of these differences will require more stringent conditions of synchrony than are now available.

The predominance of transformation seen during late G_1/early S phase in this and other studies (13, 14) suggests that transforming genes replicate early in S phase. Alternatively, regions of the DNA displaying increased sensitivity to the actions of MNNG may become more accessible during the transition from G_1 to S. Differential sensitivity of DNA is suggested by the greater level of adduct formation in linker DNA than in core DNA in human fibroblasts treated with *N*-acetoxy-2-acetylaminofluorene (38) and by the increased binding of actinomycin D to DNA during late G_1 and early S phase (39). Although gross methylation of macromolecules by MNNG appears to be independent of cell-cycle position (25), crucial sites sensitive to the action of MNNG may become exposed during late G_1 or early S phase.

Studies of concomitant induction of mutation and transformation in asynchronous systems have suggested ratios of transformation to mutation of approximately 20:1 (5). This has been explained in part as being due to a larger "target size" for transformation (7). In contrast, the results of this study demonstrate a widely varying ratio of transformation to mutation to ouabain resistance throughout the cell cycle (Table 1). This implies that fundamental differences may exist between the lesions responsible for transformation and mutation or in the processing of these lesions, and that caution must be exercised in quantitatively implicating mutagenic agents as carcinogens solely on the basis of assays of mutagenesis. Recent reports by Kennedy *et al.* (40) and Fernandez *et al.* (41) also challenge the somatic mutation hypothesis of carcinogenesis. Their studies suggest that transformation *in vitro*, rather than arising from a single mutational change, is a probabilistic event involving activation of a large fraction of the cell population upon exposure to a carcinogen. Thus, differing cell-cycle specificities of mutation and transformation might be expected due to the different mechanisms involved.

Conclusions based on the results of this study must be qualified due to a lack of absolute synchrony. As a consequence, it is impossible to state categorically whether cells transformed after treatment at 7.5 hr after release from isoleucine deprivation were in G_1 or S phase. This study was also restricted to the use of one mutagen and a single mutagenic loci.

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