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Transient Inhibition of DNA Synthesis Results in Increased Dihydrofolate Reductase Synthesis and Subsequent Increased DNA Content per Cell

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We examined the role that blockage of cells in the cell cycle may play in the stimulation of gene amplification and enhancement of drug resistance. We found that several different inhibitors of DNA synthesis, which were each able to block cells at the G_1-S-phase boundary, induced an enhanced cycloheximide-sensitive synthesis of an early S-phase cell cycle-regulated enzyme, dihydrofolate reductase, and of other proteins as well. This response was specific, in that blockage at the G_2 phase did not result in overproduction of the enzyme. When the cells were released from drug inhibition, DNA synthesis resumed, resulting in a cycloheximide-sensitive elevation in DNA content per cell. We speculate that the excess DNA synthesis (which could contribute to events detectable later as gene amplification) is a consequence of the accumulation of S-phase-specific proteins in the affected cells, which may then secondarily influence the pattern of DNA replication.

Resistance of cultured cells to methotrexate as a result of amplification of the dihydrofolate reductase gene can be enhanced by pretreatment with a variety of agents, including metabolic inhibitors of DNA synthesis such as methotrexate (22, 25) and hydroxyurea (1, 17), as well as agents that introduce adducts into DNA, including UV light and carcinogens (23; S. Lavi, in press). These diverse treatments have in common inhibition of DNA synthesis and progression of cells through the cell cycle. The question arises as to whether the two phenomena are related. That is, does transient blockage of cells in the cell cycle in some way promote the gene amplification events that are detectable after the inhibition of DNA synthesis is relieved?

In an initial approach to this question, Mariani and Schimke (17) showed that when synchronized CHO cells were treated in mid-S phase with hydroxyurea for 6 to 8 h and then DNA synthesis was allowed to resume during recovery of cells from the drug treatment, the level of dihydrofolate reductase enzyme increased in a subset of the cells. In addition, some cells displayed an increase in dihydrofolate reductase gene copy number that was associated with an enhanced resistance to methotrexate. Subsequently, Hill and Schimke (9) reported that following treatment of cells with hydroxyurea, the intensity of fluorescence staining of DNA per cell undergoes transient increases in a subset of the cell population and that such cells contain a variety of chromosomal aberrations. These observations have led to a model for gene amplification which proposes that transient perturbation of DNA synthesis during S phase results in overreplication of portions of the genome during the recovery period (20). Much of the rereplicated DNA would be unstable and rapidly lost from cells; but that portion of the rereplicated DNA which underwent successful recombination to generate functional, stable genes would, when placed under appropriate selection, result in selective gene amplification and drug resistance. Such induced enhancement in the frequency of gene amplification would necessarily represent an elevation over rates of spontaneous gene amplification (12, 14).

Nevertheless, it remains unclear how the inhibition of replicative DNA synthesis would result in an enhancement of gene amplification. Here we report the effects of variable times of DNA synthesis inhibition on the levels of dihydrofolate reductase as well as on the DNA content per cell, both during drug inhibition and following removal of the inhibitors. We found that inhibition of DNA synthesis results in a cycloheximide-sensitive accumulation of dihydrofolate reductase. Following removal of this inhibition there was a subsequent cycloheximide-sensitive increase in DNA content per cell, as determined by flow cytometry. The magnitude of the increase in DNA content was greatest following release from prolonged inhibition of synthesis and was correlated with maximal stimulation of dihydrofolate reductase enzyme accumulation.

MATERIALS AND METHODS

Growth of cells and flow cytometry. CHO cells, both sensitive (CHO K_1 [no methotrexate]) and resistant (CHO K_1B_{11} [0.5 μM methotrexate]; amplified approximately 50-fold for the dihydrofolate reductase gene) to methotrexate, were used in these studies. They were grown as described previously (12) in F12 medium (GIBCO Laboratories, Grand Island, N.Y.) with 10% fetal bovine serum (GIBCO), with 0 or 0.5 μM methotrexate, respectively. CHO cells defective in synthesis of dihydrofolate reductase (24) were also used and were grown in medium supplemented with 30 μM each of glycine, hypoxanthine, and thymidine. All cells used were verified to be free of contamination by mycoplasm. Where indicated, cells were stained with 10 to 30 μM fluorescein-ated methotrexate (13) in medium supplemented with glycine, hypoxanthine, and thymidine for 12 to 24 h prior to analysis in a FACS II instrument (Becton Dickinson FACS Division, Mountain View, Calif.); in some experiments the periods of staining of cells and of exposure to drugs over-

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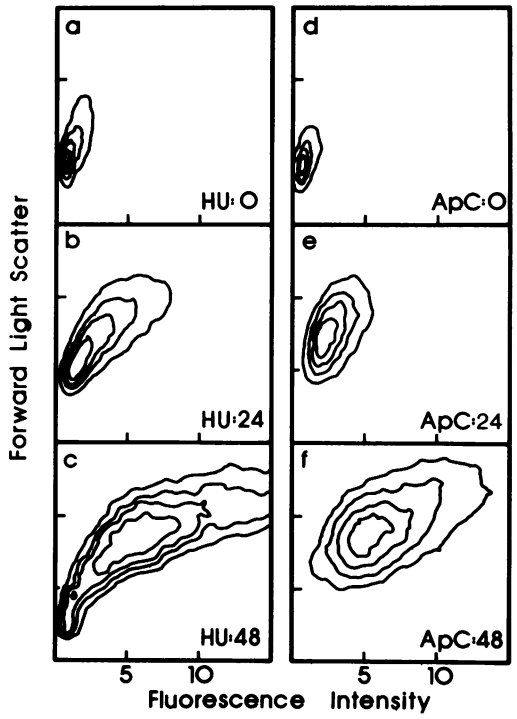

FIG. 1. Hydroxyurea (HU) and aphidicolin (ApC) increase cellular dihydrofolate reductase enzyme content. CHO cells were treated with hydroxyurea (a, b, and c) or aphidicolin (d, e, and f). Staining with fluoresceinated methotrexate was examined at 0 h (a and d), 24 h (b and e), and 48 h (c and f) of treatment. The results are presented as contour plots (20% intervals) of forward light scatter versus fluorescence intensity for populations of 10^4 cells each. Forward light scatter is a function of cell size, which increases as a result of growth during and after drug exposure. Fluorescence intensity is calibrated so that one unit indicates the mean fluorescence of control cell populations (a and d).

lapped. For analysis and sorting of cells by DNA content, living cells were stained for 1 h prior to analysis with 30 μM Hoechst 33342 dye (Calbiochem-Behring, La Jolla, Calif.) (18). DNA content was also analyzed in fixed cells after they were stained with 30-μg/ml chromomycin A_3 (Sigma Chemical Co., St. Louis, Mo.) (18). In addition, independent analyses of DNA content in sorted cells were made by spectrofluorimetry. Known numbers of cells (determined by hemocytometer counts) were lysed in NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris hydrochloride [pH 8]) with 0.5% sodium dodecyl sulfate. Fluorescence was measured in a spectrofluorimeter (excitation wavelength, 365 nm; emission wavelength, 454 nm; The Perkin-Elmer Corp., Norwalk, Conn.), after the addition of sample to NET buffer containing Hoechst 33342 dye at 1.5×10^{-6} M. The final sodium dodecyl sulfate concentration was ≤0.01%. DNA content in treated cells is expressed as a ratio with respect to the level in untreated cells in the G_1 phase of the cell cycle.

Cell selection and drug treatment. Cells were treated with 1 mM hydroxyurea (1), 5 μg of aphidicolin per ml (except as indicated [8]), 10 μg of cycloheximide per ml (16), 0.5 μM methotrexate, or 0.1 μg of Colcemid per ml. All chemicals were obtained from Sigma, except for methotrexate (National Cancer Institute) and Colcemid (GIBCO). During recovery from drug exposure, cells were rinsed at least four times; during recovery of cells from treatment with methotrexate, the culture medium was also supplemented with glycine, hypoxanthine, and thymidine, as described above. In some experiments the degree of inhibition of DNA synthesis was measured by incorporation of [^{3}H]thymidine, administered for 0.5 h at 1 μCi/ml.

Coculture experiments. In coculture experiments, dihydrofolate reductase-negative CHO (24) were cocultured with cells of the C3 subclone of mouse L5178Y lymphoma cells (9). The mixed cell population was exposed to hydroxyurea for 6 h, and then coculturing was continued in the absence of the drug for an additional 12, 24, or 72 h. Following this, the nonadherent C3 cells were removed by extensive rinsing of the cultures with culture medium. Incorporation of functional mouse dihydrofolate reductase genes by the CHO cells was then measured by examining their acquisition of competence to form colonies during growth for 3 weeks in medium not supplemented with glycine, thymidine, or hypoxanthine (7). As a control, the CHO cells were transfected directly with DNA prepared from the C3 cells, as described previously (7).

In parallel experiments, C3 cells were prelabeled for 36 h with [^{3}H]thymidine (0.025 μCi/ml) and then cocultured for 12 to 72 h with hydroxyurea-treated dihydrofolate reductase-negative CHO cells. After removal by rinsing of the nonadherent C3 cells, the CHO cells were arrested in metaphase with Colcemid (2 h), and chromosome spreads were prepared for autoradiography (Kodak NTB-2 emulsion). Transfer of labeled DNA was measured by counting silver grains over chromosomes. For each sample, 50 to 200 metaphase spreads were counted.

Measurement of dihydrofolate reductase enzyme and mRNA. To measure dihydrofolate reductase synthesis, cells were pulse-labeled for 0.5 h with [^{35}S]methionine as described previously (18) and proteins were separated electrophoretically by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by autoradiography. Autoradiographic signals were quantitated by integrating densitometry on a Joyce-Loebl Chromoscan III with background correction. mRNA content was determined by the isolation of total RNA from treated or control cells, separation by electrophoresis in formaldehyde-agarose gels, and hybridization to ^{32}P-labeled mouse dihydrofolate reductase probe (2).

RESULTS

Increase in dihydrofolate reductase levels during prolonged S-phase block. The experiments cited above (1, 17) are consistent with a simple model in which the enhanced amplification of the dihydrofolate reductase genes during recovery from drug exposure leads to subsequent increased dihydrofolate reductase mRNA and enzyme synthesis. However, the experiments did not exclude the possibility that the increase in enzyme content resulted from a process that did not strictly depend on the generation of new genes. To examine this possibility, we used hydroxyurea or aphidicolin to inhibit DNA synthesis in CHO cells for various periods of time and determined the cellular content of dihydrofolate reductase by measuring the intensity of staining of the cells with fluoresceinated methotrexate, which quantitatively binds to dihydrofolate reductase (13).

In Fig. 1 it is shown that prolonged treatment of cells with either drug results in a progressive increase in staining for dihydrofolate reductase that is a function of the duration of drug exposure. This increase was observed, even though DNA synthesis (as determined by incorporation of [^{3}H]thymidine during the first 30 min of drug exposure) was

reduced to 6% or less of the level of untreated cells. The increase in mean fluorescence intensity of populations of treated cells was as great as sevenfold when the inhibition was maintained for 48 h (Fig. 1c and f). This increase was observed both in parental cells that were sensitive to methotrexate, as well as in methotrexate-resistant cells with high enzyme levels (and amplified dihydrofolate reductase genes); in each case the relative enhancement in staining intensity during drug exposure was comparable (Fig. 2). In addition, we have examined the behavior of cells that were defective for the dihydrofolate reductase gene (24). Such cells do not display drug-induced elevations in staining intensity when stained with fluoresceinated methotrexate (Fig. 2), indicating that the increases in fluorescence staining is a true function of dihydrofolate reductase enzyme content. We also observed that prolonged treatment of cells with methotrexate results in a similar marked increase in staining intensity with dihydrofolate reductase (Fig. 2). We therefore conclude that a variety of agents, each acting by different mechanisms to inhibit DNA synthesis and to block the progression of cells through the S phase of the cell cycle, is capable of increasing dihydrofolate reductase levels.

To verify that the increase we observed in dihydrofolate reductase content by flow cytometry was not an artifact of staining with fluoresceinated methotrexate, we analyzed protein synthetic patterns in hydroxyurea-treated cells that were pulse-labeled with [^{35}S]methionine (Fig. 3). Cells with amplified dihydrofolate reductase genes were used for this analysis because the levels of enzyme in nonamplified cells were too low to visualize readily by one-dimensional gel electrophoresis. In Fig. 3 it is shown that rates of synthesis of dihydrofolate reductase increased over sevenfold following 12 h of inhibition of DNA synthesis and that the degree of increase was greater with prolonged exposure to drug. The rate of dihydrofolate reductase synthesis was elevated as early as 6 h after the onset of drug exposure. The onset of this increase was earlier than that detectable in these cells when they were stained with fluoresceinated methotrexate

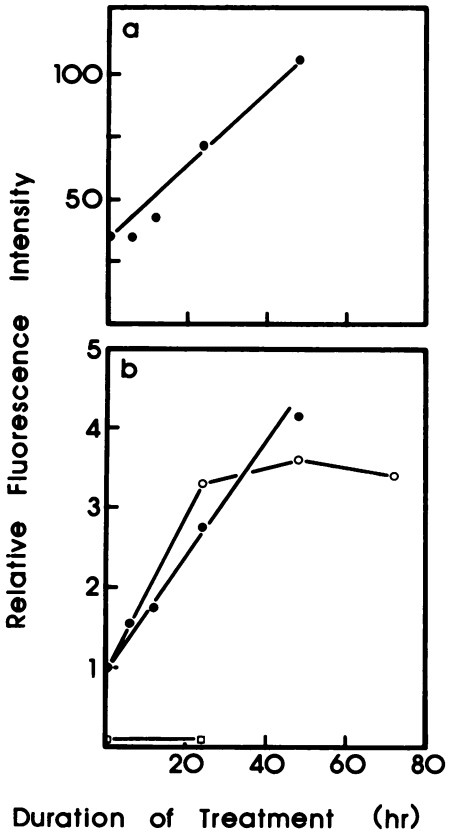

FIG. 2. Dihydrofolate reductase content increases with duration of drug treatment. Cells were treated with hydroxyurea, and the mean population fluorescence measured at the indicated times. The cells used were CHO K_1 (●, panel b; no methotrexate treatment), CHO K_1B_{11} (●, panel a; treatment with 0.5 μM methotrexate), and dihydrofolate reductase-deficient cells (□, panel b). In one experiment, CHO K_1 cells were treated instead with methotrexate (○, panel b). In each case the fluorescence intensity was calibrated so that one unit indicates the mean fluorescence of untreated CHO K_1 cells.

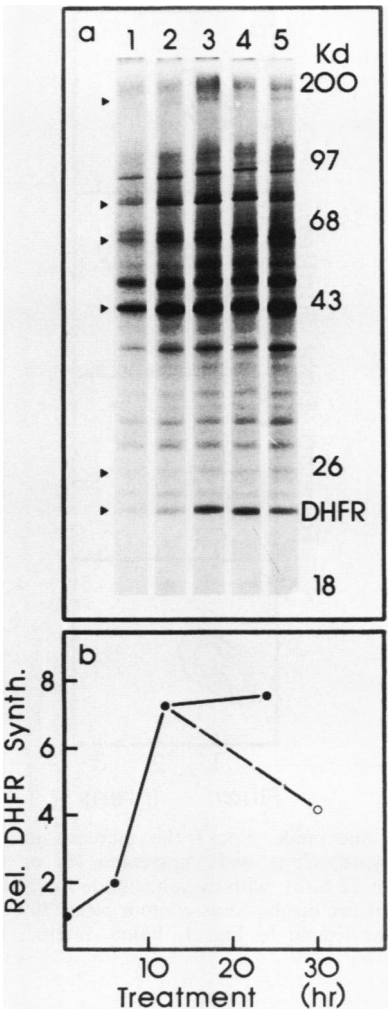

FIG. 3. Hydroxyurea increases synthesis of dihydrofolate reductase. (a) CHO K_1B_{11} (0.5 μM methotrexate treatment) cells were treated with hydroxyurea for 0 h (lane 1), 6 h (lane 2), 12 h (lane 3), or 24 h (lane 4) and pulse-labeled with [^{35}S]methionine. In one sample, cells were treated for 12 h and then allowed to recover in the absence of hydroxyurea for 18 h (lane 5). Autoradiograms of electrophoresed proteins are shown with molecular mass markers indicated at the right. Arrowheads to the left of the gels indicate major proteins, including dihydrofolate reductase, the synthesis of which increased during hydroxyurea treatment. Abbreviations: Kd, Kilodaltons; DHFR, dihydrofolate reductase. (b) Relative synthetic rates of dihydrofolate reductase (Rel. DHFR Synth.), as indicated by densitometry of the autoradiograms.

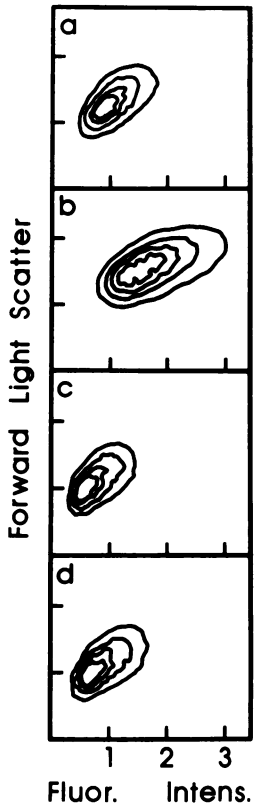

FIG. 4. Cycloheximide blocks the increase in dihydrofolate reductase enzyme. Cells were untreated (a) or treated with hydroxyurea for 12 h (b), with cycloheximide for 12 h (c), or with both (d). Results are displayed as contour plots (20% intervals), as described in the legend to Fig. 1. Fluor. Intens., Fluorescence intensity.

(Fig. 2a). This slight discrepancy may be accounted for by the long incubation period required for the equilibration of fluoresceinated methotrexate across the cell membrane. On removal of hydroxyurea from the culture medium, the rate of dihydrofolate reductase synthesis gradually returned to control levels, but even after 18 h of recovery this rate was still elevated fourfold (Fig. 3a, lane 5). In addition to dihydrofolate reductase, at least five other bands (the identities of which are presently unknown) showed an increase in labeling intensity in drug-treated cells (Fig. 3a, arrowheads). Thus, the effect of the inhibition of DNA synthesis is not limited to dihydrofolate reductase.

The increase in dihydrofolate reductase content during treatment with hydroxurea can be blocked by cycloheximide. Cells were treated for 12 h with hydroxyurea in the presence or absence of cycloheximide; the brief period of treatment was necessary to avoid cytotoxic effects of the cycloheximide. Hydroxyurea treatment induced, as before, an increase in fluoresceinated methotrexate staining intensity (Fig. 4a versus b), which was abolished by cotreatment with cycloheximide (Fig. 4d). Cycloheximide alone had no effect on fluorescence staining of these cells (Fig. 4c).

S phase block increases dihydrofolate reductase mRNA levels. The amount of dihydrofolate reductase mRNA in cells treated with hydoxyurea is shown in Fig. 5. The level of mRNA increased dramatically in cells treated for 24 h, an elevation which is consistent with the increased rate of enzyme synthesis (Fig. 3) and accumulation of enzyme (Fig. 2). When hydroxyurea was removed, the level of dihydrofolate reductase mRNA remained elevated for at least 24 h. In contrast, we observed no enhancement in levels of hydroxymethylglutaryl coenzyme A reductase or c-*myc* mRNA levels but found an approximate twofold increase in β-actin mRNA content in treated cells (data not shown). The amount of dihydrofolate reductase mRNA also increased in cells treated for 24 h with aphidicolin, although the degree of the increase was not as great (four- to fivefold; data not shown) as that seen when cells were treated with hydroxyurea.

Cells treated with hydroxyurea have an increased DNA content following resumption of DNA synthesis. A prediction arising from previous studies (17) was that conditions favoring enhanced gene amplification may give rise to cells with measurable increases in total DNA content. To test this, we treated asynchronously growing cell populations with hydroxyurea or aphidicolin for various periods of time, as shown in Fig. 1, and then prepared cells for flow cytometric measurement of DNA content either immediately or after a period of recovery from the inhibition of DNA synthesis. As expected, prolonged inhibition (24 h) of DNA synthesis, followed by recovery (24 h) of synthesis, resulted in a significant increase in DNA fluorescence staining intensity per cell (Fig. 6d versus f), with some cells showing elevations in DNA staining to three of more times the level during the G_2 phase (Fig. 6f). Brief inhibition (1 h) of DNA synthesis, followed by recovery for 24 h (Fig. 6e) did not elicit a measurable increase in DNA staining, however.

Surprisingly, cells exposed to prolonged treatment with inhibitors of DNA synthesis displayed measurable increases in DNA staining intensity, even without periods of recovery from drug exposure. When aphidicolin was continuously applied for 42 h and DNA staining was examined immediately without recovery of the cells in the absence of drug, the staining intensity of DNA was nevertheless elevated (Fig. 6a versus c). Even with only 24 h of exposure to the inhibitor, DNA staining intensity was also elevated, although the enhancement was not as great (Fig. 6b).

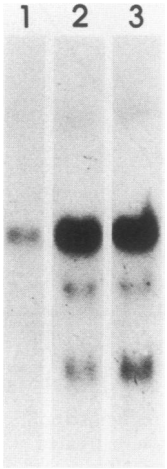

FIG. 5. Hydroxyurea increases dihydrofolate reductase mRNA content. Total RNA was isolated from untreated CHO K_1B_{11} (0.5 μM methotrexate treatment) cells (lane 1), from cells treated with hydroxyurea for 24 h (lane 2), or from cells treated for 24 h and then allowed to recover for a further 24 h (lane 3). Autoradiograms of samples after electrophoresis and hybridization of the RNA to ^{32}P-labeled dihydrofolate reductase probe are shown.

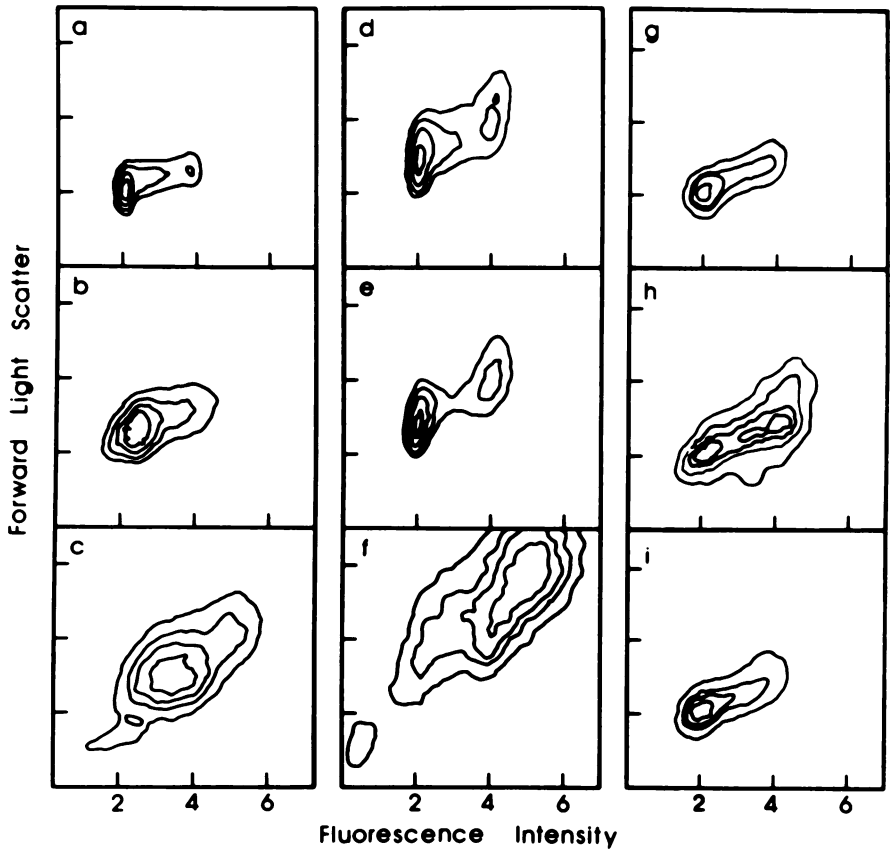

FIG. 6. Hydroxyurea or aphidicolin treatment induces increases in DNA content. DNA fluorescent staining intensity was measured in cells that were treated with aphidicolin for 0 h (a), 24 h (b), or 42 h (c), with replenishment of the drug occurring every 12 h. In a separate experiment, DNA fluorescence staining was measured in untreated cells (d) or in cells treated with hydroxyurea for 1 h (e) or 24 h (f); in this case, the treated cells recovered from the hydroxyurea treatment for 24 h prior to fluorescence analysis. In a third experiment, cells were untreated (g) or treated with aphidicolin (1 μg/ml) plus cycloheximide for 18 h and then allowed to recover from the drug exposure for 24 h (h) or 72 h (i) prior to analysis for DNA content (after staining of fixed cells with chromomycin A_3). The results are presented as contour plots (20% intervals), with 2 and 4 units of fluorescence indicating the mean DNA staining intensity of cells in G_1 and G_2-M phases of the cell cycle, respectively.

That DNA staining intensity should increase during prolonged inhibition of DNA synthesis is seemingly paradoxical. To resolve this issue we monitored the degree of hydroxyurea inhibition of DNA synthesis by measuring incorporation of [^{3}H]thymidine (triplicate samples of 10^5 cells each, pulse-labeled for 0.5 h). Although 1 mM hydroxyurea initially reduced thymidine incorporation to 6% or less of control values, this effectiveness diminished with time, even when the hydroxyurea was replenished at 12-h intervals. By 24 h of continuous exposure to the drug, thymidine incorporation increased sevenfold relative to the first hour (to 41% of the control value), suggesting that the cells developed a degree of resistance to the effects of hydroxyurea. If the drug was then removed, incorporation of thymidine was markedly increased to 130% of the level in untreated control cells. The partial resumption of DNA synthesis (1) was also observed when cells were treated with aphidicolin (data not shown). Such a partial resumption in DNA synthesis, if it occurs in cells that are unable to undergo normal mitosis, may account for the abnormal generation of increased amounts of DNA per cell.

We also wished to determine whether the elevation in DNA staining intensity following administration of inhibitors of DNA synthesis could be blocked by protein synthesis inhibitors. We therefore treated cells with both aphidicolin and cycloheximide for 18 h and then compared DNA staining profiles after 24 h (Fig. 6h) or 72 h (Fig. 6i) of recovery in the absence of either drug with the DNA staining profile of control untreated cells (Fig. 6g). We found that the increase in DNA staining intensity after 24 h of recovery was almost completely abolished by the cycloheximide, and the fluorescence profile returned to control levels by 72 h. The slight shift in staining observed in Fig. 6h may be accounted for by the partial synchronization of the cell population under these conditions. At neither time was the dramatic increase in cell size or DNA content observed to be at the level as that which occurred in the absence of the cycloheximide cotreatment (Fig. 6f).

Elevation in DNA content does not reflect cell fusion or uptake of DNA from damaged cells. A possible explanation for the apparent elevation in cellular DNA content and in frequency of gene amplification (see below) (17) that we observed is that it might represent an artifact of cell fusion or uptake of DNA released from dying cells. To examine this point, we conducted several tests. First, we determined whether the experimental conditions employed here permitted the transfer of dihydrofolate reductase genes among cells. Dihydrofolate reductase-negative CHO cells (24) were cocultured as follows with C3 mouse lymphoma cells that were highly amplified for the dihydrofolate reductase gene.

TABLE 1. Uptake of labeled C3 DNA by dihydrofolate reductase-negative cells

Cell line[a]	Treatment	No. of silver grains per metaphase (mean ± SD)
C3	[^{3}H]thymidine prelabel	53 ± 3
DHFR$^-$	Hydroxyurea, 0 mM; C3 cells, none	2.1 ± 0.1
DHFR$^-$	Hydroxyurea, 1 mM; C3 cells, none	2.9 ± 0.1
DHFR$^-$	Hydroxyurea, 1 mM; C3 cells, 12 h	1.8 ± 0.4
DHFR$^-$	Hydroxyurea, 1 mM; C3 cells, 24 h	3.1 ± 0.1
DHFR$^-$	Hydroxyurea, 1 mM; C3 cells, 72 h	3.5 ± 0.5
DHFR$^-$	Hydroxyurea, 0 mM; C3 cells, 24 h[b]	1.9 ± 0.8

[a] DHFR$^-$, Dihydrofolate reductase negative.
[b] C3 cells were pretreated with hydroxyurea.

Both types of cells were treated for 6 h with hydroxyurea and were cocultured (5×10^5 cells of each cell type per plate) in the absence of drug for 12, 24, or 72 h. In control experiments, CHO cells were either treated or untreated with hydroxyurea and then cultured without C3 cells. Alternatively, C3 cells were treated with hydroxyurea and then added to untreated CHO cells. Finally, CHO cells were transfected directly with calcium phosphate-precipitated DNA (40 μg of C3 DNA per 3×10^6 CHO cells) purified from C3 cells (7). Efficiency of uptake of dihydrofolate reductase genes was determined by rinsing away the nonadherent C3 cells and growing the remaining CHO cells in medium lacking glycine, hypoxanthine, and thymidine, in which only dihydrofolate reductase-positive cells can survive to form colonies. Dihydrofolate reductase-positive colonies arose at a frequency of 1.6×10^{-5} when the CHO cells were transfected directly with C3 DNA. However, no colonies were detected in any of the other experiments, indicating a frequency of transfer of less than 10^{-7}. Thus, the dihydrofolate reductase-negative cells did not appreciably take up functional genes from the C3 cells as a result of exposure to hydroxyurea.

In a second series of experiments we determined whether bulk transfer of DNA could occur among cells under these experimental conditions. C3 cells were prelabeled with [^{3}H]thymidine and then cocultured with dihydrofolate reductase-negative CHO cells for 12 to 72 h. Autoradiography performed on Colcemid-arrested metaphase spreads prepared from the CHO cells revealed that no elevation above background could be detected (Table 1), indicating that less than 5% of a genomic equivalent of C3 DNA was transferred per cell; this was not enough to account for the elevation in apparent DNA content of the CHO cells by up to 300% or greater (Fig. 6).

Finally, we used spectrofluorimetric methods to verify the apparent increases in DNA content per cell revealed by flow cytometry. CHO cells were treated with aphidicolin (1 μg/ml) for 18 h, released from the inhibition for 24 h, stained with Hoechst 33342 dye, and sorted on the basis of DNA content. Samples that were analyzed included a pooled population of cells with apparent DNA staining intensities greater than 110% of the G_2-phase peak, as well as untreated cells sorted into G_1- and G_2-phase populations. Spectrofluorimetry of cell lysates after staining with Hoechst 33342 dye confirmed an elevation of DNA-specific fluorescence staining for the treated and sorted cells to a level of 2.9 ± 0.2 times the G_1-phase level (with G_2-phase cells at 2.0 times the G_1-phase level). Cytological analyses of the sorted cells with apparent elevations in DNA content revealed multiple chromosomal abnormalities (9; S. W. Sherwood, A. B. Hill, and R. T. Schimke, manuscript in preparation). Although a small fraction of the cells (5% or less) displayed tetraploidy or endoreduplication, use of this mechanism was unable to account for the large increase in apparent DNA content. Instead, much of the extra DNA may have arisen from abundant extrachromosomal DNA that was visible in the microscope.

Treatment of cells with Colcemid does not induce an increase in dihydrofolate reductase content. To determine whether blockade at other points in the cell cycle would induce responses similar to those described above, asynchronously growing populations of cells were treated with Colcemid for 18 h and then analyzed for staining with fluoresceinated methotrexate or Hoechst 33342 dye. Although this concentration of Colcemid was sufficient to completely prevent mitosis, resulting in an accumulation of cells in the G_2 phase of the cell cycle (Fig. 7d versus c), no increase in DNA staining was observed above the normal G_2-phase level. Similarly, the staining with fluoresceinated methotrexate also showed no increase above the level to be expected from an increase in cell size (Fig. 7b versus a). This result indicates that the increase in fluorescence staining that we observed is not simply a consequence of a prolonged block in the progression of cells through the cell cycle. Rather, it arises specifically from the blockage of cells in the S phase of the cell cycle.

Frequency of formation of methotrexate-resistant colonies increases with hydroxurea treatment. We wished to establish a relationship between the development of resistance to methotrexate and the increase in DNA content following the exposure of cells to hydroxyurea. Cells were treated with hydroxyurea for 18 h, allowed to recover in the absence of hydroxyurea for 12 h, sorted according to DNA staining intensity, and then exposed to selection with 100 nM methotrexate. When colonies resistant to methotrexate were counted after 3 weeks, we found that the cell population displaying increased DNA content showed a 10-fold enhancement in the frequency of resistance when compared with cells not exposed to hydroxyurea (Table 2).

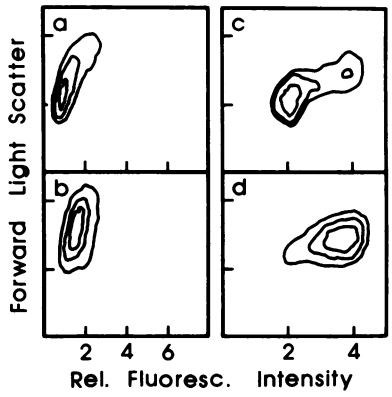

FIG. 7. Colcemid does not induce increases in cellular fluorescence staining. Cells were analyzed for staining with fluoresceinated methotrexate (a and b) or with Hoechst 33342 dye (c and d), indicating dihydrofolate reductase enzyme and DNA content, respectively. Cells were either untreated (a and c) or treated with Colcemid for 18 h (b and d). Results are presented as contour plots (25% intervals), with fluorescence scales calibrated as described in the legends to Fig. 1 and 6. In this experiment, treatment of cells with hydroxyurea induced a sevenfold elevation in dihydrofolate reductase enzyme content (data not shown). Rel. Fluors., Relative fluorescence.

DISCUSSION

Results of previous studies from our laboratory have shown that treatment of cells with hydroxyurea enhances the frequency of dihydrofolate reductase gene amplification in mouse 3T6 cells (1), CHO cells (17), and mouse L5178Y cells (9). Mariani and Schimke (17) provided evidence that when CHO cells are treated with hydroxyurea for 6 h, beginning soon after the onset of the S phase, DNA sequences that were already replicated prior to drug treatment, including the gene for dihydrofolate reductase, were preferentially rereplicated during the recovery from drug exposure. A subpopulation of the treated cells with both enhanced dihydrofolate reductase enzyme content and elevated dihydrofolate reductase gene copy number was also enriched for cells resistant to methotrexate. Similarly, Hill and Schimke (9) treated asynchronously growing L5178Y cells with hydroxyurea for 6 h and found that after recovery some cells had elevated DNA content. It was this subset of cells that displayed frequent chromosomal aberrations in the first M phase following resumption of DNA synthesis, and it was from these cells that a high frequency of methotrexate resistance was generated.

One explanation of these results is that the initial event in the induction of gene amplification by hydroxyurea is the rereplication of the dihydrofolate reductase genes, from which elevated levels of dihydrofolate reductase and methotrexate resistance subsequently derive (17). Another explanation, not mutually exclusive of the first, is that some of the increase in dihydrofolate reductase could instead arise from altered expression patterns of the parental copies of dihydrofolate reductase genes. The results of the study presented here are consistent with the second of the two possible explanations.

We found that prolonged inhibition of DNA synthesis with aphidicolin, hydroxyurea, or methotrexate resulted in the cycloheximide-sensitive accumulation of dihydrofolate reductase (Fig. 1, 2, and 4). This accumulation was a consequence of elevated dihydrofolate reductase synthetic rates (Fig. 3), which in turn resulted from an increase in dihydrofolate reductase mRNA levels (Fig. 5). The increase in dihydrofolate reductase content was not observed, however, when cells were blocked in the G_2 phase of the cell cycle by treatment with Colcemid (Fig. 7). Previous workers (3) have also noted a methotrexate-inducible accumulation of dihydrofolate reductase enzyme, although the degree of enhancement was not as great as that reported here (presumably because of the brief exposure of their cells to methotrexate). Our results considerably extend their observations, and we argue that the basis for the increase in enzyme levels rests at least in part with the marked elevation in dihydrofolate reductase mRNA levels in the treated cells (Fig. 5). However, we cannot exclude the possibility that altered rates of translation or of degradation of the dihydrofolate reductase protein may also contribute to the enhancement that we observed.

Similarly, we do not yet know whether the increase in the dihydrofolate reductase mRNA levels reflects an induction by drug treatment of increased transcription, decreased degradation, or accumulation due to prolonged periods of synthesis of mRNA at normal rates. Some insight into these questions may be gained from a consideration of the cell cycle regulation of the dihydrofolate reductase gene. The rate of synthesis of dihydrofolate reductase protein is low during the G_1 phase and increases markedly at the onset of the S phase (18). Results of recent studies (5) have indicated that there is an approximate 2- to 3-h window starting at the G_1-S-phase boundary when the dihydrofolate reductase gene (which has upstream binding sites for transcription factor Sp1 [4]) is transcriptionally activated. It is possible, then, that disruption of the cell cycle by agents that block cells at the G_1-S-phase boundary could result in preferential accumulation of proteins expressed from such cell cycle-regulated genes, which would thereby be activated for an artifically prolonged period.

TABLE 2. Frequency of resistance to methotrexate

Cell population[a]	Plating efficiency (%) in the following methotrexate concn:		Relative plating efficiency (%)[b]
	0 nM	100 nM	
Control	57 ± 11	0.06 ± 0.01	0.10 ± 0.02
G_2	26 ± 4	0.12 ± 0.06	0.5 ± 0.2
$>G_2$	23 ± 5	0.24 ± 0.06	1.1 ± 0.4

[a] CHO cells were treated with 1 mM hydroxyurea for 18 h and then allowed to recover in the absence of hydroxyurea for 12 h. Cells were stained with Hoechst 33342 dye and sorted according to DNA content into G_2- or $>G_2$-phase subpopulations. Control cells were not treated with hydroxyurea and were sorted for G_1-phase content of DNA. Sorted cells were plated in triplicate at 0 (10^2 cells each) or 100 nM (10^4 cells each) methotrexate, and colonies were counted after 5 or 20 days, respectively.

[b] Relative plating efficiency is given as the fraction of cells surviving at 100 nM methotrexate with respect to survival at 0 nM methotrexate.

An additional point is that the concentrations of hydroxyurea and aphidicolin used in these experiments do not inhibit DNA synthesis completely. Indeed, on prolonged inhibition there was a progressive recovery of [^{3}H]thymidine incorporation into DNA (1). The mechanism for this apparent resumption of DNA synthesis is not known, although two possibilities may be considered. First, alterations in nucleotide pools that occur normally during the S phase (10) and during drug treatments (19) may decrease the effectiveness of inhibition by hydroxyurea and aphidicolin. DNA polymerase alpha inhibition by aphidicolin is competitive with deoxynucleotide triphosphates (11), and a similar phenomenon may occur by an unspecified mechanism for hydroxyurea inhibition of ribonucleotide reductase. A second possibility is that ribonucleotide reductase and DNA polymerase alpha enzyme levels might also increase in a fashion similar to that of dihydrofolate reductase during periods of inhibition of DNA synthesis, thus overcoming the inhibitory effects of these drugs.

In either case, we propose that treatment of cells with inhibitors of DNA synthesis results in the generation of cells which are arrested at a point in the cell cycle when the dihydrofolate reductase gene is transcriptionally activated. Indeed, P. J. Farnham and R. T. Schimke (submitted for publication) have shown that nuclei prepared from cells blocked with aphidicolin initiate transcription in vitro of the dihydrofolate reductase gene 10 to 50 times more actively than do nuclei from untreated cells. Cells blocked at this point in the cell cycle may have increased transcriptional capacity for a battery of genes, of which the dihydrofolate reductase gene is only one (in Fig. 3 enhanced synthesis of several unknown abundant proteins is shown) and the products of which may participate in the initiation and progression of DNA synthesis in the cell cycle. When such enzymes are present in excess, overreplication of DNA may occur when synthesis of DNA is allowed to resume.

Consistent with this, we also found that on release of cells from drug inhibition the resumption of DNA synthesis is associated with the cycloheximide-sensitive elevation of

levels of DNA fluorescence staining (Fig. 6). We believe that this represents a true increase in DNA content per cell and is not an artifact of the experimental conditions we employed. Thus, the increase is detected as enhanced cellular staining by either of two different DNA-specific fluorescent dyes. In addition, the increase cannot be accounted for either by fusion of treated cells or by uptake of DNA released from dying cells, nor is it consistent with enhanced binding of fluorescent dyes by damaged or unfolded DNA (15); it is instead associated with a variety of chromosomal abnormalities that include the appearance of abundant extrachromosomal material visible in the light microscope (9). In other studies, antibody directed against bromodeoxyuridine incorporated into DNA has been used to demonstrate overreplication following exposure of cells to hypoxia (19a), and microspectrophotometric analyses of cells exposed to methotrexate also have revealed net increases in DNA content (6). We therefore consider the increase in DNA fluorescence staining most likely to represent true increases in DNA content in the treated cells. In addition, the increase in DNA content is maximal during the recovery from prolonged inhibition of DNA synthesis and is correlated with maximal enhancement of dihydrofolate reductase enzyme levels. That is, DNA content does not increase following brief periods of inhibition of DNA synthesis (Fig. 6e), nor following cell cycle block in the G_2 phase (Fig. 7d); these are conditions that do not result in elevated levels of dihydrofolate reductase.

The conditions of drug exposure employed here included some that were more extreme than those used earlier to elicit dihydrofolate reductase gene amplification and methotrexate resistance (1, 17). Thus, although these conditions are effective in inducing marked increases in dihydrofolate reductase and DNA content, they also cause considerable cell death (Table 2). It was therefore necessary to verify that even these harsh treatments could still enhance the generation of drug resistance in cells. We found that when CHO cells were treated with hydroxyurea and then sorted for elevated DNA content, such cells were enriched for subsequent resistance to methotrexate (Table 2). This result confirms results of earlier work with mouse L5178Y cells (9).

The data presented here raise a fundamental question concerning an aspect of the regulation of DNA replication: What determines the number and timing of initiations of DNA replication once a cell is committed to the S phase and, in particular, when DNA replication patterns are perturbed by cytotoxic drugs or environmental agents? We have presented elsewhere (21) a model for the generation of chromosomal aberrations and rearrangements based on initial overreplication of DNA and subsequent recombination events, including, but not limited to, gene amplification. We suggest that disruptions in DNA synthesis, with consequent accumulations of cell cycle-regulated enzymes involved in DNA synthesis, may actually facilitate the later DNA overreplication and chromosomal abnormalities that accompany these events.

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