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Enhancement of Methotrexate Resistance and Dihydrofolate Reductase Gene Amplification by Treatment of Mouse 3T6 Cells with Hydroxyurea

PETER C. BROWN,* THEA D. TLSTY, AND ROBERT T. SCHIMKE
Department of Biological Sciences, Stanford University, Stanford, California 94305

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We investigated various parameters associated with the initial selection of mouse 3T6 cells for resistance to single concentrations of methotrexate and characterized resistant colonies for the presence of additional (amplified) copies of the dihydrofolate reductase gene. Our results indicate that the frequency of occurrence of dihydrofolate reductase gene amplification varies with the selecting concentration of methotrexate and is highly variable between clonally derived sublines of mouse 3T6 cells. Second, we increased the frequency of occurrence of cells with amplified dihydrofolate reductase genes by transiently inhibiting DNA synthesis with hydroxyurea before the selection of cells in single concentrations of methotrexate. This effect was dependent on the concentration of hydroxyurea, the time of exposure to the drug, and the time interval between the removal of hydroxyurea and the selection of cells in methotrexate.

It has now been well documented that selective gene amplification is one mechanism by which cultured mammalian cells become resistant to cytotoxic drugs (2, 4, 6, 48). Overproduction of specific intracellular proteins which are targeted by such agents results from the acquisition of additional functional copies of the gene coding for that protein. Methotrexate (MTX), a 4-amino analog of folic acid, is one such compound to which cellular resistance is mediated by gene amplification. MTX specifically inhibits the enzyme dihydrofolate reductase (DHFR) (EC 1.5.1.3), and this inhibition leads to cell death through the depletion of reduced folates, which are required for the synthesis of thymidylate, purines, and glycine (11). MTX-resistant cells have been isolated and characterized by a number of investigators who have shown that resistant cells may have copies of the DHFR gene that are many times in excess of the DHFR gene copy number of the original population (2, 7, 10, 14, 26–28, 30, 46). In addition, it has recently been shown that amplification of the DHFR gene had occurred in neoplastic tissue from an individual with disseminated small-cell lung cancer after relapse during chemotherapy with MTX (9).

With one exception (47), the efforts by various investigators studying gene amplification in mammalian cells have focused on documentation and characterization of the event. In the first part of this study, we analyzed the clonal nature of MTX resistance and DHFR gene amplification and, in addition, assessed the relative contribution of gene amplification in the initial selection of cells for resistance to MTX. In the latter portion of the study, we increased the apparent frequency of DHFR gene amplification by pretreatment of cells from a clonally derived cell line with hydroxyurea (HU). That modulation of the frequency of gene amplification in this manner was possible suggests that the process of gene amplification is in part an induced event and not simply a selection for preexisting variants. Second, these findings indicate that the perturbation of DNA synthesis by antimetabolic agents may promote the process of gene amplification in general and enhance the frequency of occurrence of resistance to the initial agent and to subsequent agents as well.

MATERIALS AND METHODS

Cells and culture conditions. Uncloned mouse 3T6 cells (from Vera Morhenn, Stanford University) were grown in Dubecco modified Eagle medium, supplemented with 10% dialyzed, heat-inactivated newborn calf serum (K. C. Biologicals), 2 mM sodium pyruvate, and 2 mM additional glutamine, penicillin, and streptomycin (complete medium). Clones derived from single cells were obtained by plating dilute suspensions of cells in complete medium into 96-well microtest dishes (Falcon Plastics) and stored in liquid nitrogen within 2 months of their isolation. Stock cultures of these cells were used within 10 passages from this point. Plating efficiencies were determined by inoculating 100-mm petri dishes (Falcon Plastics) with 100 to 500 cells in complete medium (10 ml). After 6 to 8 days, the

colonies were fixed with a mixture of methanol and acetic acid (3:1), dried, stained with 2% Giemsa stain, and counted.

Determination of resistance to MTX was essentially as described previously (13). Lyophilized samples of MTX stocks (neutralized with NaOH) were rehydrated in complete medium, used for 4 to 6 days, and discarded (MTX was supplied by the National Cancer Institute). Trypsinized suspensions of appropriate numbers of cells in complete medium (5 ml) were added to an equal volume of medium supplemented with MTX at twice the desired concentration in 100-mm petri dishes. The density of cells inoculated at each concentration of MTX was critical in obtaining reproducible survival curves. Sparing effects at high-cell densities under selective conditions have been described previously and are attributed to cross-feeding as well as normal contact inhibition (44). Generally, selections of 3T6 cells, clone 5, and clone 10 were performed at or below the following cell densities in 100-mm petri dishes for each concentration of MTX: 40 nM MTX, 2×10^3 cells; 80 nM, 5×10^3 cells; 120 nM, 2×10^5 cells; 160 nM, 5×10^5 cells; 200 nM, 1×10^6 cells. Under these conditions, no more than 50 colonies per plate were routinely encountered. For clones 6 and 7, these maximum cell densities were reduced 10-fold. MTX-supplemented medium was changed every 2 to 3 days for a period of 10 days to remove cell debris which could reverse MTX toxicity by providing thymidine and preformed purines. To reduce the possibility of satellite colony formation with the addition of new medium, plates were agitated by hand before the complete removal of growth medium. After the final change of medium, cells were incubated an additional 4 to 8 days and either fixed, as above, or colonies selected at random were isolated by using cloning cylinders (18) and transferred into individual chambers of a 24-well culture plate (Costar). To optimize recovery of resistant subclones, we reduced the MTX concentration at this point to one-half of the concentration in which the colonies were originally selected. After 4 to 10 days, these subclones were transferred to 25-cm^2 flasks (Costar) and restored to the original concentrations of MTX in complete medium. After one or more transfers, a pellet of 2×10^5 cells was prepared and stored at −80°C until it was processed for determination of DHFR gene amplification. The remaining cells were passaged once, frozen, and stored in liquid nitrogen. Relative plating efficiencies were calculated by dividing the number of stained colonies (>50 cells per colony) per dish by the number of cells in the original inoculum and normalizing for the plating efficiency of these cells in the complete medium without MTX.

For the pretreatment of cells with HU (Sigma Chemical Co.), freshly prepared concentrated stocks of HU were filter sterilized and added to exponentially growing cultures growing in complete medium. After various intervals (12 to 18 h), this medium was removed, and the cells were washed with prewarmed, complete medium and grown for various periods in a 1:1 mixture of conditioned and complete medium. After 6 to 96 h, the cells were trypsinized, counted, and plated into both complete medium (to determine plating efficiency) and MTX-supplemented medium (minimum of three dishes for each experimental point).

Assessment of DHFR gene amplification. The dot hybridization assay (22) was extensively modified (R. Jorgenson, personal communication) to determine the presence of amplified DHFR genes in MTX-resistant subclones. Trypsinized cells were suspended in Hanks balanced salt solution (HBSS) with 1% serum, and 2×10^5 cells were pelleted in 1.5-ml conical polypropylene tubes (2,000 rpm for 5 min) and stored at −80°C until used. Cell pellets were lysed with the addition of 10 μl of 0.2% sodium dodecyl sulfate (SDS; BDH) in $TE_{10}N$ (0.01 M Tris-hydrochloride [pH 8.0], 0.01 M EDTA, 0.01 M NaCl) at 37°C for 15 min with mixing. After dilution with 180 μl of $TE_{10}N$, RNase A (Sigma Chemical Co.; heated to 80°C for 10 min to inactivate DNase) was added to a concentration of 20 μg/ml, and the mixture was incubated for 1 h at 37°C. Proteinase K (Beckman Instruments, Inc.) was then added to a concentration of 50 μg/ml, and the incubation continued for 1 h. NaCl was added to a concentration of 0.1 M, and the mixture was sequentially extracted with equal volumes (220 μl) of phenol and chloroform (1:1, saturated with 0.05 M Tris-chloride [pH 7.5]–0.001 M EDTA) and then with chloroform alone. Care was taken to remove only the organic (bottom) phase after centrifugation (Eppendorf Microcentrifuge for 1 min) of this first extraction mixture. After the second extraction, the aqueous phase was removed (200 μl) and transferred to 6-ml polypropylene tubes (Falcon Plastics). With care, this extraction procedure is essentially quantitative for the recovery of DNA (data not shown).

DNA was denatured by the addition of 1/10 volume of 3.0 M NaOH for 10 min at room temperature. The mixture was neutralized by the addition of an equal volume of 2.0 M ammonium acetate and immediately applied to a nitrocellulose filter in duplicate in the apparatus described below. A template was machined from 0.375-inch (ca. 0.95-cm) Lucite (Steed Engineering, Palo Alto, Calif.) with parallel rows of polished conical slots tapering to 1 by 6 mm. To minimize adsorbtion of denatured sample DNA to the plastic template, the slots were prefilled for 15 to 30 min with a solution of single-stranded DNA (salmon sperm, 100 μg/ml) in 1 M ammonium acetate and thoroughly washed with this solution minus DNA. The template was firmly clamped to (in order): nitrocellulose (Schleicher & Schuell BA-85, prewetted) backed by a second sheet of nitrocellulose (to avoid contact of the first sheet of nitrocellulose with backing paper); three sheets of Whatman 3MM paper; approximately 1 cm of dry paper towels; and finally, a rigid backing plate of 0.25-inch (ca. 0.64-cm) Lucite. Before the application of sample DNA, the wells of the assembled apparatus were prewashed with 1.0 M ammonium acetate (100 μl per well). One half of the sample DNA (denatured and neutralized) was applied to each of the pairs of slots in the assembled template and allowed to filter through the nitrocellulose membrane. Each well was washed with 200 μl of 1.0 M ammonium acetate, and, after complete absorbtion, the apparatus was disassembled, and the uppermost nitrocellulose filter containing sample DNA was soaked in 5× SSC for 5 min (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 6.5), air dried for 15 to 30 min, and baked for 2 h in vacuo at 80°C. The filter was then cut into halves (parallel to the rows of slots), and each half was hybridized with cloned ^{32}P-labeled DHFR cDNA (7)

or mouse alpha-fetoprotein (α-FP) cDNA (24). At the end of the hybridization period (48 h with intermittent mixing [7]), the filters were washed once with prehybridization buffer (50 ml) at 68°C and then for 1 h each with two changes of 250 ml of 5× SSC, 2× SSC, and 1× SSC, all at 68°C. After a final wash in 0.1× SSC at 50°C, the filters were dried, rejoined, exposed to preflashed X-ray film, and developed (29).

Determination of DNA synthesis. At various periods after the removal of HU, cells were labeled with [^{3}H]thymidine (Amersham Corp.; 6.7 Ci/mmol, 0.5 μCi/ml) for 30 min, washed with ice-cold HBSS supplemented with 10^{-5} M thymidine, trypsinized (GIBCO trypsin-EDTA) at room temperature, and suspended in cold HBSS. A sample of these cells was centrifuged and lysed with 0.5% SDS in TEN_{100} (TEN with 100 mM NaCl). Incorporated radioactivity was determined in a sample of the cell lysate (8), whereas DNA content was determined by fluorimetric analysis (7), using Hoechst 33258 (Calbiochem) with some modifications. Since SDS in excess of 0.005% interfered with the determination of nanogram quantities of DNA, 20 μl of cell lysate was diluted with mixing into 2 ml of TEN with Hoechst 33258 (5×10^{-8} g/ml). Fluorescence was measured and compared with a standard curve prepared from SDS lysates of known numbers of 3T6 cells. From this curve, the cellular equivalents of DNA in each sample were determined and used to normalize acid-precipitable radioactivity. By this technique, the DNA content of a minimum of 10^3 cells could be determined.

The remaining sample of washed cells was processed for autoradiography. Cells were pelleted through 0.3 M sucrose, washed with HBSS, fixed with methanol-acetic acid (3:1), and deposited on a clean microscope slide. The slides were dried, washed in 2× SSC, redried, and dipped in Kodak NTB-2 photographic emulsion. After 1 to 3 days, the slides were developed in Dektol (Kodak), stained with 2% Giemsa stain, and examined. Cells with grain counts three times greater than background (three to seven grains on control cells labeled for approximately 5 s at room temperature) were scored as labeled nuclei. A minimum of 100 cells per point was examined.

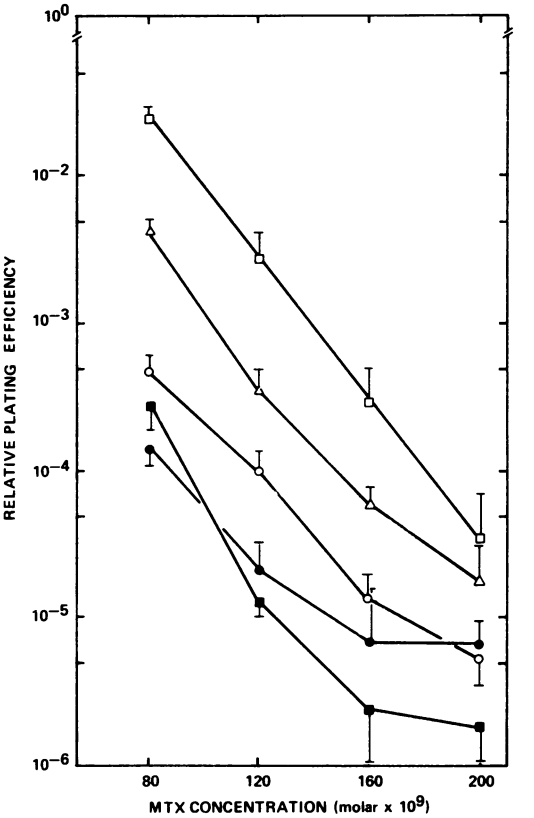

FIG. 1. Resistance to MTX as measured by colony formation. Clonally derived sublines of 3T6 cells were trypsinized and plated into medium containing various concentrations of MTX. After approximately 3 weeks, colonies (>50 cells) were fixed, stained, and counted. Symbols: ●, parental 3T6 cells; ○, clone 5; □, clone 6; △, clone 7; and ■, clone 10. Bars, Standard deviations.

RESULTS

MTX resistance and corresponding DHFR gene amplification in clonally derived mouse 3T6 cells. Clonally derived lines of 3T6 cells were tested for their ability to form colonies in single-step selections in various concentrations of MTX. Although 10 sublines were tested in this manner, the data for 4 representative sublines as well as for the uncloned parental 3T6 cell line are presented in Fig. 1. Plating efficiencies at lower MTX concentrations have been omitted, but the concentration of MTX that inhibited the formation of colonies by 50% for the uncloned parental population and for clone 5 was about 20 nM MTX. Figure 1 shows that, with the exception of the 200 nM selection, the number of surviving colonies varied over 100-fold among sublines at each concentration of MTX. Similar results have been obtained with clonally derived sublines of Chinese hamster ovary cells (R. Johnston, unpublished observation). Of the clones depicted in Fig. 1, clone 5 was chosen for further characterization because of its high plating efficiency (80 to 90%), rapid growth (ca. 16-h cell cycle), uniform colony morphology, and MTX sensitivity, which was similar to that of the uncloned parental 3T6 cell line.

A rapid assay for the occurrence of DHFR gene amplification in MTX-resistant subclones was developed which was an extensive modification of the dot hybridization technique (22). To determine the sensitivity of the slot hybridization assay, we made various reconstruction series from 3T6 clone 5 cells (MTX sensitive and the 3T6 R50 cells [7]). These latter cells are resistant to 50 μM MTX and have a DHFR gene copy number that is 40- to 50-fold higher than MTX-sensitive parental cells. Cells (2×10^5)

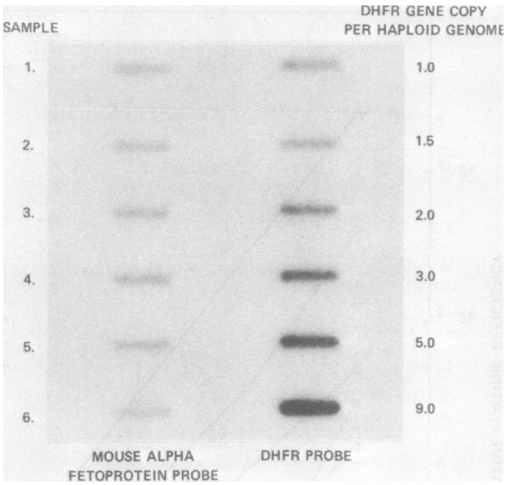

FIG. 2. Sensitivity of slot hybridization assay. Reconstructed standards were prepared from mixtures of amplified and wild-type cells. MTX-resistant 3T6 R50 cells, which have approximately 50 copies of the DHFR gene in excess of wild type, and uncloned 3T6 cells were combined in various proportions, and 2×10^5 cells of each mixture were individually processed for the slot hybridization assay. Sample 1, parental 3T6 cells, assumed to have one copy of the DHFR gene per haploid genome. The ratios of resistant to sensitive cells in the reconstructed samples are: 1:100, sample 2; 1:50, sample 3; 1:25, sample 4; 1:12, sample 5; 1:6, sample 6. Purified DNA from each sample was applied in duplicate to nitrocellulose filters. The samples on the left half of the filter were hybridized with mouse α-FP cDNA, and the samples on the right half were hybridized with DHFR cDNA, both labeled with ^{32}P by nick translation.

from each reconstruction were individually processed as described above and hybridized with the α-FP and DHFR probes. The exposures resulting from these hybridizations are shown in Fig. 2. From three separate reconstruction experiments (only one of which is shown), it was possible to visually distinguish the samples with a minimum of 2 to 3 DHFR genomic equivalents in excess of sensitive cells from the samples prepared from the parental MTX-sensitive cells alone. This discrimination was based on the differences in relative intensities of the exposures from the α-FP and DHFR probes. Visual discrimination of DHFR gene amplification from multiple exposures, each taken for different lengths of time, proved as reliable as densitometric analysis of slot intensity.

Representative experiments with independent isolates which were selected in various concentrations of MTX are shown in Fig. 3. Resistant subclones were derived from randomly selected colonies from both untreated 3T6 clone 5 cells (Fig. 3A) and from these cells exposed to either 0.2 mM HU (Fig. 3B, left-most pairs of slots) or 0.3 mM HU (Fig. 3B, right-most pairs of slots). Only subclones that showed a differential hybridization intensity equal to or greater than that of the reconstruction sample number 4 (Fig. 2) were scored as positive for gene amplification. We have not rigorously quantitated DHFR gene copy number in each of the samples; it is apparent from Fig. 3, however, that the levels of DHFR amplification in cells after pretreatment with HU and selection in 120 mM MTX were less than the maximal 5- to 10-fold amplification seen in control cells selected in parallel. It is important to note that samples with a level of DHFR gene amplification below two- to three-fold would remain undetected.

Table 1 summarizes the occurrence of DHFR gene amplification in randomly chosen MTX-resistant subclones of clonally derived 3T6 cells described in Fig. 1. Clone 5 was the most extensively characterized, and slightly more than half of the colonies selected in 120 and 160 nM MTX showed DHFR gene amplification, whereas no amplified colonies were detectable at 40 nM and only one was seen at 80 nM. In striking contrast, clones 6 and 7, which displayed the greatest inherent MTX resistance (Fig. 1), showed only one instance of DHFR gene amplification between them at 120 nM. At 160 nM, however, amplified colonies in clones 6 and 7 were found in approximately 50% of the subclones analyzed. Thus, we conclude that DHFR gene amplification did occur in each of these three clones, although the frequency of occurrence at each concentration of MTX varied significantly between clones. These results suggest cautious interpretation of data which compare frequencies of resistance and gene amplifications between unrelated cell lines as well as cells derived from a common line. We assume that the nonamplified resistant colonies evident in Table 1 represent either transport alterations, enzyme variants with decreased affinities for MTX (or combinations of both), or have a level of DHFR gene amplification below that which we can detect.

Enhancement of MTX resistance and DHFR gene amplification by pretreatment with HU. Although HU and MTX differ in their specific mechanisms of action, the drugs are similar in that both effect rapid and reversible inhibition of DNA synthesis. To test the hypothesis that transient interruption of DNA synthesis facilitates the emergence of drug resistance and gene amplification, we exposed asynchronous 3T6 clone 5 cells to various concentrations of HU for about one cell cycle, washed the cells free of drug, and grew them in drug-free medium for 6 h before they were challenged with 120 nM MTX. At this concentration of MTX, the maximum

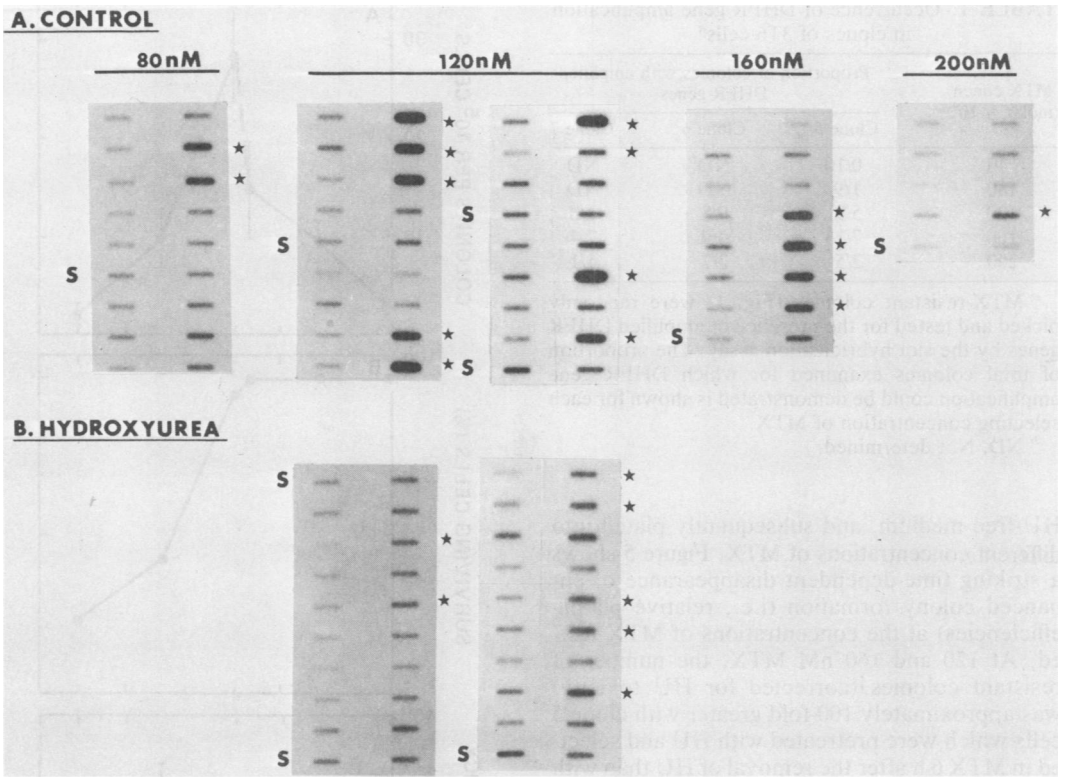

FIG. 3. DHFR gene amplification in MTX-resistant colonies of 3T6 clone 5 cells. Randomly picked colonies resistant to 80 to 200 nM MTX (Fig. 1) were grown a minimum of 10 to 15 generations in medium supplemented with the concentration of MTX in which the colonies were originally selected. Each subclone was tested for amplification of the DHFR gene by slot hybridization assay when a minimum of 2×10^5 cells became available (2 to 6 weeks). Hybridization to the DNA from each sample is represented by adjacent pairs of slots; the left half of each pair was hybridized with the α-FP probe and the right half with the DHFR probe. Parental 3T6 clone 5 cells are marked (S) to the left of each sample. Those samples showing amplification of the DHFR gene (2 to 3 times sensitive cells, Fig. 2) are marked (★) to the right of each sample. (A) 3T6 clone 5 cells without HU pretreatment. (B) 3T6 clone 5 cells pretreated with either 0.1 mM HU (left-most pair of slots) or 0.3 mM HU (right-most pair of slots).

occurrence of amplified, MTX-resistant subclones was seen (Table 1). Experiments with exposure of cells to HU for less than 14 to 16 h resulted in diminished effects on subsequent MTX resistance (data not shown).

Figure 4A shows that the number of surviving colonies was increased in a dose-dependent manner until 0.1 mM HU (29 colonies per 10^5 cells) and declined thereafter as the toxicity of HU increased. This toxicity was reflected in reduced plating efficiencies of cells exposed to concentrations of HU above 0.1 mM (Fig. 4B). Relative plating efficiencies (corrected for this toxicity) in 120 nM MTX continued to increase to 0.3 mM and declined slightly thereafter (Fig. 4C). Thus, after pretreatment of cells with 0.3 mM HU, the resistance of 3T6 cells to MTX, as measured by relative plating efficiency, was increased approximately 50-fold (1.6×10^{-3} versus 3.9×10^{-5} colonies per viable cell plated). When clones 6 and 7 (Fig. 1) were pretreated identically with 0.3 mM HU, qualitatively similar effects on resistance were observed (data not shown). Because of the substantial correction factor for toxicity at 0.3 mM HU (less than 5% survival) in the calculation of relative plating efficiency, we were concerned that pretreatment with HU was merely selecting a subpopulation of cells that was inherently resistant to MTX. That this was not the case was seen in control experiments (not shown) which clearly indicated that previously selected MTX-resistant cells were as sensitive to the toxic effects of HU as were parental MTX-sensitive cells.

We next investigated the effects of varying the time interval between the removal of HU and the selection in MTX. Cells were exposed to HU (0.3 mM) for 17 h, grown for various periods in

TABLE 1. Occurrence of DHFR gene amplification in clones of 3T6 cells[a]

MTX concn (molar × 10^9)	Proportion of colonies with amplified DHFR genes		
	Clone 5	Clone 6	Clone 7
40	0/10	ND[b]	ND
80	1/9	ND	ND
120	5/9	0/8	1/8
160	7/13	4/9	2/4
200	2/5	2/2	1/1

[a] MTX-resistant colonies (Fig. 1) were randomly picked and tested for the presence of amplified DHFR genes by the slot hybridization assay. The proportion of total colonies examined for which DHFR gene amplification could be demonstrated is shown for each selecting concentration of MTX.

[b] ND, Not determined.

HU-free medium, and subsequently plated into different concentrations of MTX. Figure 5 shows a striking time-dependent disappearance of enhanced colony formation (i.e., relative plating efficiencies) at the concentrations of MTX tested. At 120 and 160 nM MTX, the number of resistant colonies (corrected for HU toxicity) was approximately 100-fold greater with clone 5 cells which were pretreated with HU and selected in MTX 6 h after the removal of HU than with untreated control cultures. After 48 h of growth in the absence of HU, this enhancement was reduced to about 20-fold; after 96 h, the enhancement of resistance was reduced still further to about 5-fold. Clone 5 cells which were treated with HU (Fig. 5) and grown for 10 days in drug-free medium were no more resistant to MTX than control cells nor less sensitive to the toxic effects of a second exposure to HU (data not shown). Thus, the enhancement of MTX resistance by pretreatment of cells with HU is transient, and no permanent disposition toward MTX resistance or HU insensitivity resulted from this pretreatment.

We next examined the occurrence of DHFR gene amplification in subclones derived from MTX-resistant colonies which were selected after pretreatment of clone 5 cells with HU. These results are shown in Table 2 for three separate experiments, two of which are also shown in Fig. 3. Although other concentrations of MTX were studied, for simplicity, only the colonies which emerged in 120 nM MTX are represented. The relative amplification frequency at each concentration of MTX is defined as the numerical product of the relative plating efficiency and the proportion of subclones which showed amplification of the DHFR gene by slot hybridizations. Whereas pretreatment with 0.1 and 0.2 mM HU increased the relative amplification frequency about 10-fold, pretreatment with 0.3

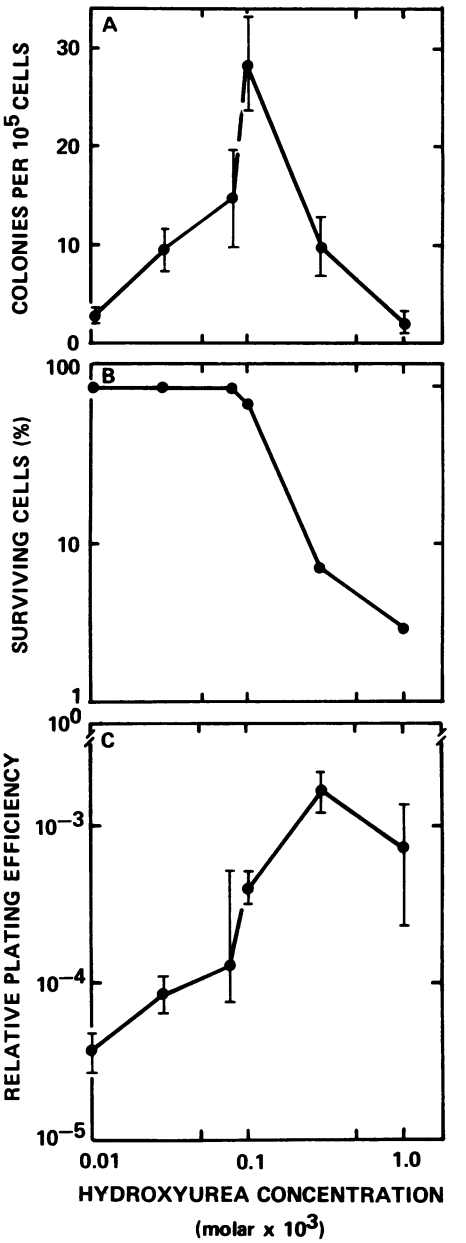

FIG. 4. Effects of pretreatment with HU on resistance to MTX. Exponentially growing cultures of 3T6 clone 5 cells were treated with various concentrations of HU for 17 h, washed free of the drug for 6 h, trypsinized, and plated into either 120 nM MTX (A) or complete medium without MTX (B). Colonies were fixed, stained, and counted after approximately 1 week (control cells, B) or 2 weeks (MTX-resistant cells, A). (A) Surviving colonies in 120 nM MTX from 10^5 cells plated per petri dish. (B) Surviving cells (i.e., colonies) expressed as a percentage of cells initially plated (100 to 500 cells). (C) Relative plating efficiency is the data in (A) normalized for the reduced viability of cells after treatment with HU in (B). Bars, Standard deviations.

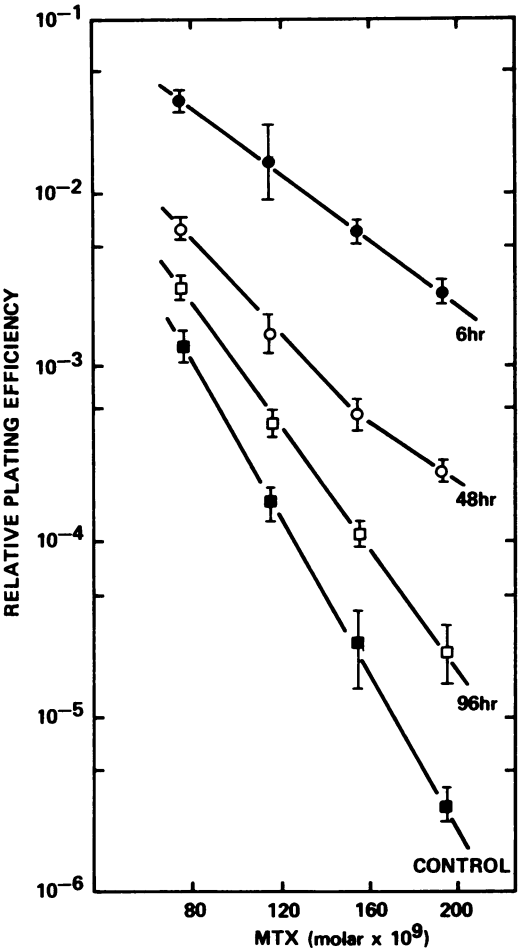

FIG. 5. Effects of recovery time after pretreatment with HU on MTX resistance. Exponentially growing 3T6 clone 5 cells were treated with 0.3 mM HU for 17 h. Cells were washed free of the drug, grown for 6, 48, or 96 h before trypsinization, and plated into MTX-supplemented medium. Relative plating efficiencies were determined after about 3 weeks. Bars, Standard deviations.

mM HU resulted in a 70-fold enhancement of the relative amplification frequency at 120 nM MTX. Table 2 (and Fig. 3) also shows that the incidence of apparently nonamplified, MTX-resistant cells increased concordantly inasmuch as the relative proportions of amplified and nonamplified samples remained about the same in controls and in cells pretreated with HU. This point will be discussed in more detail below.

Effects of HU pretreatment on DNA synthesis. HU is a potent and reversible inhibitor of ribonucleotide reductase (EC 1.17.4.1). The inhibition of ribonucleotide reductase results in depletion of deoxynucleoside diphosphate pools and, in the absence of salvaged deoxypurines and deoxypyrimidines, the cessation of DNA synthesis (42, 45). It was therefore of interest to examine DNA synthesis during the pretreatment of clone 5 cells with HU and before selection in MTX under the conditions which resulted in the enhancement of DHFR gene amplification. Figure 6A shows that after 1 h of treatment with 0.1 or 0.3 mM HU, the incorporation of labeled thymidine into acid-precipitable material was reduced 87 or 98%, respectively. Control experiments (not shown) indicated that the inhibition of thymidine incorporation was maximal at these concentrations of HU after 1 h. Figure 6B shows the percentages of cells that were engaged in DNA synthesis at time points corresponding to those in Fig. 6A. Although total thymidine incorporation was dramatically inhibited by HU, the proportion of cells that were still synthesizing low levels of DNA was reduced by less than a factor of 2 for 0.3 mM HU and even less for 0.1 mM HU.

Although HU was maintained in the culture continuously for 15 h, spontaneous reversal of inhibition had occurred by the end of the pretreatment period, and levels of thymidine incorporation were approximately equivalent to those of control cells at the end of the pretreatment period. This spontaneous reversal of inhibition was not unexpected inasmuch as the concentrations of HU used were not excessive and HU in cell culture conditions is somewhat unstable (36). At higher concentrations of HU, inhibition of DNA synthesis was constant for the duration of the treatment, and these higher concentrations have been used to partially synchronize cell populations at the beginning of the S phase (1). At least partial synchronization by HU of the cell population shown in Fig. 6A is suggested by the rise and fall of thymidine incorporation relative to controls after removal of the drug. This overall pattern of DNA synthesis is characteristic of the progression of cells in early S phase as these cells traverse S phase. That the total incorporation of radioactivity in cells pretreated with 0.3 mM HU was less than in cells exposed to 0.1 mM HU may also reflect the increased toxicity encountered with the use of higher concentrations of HU (Fig. 4B) (33, 34). In this respect, it might be argued that the ability of a cell to amplify the DHFR gene depends on the position of the cell in the cell cycle upon encountering MTX. That this is not a significant factor in the emergence of MTX-resistant cells was shown with synchronized Chinese hamster ovary cells which were selected by mitotic detachment and subsequently challenged with MTX at various points in the cell cycle (T. Tlsty, unpublished observation).

TABLE 2. Effects of pretreatment with HU on frequency of DHFR gene amplification[a]

Pretreatment	Amplified/ total[b]	Relative plating efficiency	DHFR amplification frequency[c]	Fold increase[d]
Expt III-I				
0.1 mM HU	6/9	4.2×10^{-4}	2.8×10^{-4}	10
None	ND[e]	3.7×10^{-5}	2.4×10^{-5}[f]	
Expt II-7				
0.2 mM HU	4/14	5.1×10^{-4}	1.5×10^{-4}	10
None	5/9	2.9×10^{-5}	1.6×10^{-5}	
Expt II-9				
0.3 mM HU	4/8	3.3×10^{-3}	1.7×10^{-3}	70
None	3/5	4.0×10^{-5}	2.4×10^{-5}	

[a] 3T6 clone 5 cells were treated with HU and selected in 120 nM MTX (Fig. 4). After approximately 3 weeks, colonies were picked at random and subsequently tested for the presence of amplified DHFR genes. The remaining colonies were fixed and counted for the determination of relative plating efficiencies.

[b] Proportion of total colonies examined for which DHFR gene amplification could be demonstrated by the slot hybridization assay.

[c] Numerical product of relative plating efficiency and the proportion of colonies with amplified DHFR genes (see footnote *b* above).

[d] Increase in the frequency of occurrence of DHFR gene amplification relative to untreated control cells.

[e] ND, Not determined.

[f] Proportion of colonies with amplified DHFR genes is taken as 8/14 and represents the cumulative data from experiments II-7 and II-9 controls (i.e., none).

DISCUSSION

In this study, we attempted to define various parameters associated with amplification of the DHFR gene in mouse 3T6 cells. In addition, we used HU as a model compound to test the hypothesis that transient inhibition of DNA synthesis facilitates the process of gene amplification. Throughout, we assessed the apparent frequency of DHFR gene amplifications through quantitative colony formation assay in single, though varied, concentrations of MTX followed by analysis of randomly chosen, resistant colonies for the presence of amplified DHFR genes. Implicit in this approach was the assumption that the amplification of the DHFR sequences in cells that were progenitors of MTX-resistant colonies was accurately reflected in the analysis of resistant subclones resulting from each colony.

MTX resistance and DHFR gene amplification. For any given phenotypic trait, variations among cells in culture appear to be of universal occurrence (20, 40). Resistance to MTX coincident with amplification of the DHFR structural gene is, from these studies, no exception to this rule. Although analysis of MTX-resistant subclones has been described previously by others (12, 13), we showed that the sensitivity of parental 3T6 cells to MTX varied over 100-fold among clonally derived sublines (Fig. 1). Furthermore, the apparent frequency of the occurrence of DHFR gene amplification in the three clones studied seemed to vary with the stringency of the selection as measured by relative plating efficiency rather than with the absolute concentration of MTX used to isolate resistant colonies (Table 1). We had hoped that clones 6 and 7 would show a frequency of DHFR gene amplification that was commensurate with their increased resistance to MTX. Since this was not the case, we speculate that intrinsic resistance to MTX among cells (15) follows some as yet undefined distribution and that gene amplification as a mechanism for additional resistance is invoked by each cell as some threshold of selection is surpassed. This threshold might be perceived as the extent to which the metabolic balance of the cell is perturbed by MTX.

Of additional interest to us was the observation that at a relatively high stringency of selection (200 nM MTX), the frequency of occurrence of DHFR gene amplification was not increased when compared to cells selected at lower concentrations of MTX. Also, the intensities of the hybridization signals from the DHFR probe from the samples selected in 200 nM MTX were uniformly decreased (Fig. 3; unpublished data). Primarily for these reasons and for reasons of economy, clone 5 cells were best tested for resistance to MTX and DHFR gene amplification at 120 nM MTX (relative plating efficiency, ca. 5×10^{-5}). Because the apparent frequency of DHFR gene amplification was not increased by higher stringencies of selection, we suggest that gene amplification in single-step selections in MTX is less efficient in providing cellular resistance above certain concentrations of MTX (i.e., stringencies of selection). Under these

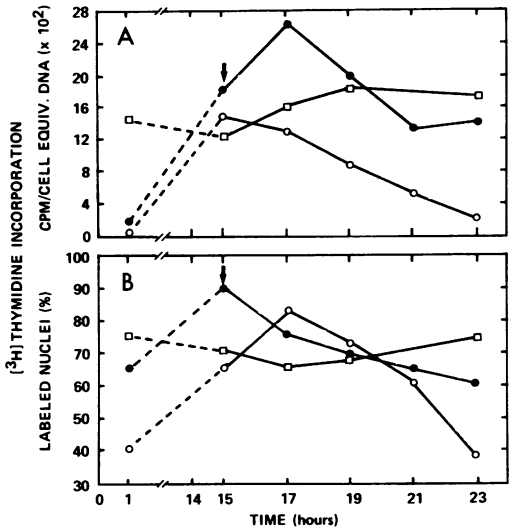

FIG. 6. DNA synthesis during and after pretreatment with HU. 3T6 clone 5 cells were treated with either 0.1 or 0.3 mM HU for 15 h, washed free of the drug with conditioned medium, and grown an additional 8 h. At various intervals, cells were pulse labeled with [^{3}H]thymidine. A portion of each sample was precipitated in CCl_3COOH, and the remainder was processed for autoradiography. The zero time point represents the time of addition of HU, whereas the arrow indicates the removal of drug (i.e., 15 h). Labeled thymidine was added 30 min before the points indicated in the figure, and thus, the 15-h point represents data taken just before the removal of HU. (A) [^{3}H]thymidine incorporation normalized for the DNA content in each sample. (B) Percentage of labeled nuclei after microscopic examination of cells exposed for 3 days to the photographic emulsion. Symbols: □, untreated control cells; ●, 0.1 mM HU; and ○, 0.3 mM HU.

conditions, cells with modes of resistance other than gene amplification predominate. Thus, it is not unreasonable that for each cell there are both upper and lower limits of the stringency of selection in which the process of gene amplification is favored. It follows, therefore, that higher levels of gene amplification might only be achieved by conventional stepwise selections in increasing concentrations of drugs (17). We estimate that the maximum level of DHFR gene amplification possible in single-step selections is less than 10-fold. This upper limit is in approximate agreement with the results of others (47).

Enhancement of gene amplification by HU. Studies with both procaryotes and eucaryotes have shown that transient inhibition and resumption of DNA synthesis by various protocols and drugs results in the premature reinitiation of DNA synthesis whereby certain segments of DNA are replicated more than once within the same S phase (5, 31, 32, 43, 49, 50). We speculated that such processes could result in cells having additional copies of the DHFR gene, among other segments of DNA, and that these cells would survive with increased frequencies relative to control cells when grown in toxic concentrations of MTX. The approach that we used was to transiently inhibit DNA synthesis with HU and assess MTX resistance and DHFR gene amplification in cells thus treated. Our results indicated that pretreatment of cells with HU did indeed increase the apparent frequency of DHFR gene amplification (Fig. 4; Table 2). Moreover, the effects of HU were transient and resulted in no permanent lesions which predisposed these cells to increased frequencies of resistance to MTX and DHFR gene amplification (Fig. 5).

Two additional observations are worthy of note. First, the levels of DHFR gene amplification in cells pretreated with HU were depressed at least twofold relative to untreated controls (Fig. 3). Second, the proportion of nonamplified, MTX-resistant cells remained approximately the same in both control and HU-treated cells (Table 2), implying that both of these classes of resistant cells (amplified and nonamplified for DHFR) were effectively increased after pretreatment with HU and selection in MTX. Without further analysis of each of the MTX-resistant variants resulting from HU pretreatment, we are unable to provide a totally satisfactory explanation for these observations. One possibility is that HU pretreatment may facilitate the development of MTX resistance by alternate mechanisms, including mutations resulting in decreased affinity of DHFR for MTX (16), alterations in MTX transport (41), or alterations in MTX metabolism (15). Alterations of these types in addition to gene amplifications are not mutually exclusive; so-called mixed MTX resistance occurs which includes both DHFR gene amplification and alterations in DHFR (16) as well as DHFR gene amplification and altered transport of MTX (unpublished observations). Such mixed resistances may account for the lower degrees of DHFR gene amplification in HU-pretreated cells noted above. Although reports have indicated that HU is not mutagenic in Chinese hamster ovary cells (21, 37), the combined effect of HU pretreatment followed by MTX in mouse cells has not been investigated. Finally, it cannot be excluded that gene amplification may account for MTX resistance resulting from transport alterations inasmuch as certain drug resistances in cultured cells have been shown to result from the overproduction of specific proteins which are probably encoded by genes residing on double minute chromosomes or within homogeneously staining regions (3, 23;

M. B. Meyers and T. L. Biedler; J. Cell Biol. 95:435a, 1982).

We chose to inhibit DNA replication with HU, although clearly MTX would also have been appropriate, but it would have been difficult to distinguish any amplification-enhancing activity from mere cell selection resulting from pretreatment with MTX. In one report, however, pretreatment of cells with the lipid-soluble antifolate DDMP ([2,4-diamino-5-(3',4'-dichlorophenyl)]-6-methylpyrimidine) increased the survival of cells when they were subsequently challenged with either DDMP or MTX (19). Concentrations of HU in the pretreatment were employed such that most, but not all, DNA synthesis was inhibited after initial exposure (Fig. 6). By allowing low levels of DNA synthesis to continue, toxicity was decreased (Fig. 4; references 33 and 34), and cells were allowed to spontaneously recover from inhibition. Such conditions undoubtedly prevail during the initial exposure of cells to MTX (15, 35). An extrapolation of our results with HU to MTX would suggest that MTX could function in an equivalent manner by enhancing the frequency of gene amplifications in general and subsequently function as a selecting agent for those cells in which amplification of the DHFR gene had occurred. An important issue yet unresolved is the extent to which spontaneous gene amplifications might also contribute to the emergence of MTX-resistant cells (39). Conventional fluctuation analyses (25) are unsuitable for this determination inasmuch as stability of the spontaneous amplification is required yet newly amplified DHFR genes are invariantly unstable (38).

A major implication of this study relates to the use of antimetabolites in cancer chemotherapy. Thus, incomplete inhibition of DNA synthesis may, in fact, facilitate gene amplification and the subsequent emergence of resistance as a result of these amplifications. As other inhibitors of DNA synthesis with different modes of action are tested, it should become apparent whether inhibition of DNA synthesis per se is causative or whether severe metabolite imbalance such as that resulting from treatment with HU or MTX is responsible for the enhancement of gene amplification frequencies.

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