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Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells

(hypoxia/gene amplification/overreplication/methotrexate resistance)

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ABSTRACT **Exposure of Chinese hamster cells to reduced oxygen partial pressure results in a marked enhancement in the frequency of methotrexate resistance and dihydrofolate reductase gene amplification. The frequency of enhanced resistance is a function of (*i*) the length of exposure to hypoxic conditions and (*ii*) the time after recovery from hypoxia when cells are plated into methotrexate-containing medium. Hypoxia results in an inhibition of DNA synthesis; upon return to normal oxygen atmosphere, >60% of cells in S phase at the time hypoxia was started subsequently undergo overreplication of DNA within a single cell cycle. The cells with the increased frequency of gene amplification are derived from this subset of overreplicated cells. These results are discussed within the context of the hypoxic state of many solid tumors and the high frequency of aneuploidy, chromosomal aberrations, and spontaneously occurring resistances to a number of cancer chemotherapeutic agents.**

Amplification of specific genes as a mechanism for the acquisition of resistance in somatic mammalian cells has become an increasingly prevalent theme, whether selected in the laboratory or studied as resistance in tumors to clinical chemotherapy (see refs. 1 and 2). Various agents have been shown to increase the frequency of gene amplification, including drugs that inhibit DNA replication (3–5) and any number of other agents (6). Our laboratory has proposed that the initial event in gene amplification is an inhibition of DNA replication, the consequence of which is the generation of increased capacity for initiation of DNA replication when cells recover from the inhibited state (i.e., overreplication) (1, 7). We have proposed further that the recombination events that occur subsequent to overreplication can result in a variety of chromosomal aberrations–rearrangements and mutational events (8). Rice *et al.* (9) have reported that a 20-hr exposure of cells to hypoxic conditions and subsequent return to normal aeration conditions results in the generation of a subset of cells with DNA content of >4 C within a single cell cycle. The generation of cells with more than normal DNA content is consistent with the concept that perturbation of DNA synthesis results in overreplication of DNA, as concluded by Mariani and Schimke (7) and Hill and Schimke (10).

In this paper, we report that subjecting cells to hypoxic conditions can markedly enhance the frequency with which they become resistant to methotrexate as a result of amplification of the dihydrofolate reductase gene.

METHODS

Cell Culture Conditions. Chinese hamster ovary AA8 cells (11) were grown under monolayer conditions in Ham's F-12 medium without glycine, hypoxanthine, and thymidine (GIBCO) and supplemented with 10% dialyzed fetal calf serum; they were passaged in midlogarithmic phase by standard trypsinization methods. Cells were plated into media containing various concentrations of methotrexate, and colony numbers were determined 3 wk later. Thymidine incorporation studies were as described by Brown *et al.* (4). The conditions for generating hypoxia were similar to those described by Rice *et al.* (9). Briefly, 2×10^6 exponential cells were plated into 60-mm^2 permanox (Lux Scientific) Petri dishes. After allowing 2 hr for attachment at 37°C, the cells were equilibrated with 95% N_2/5% CO_2 at 37°C in specially designed nylon holders.

Analysis of Cell Cycle Kinetics—Flow Cytometric Techniques. The techniques for incorporation of BrdUrd and staining with propidium iodide for DNA content and a fluorescein-conjugated BrdUrd antibody are similar to those described by Rice *et al.* (9). Cells were labeled for 10 min at 37°C with 30 μM BrdUrd. After fixation in 70% EtOH, cells were treated with RNase at 1 mg/ml for 1 hr, followed by an extraction on ice with 1.5 M HCl to remove histones. After a 5-min denturation in water at 100°C, the cells were stained with a monoclonal anti-BrdUrd antibody (clone IU-2, a gift from Frank Dolbeare), followed by a fluorescein-conjugated anti-mouse IgG (Sigma). DNA was stained with propidium iodide (Sigma) at 10 μg/ml. Cells were analyzed by 488-nm laser excitation on an EPICS flow cytometer using a 514-nm band-pass filter for fluorescein and a 600-nm wavelength-pass filter for propidium iodide fluorescence. For the viable cell-sorting experiments, cells were stained with 5 μM Hoechst 33342 (Calbiochem) for 30 min at 37°C and analyzed with 50-mW laser excitation, and the emission was integrated above 425 nm as described in detail (12).

Gene Copy Analyses. Various methotrexate-resistant colonies were cloned by use of cloning rings and were grown to sufficient numbers (10^7 cells) for DNA isolation by standard methods of extraction with phenol and chloroform and precipitation with ethanol (23). Gene copy numbers were estimated by a slot-blot method with an α-fetoprotein cDNA sequence as a control (4). Either nitrocellulose filters were counted directly on an AMBIS β scanning device or radiographs were integrated on an E-C Apparatus (St. Petersburg, FL) densitometer.

RESULTS

Hypoxia Increases the Frequency of Methotrexate Resistance. Fig. 1 shows the frequency of methotrexate resistance as a function of the time cells were exposed to hypoxic conditions. In this experiment the cells were plated into various methotrexate concentrations immediately after return to normal aeration conditions. Fig. 1 *Inset* shows that cell survival was not affected by exposure to hypoxia for up to 28 hr, but by 72 hr of hypoxia, cell survival was reduced to 6% of control values. All values in Fig. 1 were corrected for cell killing by hypoxia. The length of time the cells were

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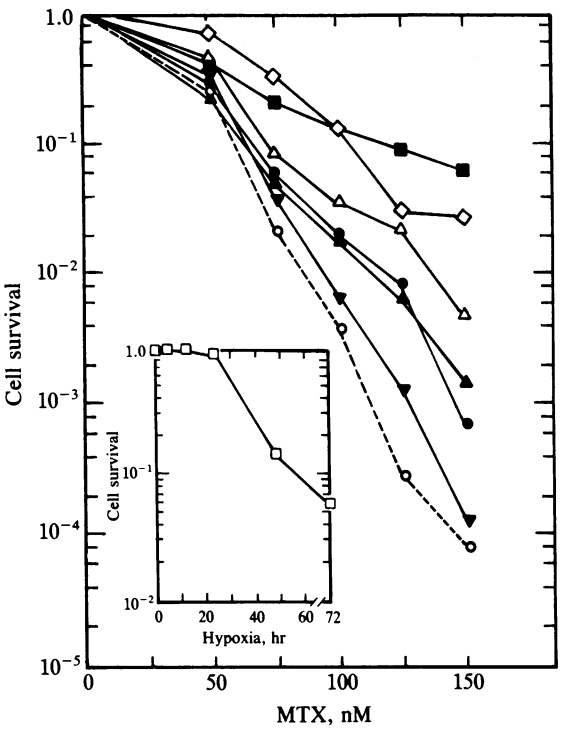

FIG. 1. Frequency of methotrexate (MTX) resistance as a function of time in hypoxia. Chinese hamster AA8 cells were placed under hypoxic conditions for 4 hr (▼), 24 hr (●), 12 hr (▲), 12 + 12 hr (△), 48 hr (◇), and 72 hr (■); trypsinized; and plated immediately into methotrexate-containing media. All cell-survival curves are normalized to the relative plating efficiency in normal medium (*inset*). The control curve (○---○) is without prior treatment. The 12 + 12 curve represents cells treated for two 12-hr intervals of hypoxia interrupted by a 24-hr period of normal aeration.

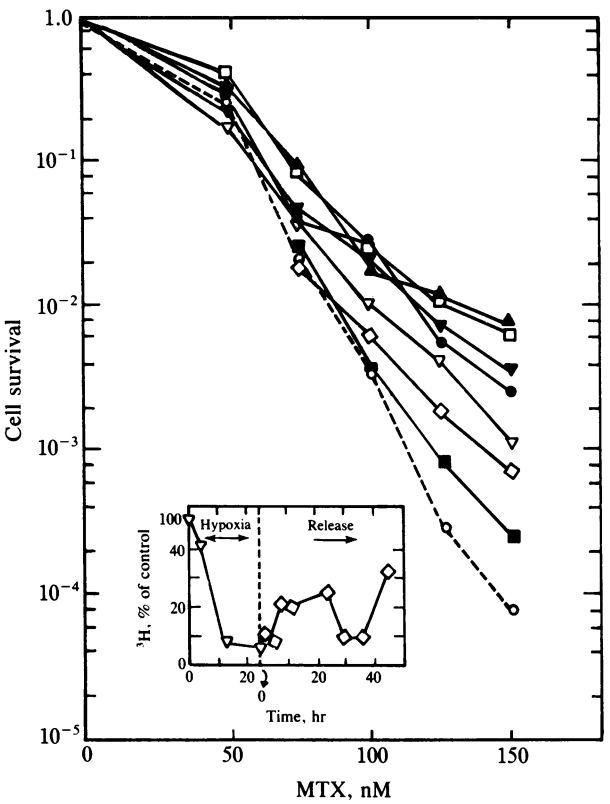

FIG. 2. The effect of recovery time after pretreatment with hypoxia on the frequency of methotrexate (MTX) resistance. Cells were subjected to hypoxia for 24 hr and placed under normal aeration conditions for 0 hr (▽), 4 hr (▼), 8 hr (□), 12 hr (▲), 24 hr (●), 48 hr (◇), and 72 hr (■) prior to addition to methotrexate. Control (○---○) constitutes no hypoxia treatment. Zero hour constitutes the addition of methotrexate immediately after 24 hr of hypoxia. (*Inset*) [^{3}H]Thymidine incorporation into acid-insoluble precipitates. Cells were pulse-labeled with [^{3}H]thymidine for 10 min at the times indicated during hypoxia or after the return to normal aeration conditions after 24 hr of hypoxia.

subjected to hypoxic conditions had a profound effect on the frequency of methotrexate resistance. Four hours of treatment had essentially no effect. Progressively longer periods of hypoxia increased the frequency of resistant colonies. A 24-hr exposure to hypoxia increased the frequency 10-fold. This constituted an absolute increment in resistance frequency, inasmuch as 24 hr of hypoxia had no effect on cell survival. The greatest differential between control and treated cells was with those cells subjected to 72 hr of hypoxia, where the frequency corrected (for cell killing) was enhanced 1000-fold over control values. From the cell-killing data (Fig. 1 *Inset*), it is readily obvious that virtually all cells subjected to 72 hr of hypoxia that can form colonies became resistant to methotrexate. Also, a 24-hr period of hypoxia that was interrupted after 12 hr by 24 hr of normal aeration resulted in a higher frequency of methotrexate resistance than when the 24-hr period of hypoxia was a single, continuous period (Fig. 1, 12 + 12 curve).

Fig. 2 *Inset* provides data showing the effect of hypoxia and return to aerobic conditions on thymidine incorporation into DNA. When cells were placed in hypoxic conditions, incorporation of thymidine into DNA declined so that after 10 hr it was 8% of normal values. This inhibition involved all S-phase cells and not a subset of such cells (9). Upon return to normal aeration, there was an increase in the extent of incorporation in a complex fashion. There was an initial recovery of thymidine incorporation and a secondary period of reduced incorporation, followed by a secondary increase. These results can be explained as a hypoxia-induced partial synchronization of cells at the 40-hr time period after aeration (G.C.R., unpublished data).

Fig. 2 shows that the time of placement of cells in methotrexate following the return of cells to aerobic conditions profoundly affected the estimated frequency of enhanced methotrexate resistance. The optimal time for placing cells in methotrexate was 12 hr; after that time, there was a progressive reduction in the frequency with which colonies emerged that were resistant to methotrexate. The 12-hr period of time for placement in methotrexate was at the peak of the restoration of DNA synthesis (Fig. 2 *Inset*). The finding of a critical time for placement of cells in methotrexate is in agreement with prior results with hydroxyurea (4) and ultraviolet light (5) enhancement of methotrexate resistance. In all cases, placing cells in methotrexate immediately after treatment reduced the frequency of resistance; enhanced resistance declined progressively when cells were placed in selective medium 24 hr or more after recovery of DNA synthesis. The results with hypoxia are consistent with the concept that gene amplification requires DNA synthesis during or subsequent to treatments and that the consequences of such treatments in terms of gene amplification events are transitory (10).

Hypoxia Induces S-Phase DNA Overreplication Within a Single Cell Cycle. Fig. 3 shows the results of sorting cells stained with Hoechst 33342, a viable DNA content dye. Fig. 3*A* shows the DNA distribution in the population of control cells, and Fig. 3*B* shows the DNA distribution of cells subjected to 18 hr of hypoxia analyzed 24 hr after the return to normal aeration. Fig. 3*B* shows a large proportion of the cells (40%) with a DNA content of >4 C (or G_2/M phase). Note also that such cells, as indicated by forward scatter, are larger than normal G_2/M cells. These cells were sorted within the selection windows shown and were plated into various

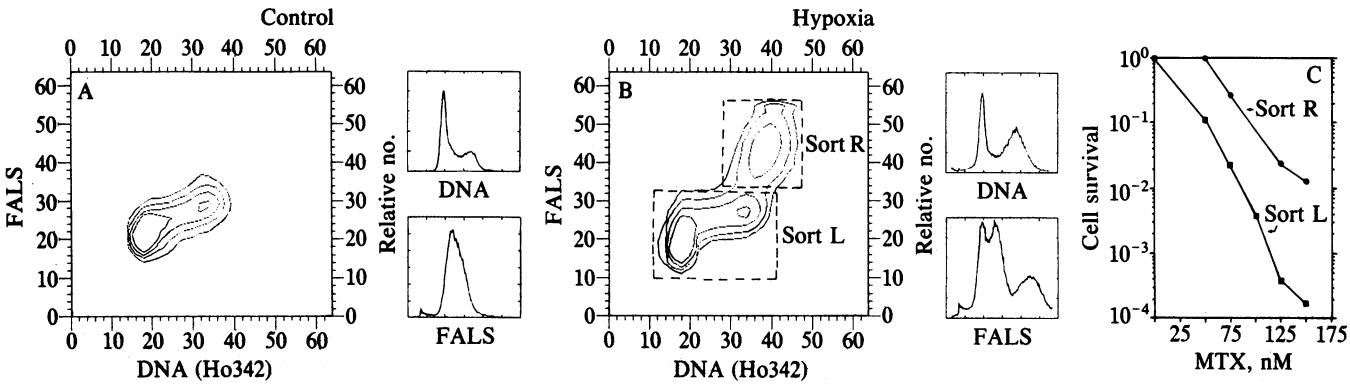

FIG. 3. Detection of DNA overreplication after hypoxia. Cells were stained with 5 μM Hoechst 33342 and analyzed for total DNA content by flow cytometry as well as forward angle light scatter (FALS). (*A*) Control cells. (*B*) Cells pretreated with 18 hr of hypoxia, followed by a 24-hr aeration period prior to analysis. Note the large fraction of cells with a DNA content of >4 C after hypoxia. (*C*) The two subpopulations marked sort L or sort R in *B* were sorted, and the frequency of methotrexate (MTX) resistance in these two subpopulations was determined.

concentrations of methotrexate. Fig. 3*C* shows that there was a markedly different survivability of cells in methotrexate as a function of whether they were sorted for DNA content of >4 C. This subset of cells had a 100-fold greater increase in the frequency of methotrexate resistance at 150 nM.

Fig. 4 shows the progression of normal and hypoxia-treated cells through a cell cycle by using the technique of Dolbeare *et al.* (13). In this technique, asynchronously dividing cell populations were pulse-labeled for 10 min with BrdUrd. The cells were fixed and stained with propidium iodide and a monoclonal anti-BrdUrd antibody as described and were analyzed simultaneously for DNA (Fig. 4, horizontal axes) and BrdUrd (Fig. 4, vertical axes). Fig. 4*A* shows cells immediately after pulse-labeling. The cell phases are identified in Fig. 4*B*. In exponential

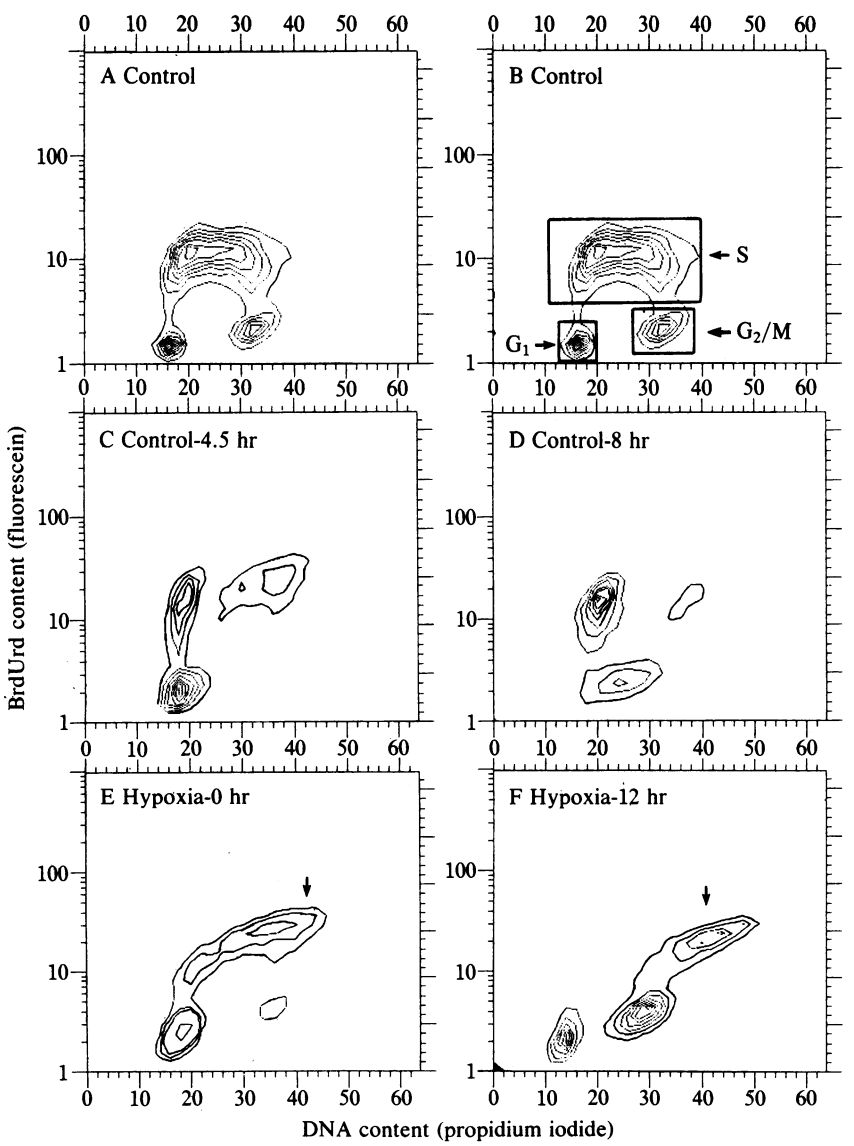

FIG. 4. Hypoxia induces S-phase overreplication within a single cell cycle, shown by simultaneous flow cytometric analysis of total DNA content and incorporated BrdUrd. (*A*) A control population given a 10-min pulse of BrdUrd. (*B*) The same cell distribution with demarkation of the individual cell cycle phases. (*C*) Cells pulsed with BrdUrd, washed, and incubated for 4.5 hr in normal medium. (*D*) Cells as in *C* but incubated for 8 hr. Note the progression of the original S-phase cells (high BrdUrd) into S phase. Also note the progression of the original G_1 cells into S phase (cells of low BrdUrd fluorescence but with a DNA content of ~2 C) and division of the original G_2/M cohort by 4.5 hr (loss of the low BrdUrd, 4 C DNA peak). (*E* and *F*) Cells that were pulsed for 10 min with BrdUrd, washed, and placed immediately into hypoxia for 24 hr. (*E*) Cells that were analyzed immediately after 24 hr of hypoxia. (*F*) Cells that were analyzed 12 hr after the return to normal aeration. Note that in the original S-phase subpopulation, there is already a detectable increase in DNA content per cell at the end of 24 hr of hypoxia as marked by the arrow (compare *A* with *E*). By 12 hr of normal aeration (*F*), this subpopulation of cells in S phase during the hypoxia treatment has increased the DNA content per cell significantly (i.e., overreplication).

growth, this cell line typically had 60% of cells in S phase, 35% in G_1, and 5% in G_2/M. Fig. 4 *C* and *D* show control cells 4.5 and 8 hr after the 10-min BrdUrd pulse. The data indicate that the BrdUrd incorporation does not alter normal cycle-progression kinetics, a finding compatible with Dolbeare *et al.* (13) using this cell line. For instance, Fig. 4*C* shows that by 4.5 hr the original G_2/M phase cells had divided and were in G_1 phase. Some of the BrdUrd-labeled S-phase cells were in G_2/M, while the remainder had divided and were in G_1 (i.e., those S-phase cells labeled with BrdUrd that were in middle-to-late S phase). By 8 hr (Fig. 4*D*), most of the original S-phase cells had progressed into G_1 (the population with G_1 DNA content and BrdUrd fluorescence). Also the original G_1 cells in which no BrdUrd was incorporated were now progressing through S phase (i.e., the population with more than G_1 DNA content but without elevated BrdUrd fluorescence). We describe this method in some detail to familiarize the reader with the manner in which various cell populations can be followed through a cell cycle.

Fig. 4*E* shows cells in which the 10 min of BrdUrd treatment was followed immediately by 24 hr of hypoxia; Fig. 4*E* should be compared with Fig. 4*B*, which shows the same cell population before hypoxia. Fig. 4*E* shows that during the 24 hr of hypoxia, cells have progressed in the cell cycle. Cells previously in G_2/M phase progressed into G_1, and cells in S phase extended their progression through S phase slightly. This is in keeping with the incomplete inhibition of DNA synthesis as produced by the hypoxic state (see Fig. 2 *Inset*). Note that 18% of the total S-phase population (10% of the total population) had a DNA content of >4 C at this time. By 12 hr after recovery from hypoxia, the pattern of cells was complex. A certain number of the cells retained the G_1 DNA content and constituted a population that was not in S phase at the time of the original BrdUrd pulse (i.e., they contained G_1 DNA content and minimal BrdUrd fluorescence). Many of the cells, including those previously in S phase, progressed through S phase (i.e., elevated DNA per cell), approaching G_2 levels and elevated BrdUrd fluorescence per cell. A significant proportion of the cells, 66% of the total S-phase population (or 34% of the total population), had a DNA content of >4 C per cell. This subpopulation constitutes cells that were actively synthesizing DNA at the time the cells were subjected to hypoxic conditions. Fig. 4 shows data from 12 hr after recovery of DNA synthesis. Fig. 3 shows results 24 hr after recovery and a greater proportion of cells with a DNA content of >4 C. Such results are in keeping with results from our laboratory (R. Johnston and S. Sherwood, unpublished data) showing that, after inhibition of DNA synthesis with aphidicolin, the generation of cells with additional DNA per cell was time dependent and was maximal at ≈24–36 hr after resumption of DNA synthesis.

Increased Methotrexate Resistance Results from Amplification of the Dihydrofolate Reductase Gene. A number of methotrexate-resistant variants were cloned and analyzed for dihydrofolate reductase gene amplification by the "slot-blot" method of Brown *et al.* (4). For this purpose, we analyzed cloned variants subjected to 24 hr of hypoxia and selected immediately at 150 μM methotrexate. Representative results of such clones are shown in Fig. 5. Of the control cell variants, two of seven contained amplified dihydrofolate reductase genes (2-fold amplification). After hypoxia, 16 of the 25 clones examined contained amplified genes (average amplification, 6-fold). The finding that 30–60% of low, single-step methotrexate-resistant variants had dihydrofolate reductase gene amplification and, upon enhancement of the frequency, this percentage did not change is consistent with prior studies from this laboratory with hydroxyurea (4) and ultraviolet light (5).

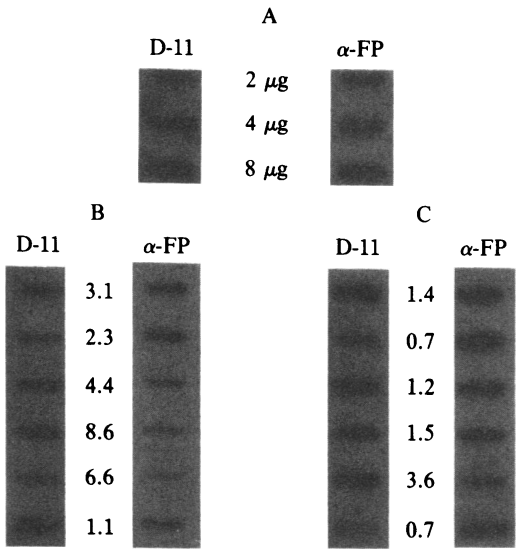

FIG. 5. Dihydrofolate reductase gene amplification in methotrexate-resistant colonies. Random colonies resistant to 150 nM methotrexate, obtained from control cells (*C*) and from cells subjected to 24 hr of hypoxia and plated into methotrexate immediately (*B*) were cloned and expanded to 10^7 in the presence of 150 nM methotrexate. DNA was isolated, and 2 μg was placed in adjacent slots and hybridized to either the dihydrofolate reductase D-11 cDNA clone (left lanes) or an α-fetoprotein cDNA clone (α-FP) (right lanes) as described. Microgram increments of DNA from the parental AA8 line are shown in *A*. The relative degree of amplification (shown between the lanes in *B* and *C*) as measured by densitometry is the average of at least two determinations with each DNA sample. In addition, random subclones were probed in an analogous manner by restriction fragment (Southern) methods of *Eco*RI digests with similar quantitative results. These are representative results from seven methotrexate clones derived from untreated cells (two of seven with amplified genes) and 25 methotrexate-resistant clones from hypoxia-treated cells (16 of 25 with amplified genes).

DISCUSSION

We reported previously that a number of treatments of cultured somatic mammalian cells increases the frequency of dihydrofolate reductase gene amplification, including drugs that inhibit DNA replication and treatments that introduce DNA adducts. Here we report that another physical perturbation of cells (i.e., hypoxia) also increases the frequency of dihydrofolate reductase gene amplification. All of these treatments have in common the transient inhibition of DNA synthesis; as a consequence during the period of recovery of inhibition of DNA synthesis, all treatments produce the appearance of a subset of cells with increased DNA content per cell (refs. 7 and 10; R. Johnston and S. Sherwood of this laboratory, unpublished observations). Here we show that after hypoxia treatment, the subset of cells with DNA content of >4 C is derived from cells that were in S phase during the time of hypoxia treatment. Data that indicate that S-phase overreplication is a generalized phenomena found with other agents that induce gene amplification (such as hydroxyurea, aphidicolin, and ultraviolet light) also were observed (C.H., unpublished data).

We have proposed that, when DNA synthesis is inhibited but RNA and protein synthesis occurs, there is an increased capacity of cells for initiation of DNA replication, resulting in overreplication of DNA in a single cell cycle (8). The phenomenon appears to be analogous to comparably perturbing treatments in prokaryotes, resulting in multiple initiations of DNA replication (14–17). We are not in a position at the present time to attempt to understand how hypoxia alters cellular metabolism such that upon reaeration there is overreplication of DNA. However, it would not appear to be the result of alterations in

pH of the media or in total glucose consumption inasmuch as changing these parameters does not itself change the frequency of methotrexate resistance or alter the effects of hypoxia (G.C.R., unpublished data).

We have proposed a model whereby overreplication–recombination results in extrachromosomal or chromosomal gene amplification (1) and have extended this model more recently to account for a variety of chromosomal aberrations and mutational events (8). The striking effects of hypoxia in inducing DNA overreplication and gene amplification may provide an understanding of certain properties of cancers and, in particular, solid tumors. There is an extensive literature documenting that solid tumors have a significant subset of cells that are hypoxic (i.e., those cells peripheral to an inner zone of necrosis but internal to those portions of a tumor with adequate capillary supply) (18–20). There is a high frequency of aneuploidy (24) and chromosomal aberrations in solid human tumors (21) and a high frequency of spontaneous resistance to a variety of cancer chemotherapeutic agents (22). We propose that these alterations may be related to a hypoxic state, in particular in those regions of a tumor where the blood supply is partially compromised and from which the progressively malignant cells are derived. Thus, the hypoxic state may well induce overreplication–recombination events leading to spontaneous drug resistance, oncogene amplification events, aneuploidy, and various forms of chromosomal rearrangements resulting in increased cellular heterogeneity and malignant progression.

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