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# Loss of unstably amplified dihydrofolate reductase genes from mouse cells is greatly accelerated by hydroxyurea

(gene amplification and loss/"curing" mammalian cells of acentric chromosomal fragments)

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**ABSTRACT** **Previous work has shown that mammalian cells that carry unstably amplified genes for dihydrofolate reductase (DHFR) gradually lose the amplified *DHFR* genes when grown in the absence of the DHFR inhibitor methotrexate (MTX). Unstably amplified genes occur on small acentric chromosomes called double minutes (DMs) or even smaller chromatin fragments, in contrast to stably amplified genes, which reside in centromere-containing chromosomes. We have found that the rate of loss of the unstably amplified *DHFR* genes can be greatly increased by growing the cells in the presence of a nonlethal concentration of hydroxyurea. For example, in one MTX-resistant subline studied, ≈90% of the original *DHFR* gene dosage is lost in 25–30 cell doublings in the absence of MTX. The same degree of loss is achieved, however, in <4 doublings if cells are grown in the presence of 50 μM hydroxyurea. This new effect of hydroxyurea does not appear to be due to changes in plating efficiency or selective cytotoxicity. In particular, no increase in cell death occurs at 50 μM hydroxyurea, and cells continue to multiply, albeit $^1/_2$ to $^2/_3$ as fast as in the absence of hydroxyurea. The ability to selectively accelerate the loss of amplified genes from mammalian cells as shown in the present work may have important implications both for the problem of drug resistance in cancer chemotherapy and for curing mammalian cells of extrachromosomally maintained DNA genomes of pathogenic viruses.**

Mammalian cells have been shown to acquire resistance to a dihydrofolate reductase (DHFR) inhibitor, methotrexate (MTX), either through an amplification of the *DHFR* gene, which leads to overproduction of the target DHFR enzyme (1–7), through mutations rendering the DHFR enzyme less sensitive to MTX, or through mutations decreasing MTX uptake by cells (1). Of these causes of MTX resistance, *DHFR* gene amplification appears to be the most frequent one under a variety of experimental conditions (1–7).

Amplified chromosomal regions other than the *DHFR* gene domain also occur in a wide variety of eukaryotic cells, usually as a result of *in vivo* or *in vitro* selection for specific properties, such as tumorigenicity (5, 6, 8) or resistance to a cytotoxic drug (1, 7, 9), but also in the course of normal development (10).

Amplified chromosomal regions occur either within the major cellular chromosomes where they are frequently recognizable as karyotypic abnormalities called "homogeneously staining regions" or as small acentric chromosomes called "double minutes" (DMs) (1, 8–11). Unlike stably amplified genes in homogeneously staining regions, which are retained for many generations in the absence of a relevant selection pressure, the unstably amplified genes in acentric DMs or smaller chromatin fragments are gradually lost from cells in the absence of selection pressure, at least in part because of the absence of functioning centromeres in DMs (1–6, 11).

It was recently reported that phorbol 12-myristate 13-acetate (PMA), a noncytotoxic, nonmutagenic phorbol ester tumor promoter, greatly increases the incidence of mouse cells with amplified *DHFR* genes under conditions of cytotoxic selection for resistance to MTX (4). Subsequent work by Tlsty *et al.* (12) has confirmed this result and in addition showed that transient cytotoxic treatment of mammalian cells (with hydroxyurea or ultraviolet light) before selection for MTX resistance also leads to an increased incidence of cells with amplified *DHFR* genes (12, 13). Noncytotoxic tumor promoters other than PMA as well as mitogenic hormones, such as insulin or epidermal growth factor, have also been shown recently to greatly increase the incidence of MTX resistance due to *DHFR* gene amplification (14). These findings have provided indirect support for an earlier hypothesis that one pathway of carcinogenesis and tumor progression may operate through a generalized increase in the frequency of disproportionate DNA replication, resulting in accelerated gene amplification (15). Targets for such "facilitated" gene amplification may include genes responsible for the initial stages of carcinogenesis or other genetic loci—for example, genes influencing metastatic potential or immunosuppressive properties of evolving tumor cells (5, 6, 8, 11, 15–17). Regardless of which of the suggested mechanisms of facilitated gene amplification is the correct one, it is clear that the probability of either gene amplification *per se* or survival of "nascent" cells bearing amplified genes can vary dramatically at least *in vitro*, depending on the conditions of selection (4, 12–14).

In several situations (for example, in cancer chemotherapy), one would like to minimize the probability of either the emergence or the persistence of cells with undesirable amplified genes. One way to approach this problem is to look for ways to increase the probability of loss of already amplified genes and to do so under otherwise noncytotoxic conditions.

We report here that when mouse cells bearing unstably amplified *DHFR* genes are grown in the presence of nonlethal concentrations of hydroxyurea, the rate of loss of the *DHFR* genes from these cells is greatly increased.

## MATERIALS AND METHODS

**Cell Lines.** Two cell lines were used: R.3, a subline of mouse 3T6 cells selected for resistance to 0.3 μM MTX (4), and R500, a subline of 3T6 cells selected for resistance to 250 μM MTX (a gift from R. Kaufman and R. Schimke). The cells were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with penicillin/streptomycin, exhaustively dialyzed 10% calf serum (4), and either 0.3 μM MTX (for R.3 cells) or 250 μM MTX (for R500 cells).

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; DMs, double-minute chromosomes; PMA, phorbol 12-myristate 13-acetate; *cis*-DDP, *cis*-diamminedichloroplatinum(II).

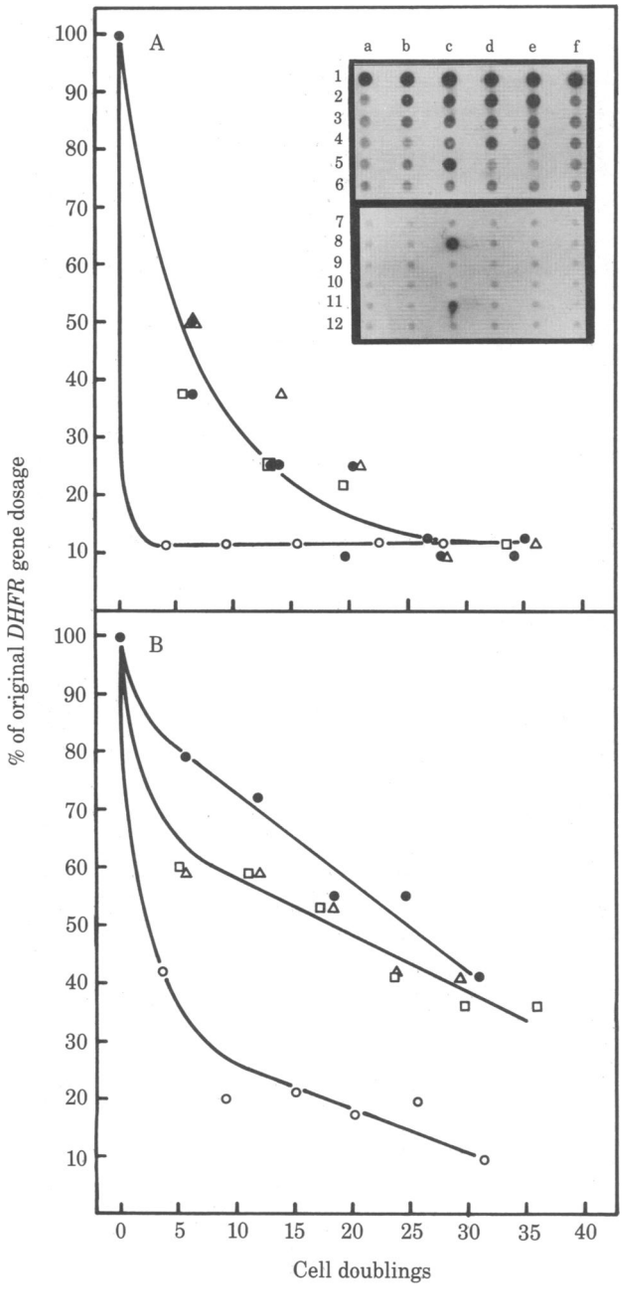


FIG. 1. Loss of unstably amplified *DHFR* genes from mouse cells as a function of cumulative cell doublings in the absence of MTX (●, control) and in the presence of either *cis*-DDP (□, 0.17 μM), PMA (△, 8 μM), or hydroxyurea (○, 50 μM). (*A*) R.3 cells (a subline of 3T6 cells selected for resistance to 0.3 μM MTX; ref. 4) were maintained in the presence of 0.3 μM MTX. To start the experiment, cells were suspended by gentle trypsinization, counted in a hemacytometer, and reseeded onto four 10-cm plates (Falcon) at $1 \times 10^5$ cells per plate in the same medium but with undialyzed calf serum and without MTX. On formation of confluent monolayers, cells in one of four plates in each series were reseeded onto four new plates under the same conditions. The remaining three plates were used for isolation of the total nuclear DNA. Relative dosages of the *DHFR* gene were determined by DNA·DNA dot-blot hybridization. (*Inset*) Example of dot hybridization data. Rows 1–6, hybridization with the DHFR cDNA probe; rows 7–12, hybridization with the $\beta^{maj}$-globin cDNA probe. Dots c5, c8, and c11 are artefacts of DNA loading (data not shown). Dots a1–f1 and a7–f7: *DHFR* and globin gene dosages, respectively, for R.3 cells at the time of removal from medium containing 0.3 μM MTX. Dots a2–a6 and a7–a12: *DHFR* and globin gene dosages, respectively, for the MTX-sensitive unselected 3T6 cells ("single-copy" control for *DHFR*). For the remaining dots, rows 2–6 and 8–12 represent *DHFR* and globin gene dosages, respectively, for successive passages in the absence of MTX (columns b and e) and in the absence of MTX and presence of either 0.17 μM *cis*-DDP (column c), 8 μM PMA (column d), or 50 μM hydroxyurea (column f). The curves in *A* and *B* were derived directly from the dot hybridization data (inset and analogous data not shown). (*B*) R500 mouse cells resistant to 250 μM MTX (a gift from R. Kaufman and R. Schimke) were grown and analyzed as described in *A* for R.3 cells.

**DNA Isolation and DNA·DNA Dot Hybridization.** Cell monolayers were rinsed with 0.14 M NaCl/5 mM Na Hepes (pH 7.5), then scraped with a rubber policeman into 0.5% Nonidet P-40/5 mM $Na_2EDTA$/5 mM Na Hepes, pH 7.5. The lysate was homogenized by four strokes of a Dounce homogenizer with a loose-fitting pestle and then centrifuged at 2,000 × *g* for 5 min. Purification of DNA from the pellet of crude nuclei was carried out as described (4). In some experiments DNA was purified in parallel from both whole cells and isolated nuclei. Cell-population doubling times were deduced from the number of cells initially seeded and the number of cells at confluence at each passage. Relative dosage of the *DHFR* gene (defined as a ratio of the *DHFR* copy number in MTX-resistant cells to that in MTX-sensitive unselected 3T6 cells) was determined by a DNA·DNA dot-blot hybridization as described (4, 18), except that a dot-blot manifold (Schleicher & Schuell) was used, and quantitation of dot hybridization patterns was carried out by cutting out and assaying individual dots of [$^{32}$P]DNA (this procedure yields more accurate results than visual comparisons of dot intensities; data not shown). The $^{32}$P-labeled hybridization probes used were a cloned mouse DHFR cDNA (pDHFR11; ref. 19; a gift from R. Schimke) and a cloned mouse $\beta^{maj}$-globin cDNA (pCRI-M9; ref. 20; donated by V. Volloch).

## RESULTS

**Search for Noncytotoxic Treatments That Accelerate Loss of Amplified Genes from Mammalian Cells: Striking Effect of Hydroxyurea.** Our strategy was to grow unstably MTX-resistant mouse cells in the absence of MTX and to monitor the decrease of their *DHFR* gene dosage as a function of cumulative cell doublings (population doublings) in the presence of a putative "gene loss-inducing" agent (Fig. 1). An important criterion used in the search for such an agent was that its effective concentration had to be nonlethal for cells on prolonged exposure. Therefore the concentration of each of the compounds to be tested in the *DHFR* gene loss assay (Fig. 1) was first optimized by growing the cells for a number of generations in the presence of different concentrations of the compound to be tested and then choosing the concentration that neither increased cell death nor slowed cell multiplication more than 2-fold (Fig. 2). Hydroxyurea, which happened to be the third agent tested, produced a striking effect (Figs. 1 and 3–5). Other compounds or treatments, some of which are known to facilitate the loss of plasmids from bacterial cells (21–25), could be tested to compare their effect on the rate of the *DHFR* gene loss with that of hydroxyurea.

Mouse R.3 cells (a subline of 3T6 cells selected for resistance to 0.3 μM MTX that contains ≈15 copies of the unstably amplified *DHFR* gene per haploid genome; see refs. 4 and 14) grown in the absence of MTX gradually lose their extra *DHFR* genes, with ≈90% of the *DHFR* gene dosage lost after 25–30 cell doublings (the fourth passage in MTX-free medium) (Fig. 1*A*). The noncytotoxic phorbol ester tumor promoter PMA (4, 26) at 8 μM and the cytotoxic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) (27, 28) at 0.17 μM had little or no effect on the rate of decrease in the *DHFR* gene copy number (Fig. 1*A*). In striking contrast, when an analogous parallel experiment was carried out in the presence of 50 μM hydroxyurea, ≈90% of the

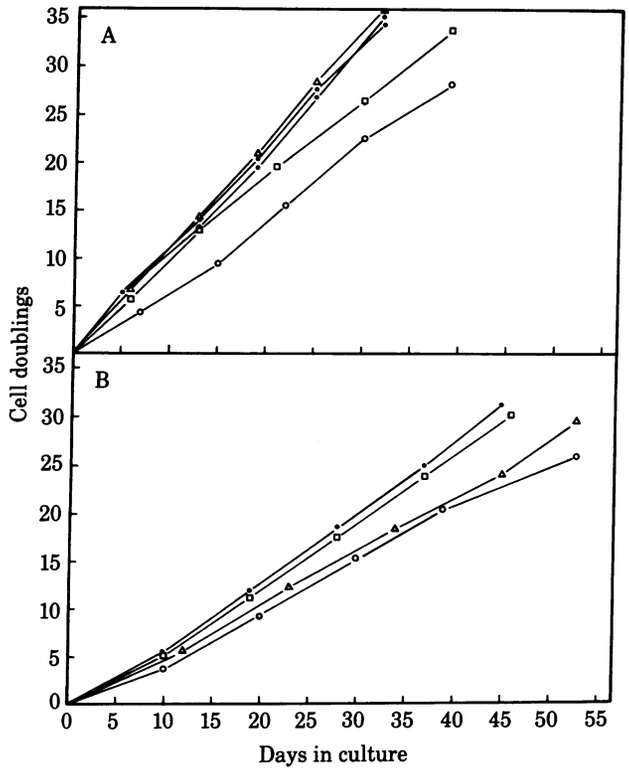


FIG. 2. Growth of R.3 cells (*A*) and R500 cells (*B*) through five passages in the absence of MTX (●) and in the absence of MTX and presence of either *cis*-DDP (□, 0.17 μM), PMA (△, 8 μM), or hydroxyurea (○, 50 μM).

*DHFR* gene dosage was lost in less than four cell doublings (first passage in MTX-free medium) (Fig. 1*A*). Similar results were obtained with another line of MTX-resistant cells, R500 (a gift from R. Kaufman and R. Schimke). These cells are resistant to 250 μM MTX and contain ≈170 copies of the *DHFR* gene per haploid genome; most but not necessarily all of these extra *DHFR* genes are amplified unstably (ref. 29; Fig. 1*B*; data not shown).

**Hydroxyurea at Concentrations That Accelerate Loss of *DHFR* Genes Allows Continued Cell Multiplication.** Cells of both sublines continued to multiply in the presence of 50 μM hydroxyurea ($^1/_2$ to $^2/_3$ as fast as in the absence of hydroxyurea), and there was no detectable increase in cell death in the presence of hydroxyurea as compared to the control medium (Fig. 2; data not shown). An apparently lower growth rate of cells in the presence of 50 μM hydroxyurea is due at least in part to a larger surface area that each cell occupies on a plate in the presence of 50 μM hydroxyurea (data not shown). The result is that the cell monolayers become confluent approximately two doublings earlier than their counterparts grown in the absence of hydroxyurea (Fig. 4*A*). The larger surface area of cells grown in 50 μM hydroxyurea is due to a hydroxyurea-mediated metabolic disturbance that leads to an increased cell volume (30).

The concentration of hydroxyurea used throughout this work (50 μM) was chosen to maximize the rate of *DHFR* gene loss without decreasing cell viability and only moderately decreasing growth rate of cells (Fig. 2). Concentrations of hydroxyurea significantly below 50 μM were much less effective in accelerating the loss of the *DHFR* genes (Fig. 3). On the other hand, concentrations of hydroxyurea significantly higher than 50 μM do result in significant cytotoxicity after prolonged exposure (data not shown).

**Hydroxyurea Effect at Low Cell-Population Doublings.** To follow the loss of the *DHFR* genes between one and four cell doublings, R500 cells maintained in the presence of 250 μM MTX were plated at $1 \times 10^5$ cells per plate in the absence of MTX. Medium in half of the plates was made 50 μM in hydroxyurea. Cultures in both hydroxyurea-lacking and hydroxyurea-containing media reached confluence at the same time, the hydroxyurea-treated cells having undergone approximately five population doublings and the untreated cells approximately seven doublings (Fig. 4*A*). The striking effect of hydroxyurea on the rate of loss of the *DHFR* genes from cells in the course of this single-passage experiment is shown in Fig. 4*B*. Considering the ways in which unstably amplified genes are thought to be lost from cells (1–6), it is interesting that the *DHFR* gene dosage in the hydroxyurea-treated cells continued to decrease between days 10 and 12, a period when the cells are at the plateau of their growth curve (Fig. 4*B*; cf. Fig. 4*A*).

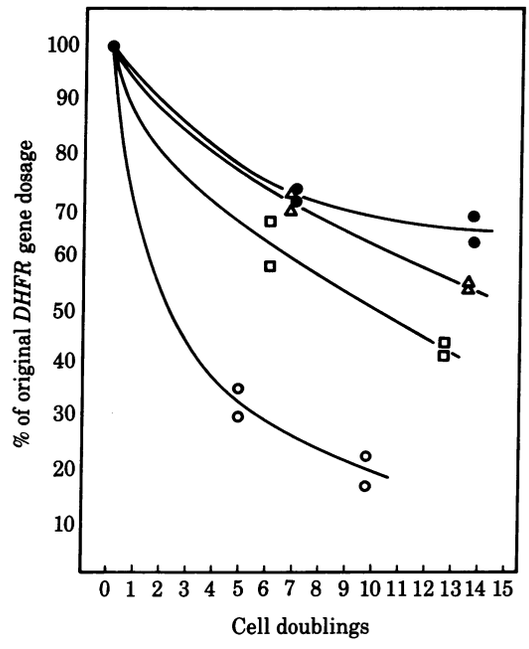


FIG. 3. Rate of loss of unstably amplified *DHFR* genes as a function of hydroxyurea concentration. For each experimental series, R500 cells maintained in the presence of 250 μM MTX were removed from their plates by trypsinization and reseeded at $1 \times 10^5$ cells per plate onto four 10-cm plates containing the same medium but without MTX. When the cells in each series reached confluence, one plate from the set of four was trypsinized, the cells were counted and passed to four new plates at $1 \times 10^5$ cells per plate, and the remaining three plates were harvested and processed for isolation of nuclear DNA. ●, No hydroxyurea; △, 10 μM hydroxyurea; □, 25 μM hydroxyurea; ○, 50 μM hydroxyurea. Data from two separate dot-blot hybridizations with the same DNA samples are shown.

**Rate of Hydroxyurea-Accelerated *DHFR* Gene Loss from R500 Cells Is the Same Whether Measured with Nuclear or Whole Cell DNA Samples.** To see if the hydroxyurea effect is due to an increased probability of postmitotic partitioning of *DHFR* gene-containing DMs into the cytoplasm through formation of micronuclei [a known pathway of DM loss (1)], we have monitored the *DHFR* gene dosage decrease at low cell-population doublings using both nuclear and whole cell DNA samples in a series of parallel measurements. The results obtained (Fig. 5) clearly did not significantly depend on the way DNA was isolated (either from crude nuclei or from whole cells). Although the data of Fig. 5 are consistent with "nonpartitioning" mechanisms of the hydroxyurea effect, they are insufficient to preclude the "partitioning" mechanisms, because several interpretations (for example, cosedimentation of nuclei and micronuclei in our preparations or rapid *in vitro* degradation of

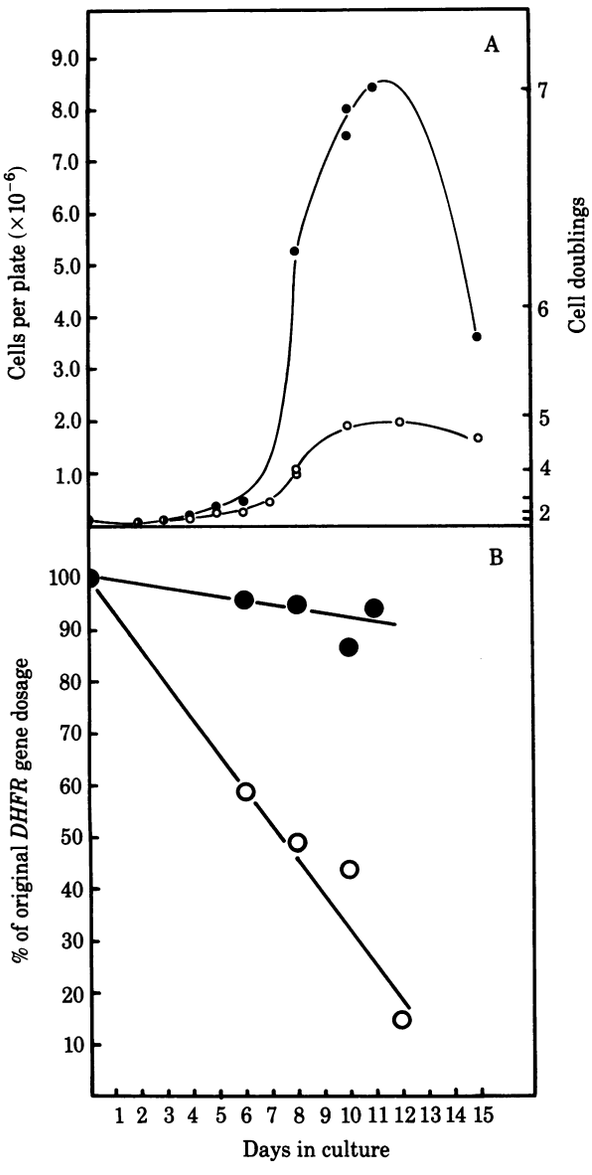


FIG. 4. Hydroxyurea-accelerated loss of *DHFR* genes during one passage of R500 cells prior to confluence. R500 cells were seeded at 1 $\times$ $10^5$ cells per 10-cm plate in the absence of MTX (●, control) or in the absence of MTX and in the presence of 50 $\mu$M hydroxyurea (○). Cell growth (*A*) and relative *DHFR* gene dosages (*B*) were monitored as described. Lower density of R500 cells at confluence in the presence of hydroxyurea is due to their increased size.

micronuclear DNA in the cytoplasm) are still compatible with the data.

**Stably Amplified *DHFR* Genes Are Not Lost in the Presence of Hydroxyurea.** When mouse L1578Y-R cells (a gift from J. Bertino), which carry ≈350 stably amplified *DHFR* genes per haploid genome (4, 31), were grown in the absence of MTX and in the presence of either 25 or 50 $\mu$M hydroxyurea for six population doublings, there was no detectable decrease in the *DHFR* gene dosage in these cells (data not shown). Thus hydroxyurea does not have a dramatic effect on stably amplified *DHFR* genes, at least during relatively short courses of treatment.

## DISCUSSION

Two major questions are raised by our results: (*i*) what is the mechanism of the hydroxyurea effect (Figs. 1 and 3–5) and (*ii*) what possible uses could this new effect be put to?

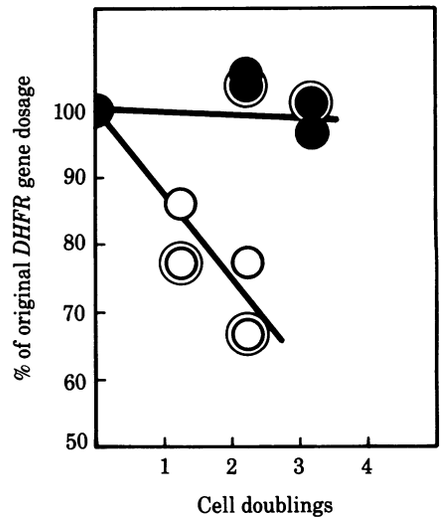


FIG. 5. Effect of hydroxyurea on *DHFR* gene dosage in R500 cells at low population doublings: identical results with DNA from whole cells and isolated nuclei. R500 cells maintained in the presence of 250 $\mu$M MTX were trypsinized and reseeded at 1 $\times$ $10^6$ cells per plate (eight 10-cm plates) and at 4 $\times$ $10^6$ cells per plate (eight 10-cm plates) in medium without MTX and with or without 50 $\mu$M hydroxyurea. When the cells of each series reached confluence, cells in two plates were trypsinized to determine cell numbers, and three plates were processed for isolation of nuclear DNA; the remaining three plates were used to isolate DNA from whole cells (detached from plates by trypsinization and washed free from the medium). No hydroxyurea, nuclear DNA (●); 50 $\mu$M hydroxyurea, nuclear DNA (○); double circles represent corresponding data for DNA from whole cells.

The absence of significant cytotoxicity of 50 $\mu$M hydroxyurea toward cells from lines bearing widely different numbers of amplified *DHFR* genes (see above) argues strongly against any mechanism for the hydroxyurea effect in which hydroxyurea preferentially suppresses growth of those cells that have higher than average numbers of the amplified *DHFR* genes.

One possible mechanism of the hydroxyurea effect may operate by increasing the probability of postmitotic partitioning of *DHFR* gene-containing DMs into the cytoplasm through formation of micronuclei [a known pathway of DM loss (1–6)]. Although our results are compatible with such a partitioning mechanism, they add a constraint that the micronuclear DHFR DNA in the cytoplasmic compartment should be degraded rapidly *in vivo* to account for indistinguishable results of monitoring the *DHFR* gene loss with either nuclear or whole-cell DNA samples (Fig. 5).

Other possibilities are that hydroxyurea preferentially inhibits replication [or decatenation (32)] of unstably amplified *DHFR* genes or induces their preferential degradation. Although it is likely that the gene loss-enhancing effect of hydroxyurea is mediated by its specific inhibition of the enzyme ribonucleoside diphosphate reductase (33, 34), a component of the replication fork (35), no direct evidence is available on this point. Use of other specific inhibitors of ribonucleoside diphosphate reductase (36) is one way to address this question. Furthermore, it is likely that unstably amplified genes other than *DHFR* are also subject to accelerated loss in the presence of hydroxyurea.

Transient treatments of mammalian cells with cytotoxic concentrations of hydroxyurea are known to increase the frequency of the *DHFR* gene amplification, apparently by increasing the probability of disproportionate DNA replication (12, 13); a possible relationship of this phenomenon to the new

effect of nonlethal hydroxyurea concentrations on the frequency of loss of the *DHFR* genes remains to be investigated.

Although hydroxyurea is a widely used antineoplastic drug, it is not understood why it is relatively effective for certain cancers, such as chronic granulocytic leukemia or polycythemia vera (37), but not for many other malignant tumors. Concentrations of hydroxyurea in patients' tissues considered to be therapeutically useful (cytotoxic) are at least an order of magnitude higher than the hydroxyurea concentration used in the present work (50 $\mu$M) (37). Hydroxyurea is also known to be a potent teratogen (38, 39). Because transient amplification of specific genes has been shown to constitute a part of normal development in at least some of the higher eukaryotic species (10), and because many cancers are known to be associated with gene amplification (1, 4–8, 39), the new effect of hydroxyurea described above may be relevant to both teratogenic and antineoplastic activities of hydroxyurea.

What are the potential applications of the ability to selectively accelerate the loss of unstably amplified genes from mammalian cells?

Previous studies have shown that the incidence of cells bearing amplified genes under conditions of cytotoxic selection can vary more than 100-fold and depends on either the presence of mitogenic substances (hormones or tumor promoters) during selection (4, 14) or a pre-exposure of cells to certain cytotoxic agents before selection (12, 13). Emergence of drug-resistant tumor cells during cytotoxic chemotherapy is one of the major unsolved problems in cancer therapy. One potential way to decrease the incidence of gene amplification-mediated drug resistance is to selectively accelerate the loss of newly acquired unstably amplified genes from "nascent" drug-resistant cells.

Unlike amplified cellular genes in tumor cells, which may be either stably or unstably amplified, chromosomes of certain DNA viruses that transform mammalian cells exist virtually exclusively in a free (unintegrated) form (40, 41). Among such viruses are papilloma viruses and possibly certain types of *Herpesvirus* (40). These viruses are associated with a wide variety of human pathologic conditions including both benign and malignant neoplastic lesions (40, 41).

If hydroxyurea should affect the stability of extrachromosomal viral DNAs in a way analogous to its striking effect on unstably amplified cellular genes, one could exploit the hydroxyurea effect for "curing" mammalian cells of extrachromosomally maintained viral DNA genomes. It should be noted in this regard that Turek *et al.* (40) have already shown that prolonged treatment of papilloma virus-transformed mouse cells with interferon results in a significant decrease in the number of extrachromosomal viral genomes per cell.

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