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Amplification and Loss of Dihydrofolate Reductase Genes in a Chinese Hamster Ovary Cell Line

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During stepwise increases in the methotrexate concentration in culture medium, we selected Chinese hamster ovary cells that contained elevated dihydrofolate reductase levels which were proportional to the number of dihydrofolate reductase gene copies (i.e., gene amplification). We studied the dihydrofolate reductase levels in individual cells that underwent the initial steps of methotrexate resistance by using the fluorescence-activated cell sorter technique. Such cells constituted a heterogeneous population with differing dihydrofolate reductase levels, and they characteristically lost the elevated enzyme levels when they were grown in the absence of methotrexate. The progeny of individual cells with high enzyme levels behaved differently and could lose all or variable numbers of the amplified genes.

The phenomenon of gene amplification as a mechanism for developing resistance to methotrexate (MTX) has been documented in our laboratory (1, 20, 23) and in other laboratories (8, 18). Characteristically, such resistance is obtained when cells are subjected to stepwise selection in growth medium containing progressively increasing concentrations of MTX. The acquisition of MTX resistance is associated with increased numbers of the gene coding for dihydrofolate reductase (DHFR), the target enzyme inhibited by MTX, and with corresponding increases in the levels of DHFR.

MTX resistance, elevated DHFR levels, and amplified DHFR genes can occur either in a stable state (i.e., the amplified genes persist when cells are grown in MTX-free medium) or in an unstable state, such that approximately 50% of the amplified DHFR genes are lost from a cell population in as few as 20 cell doublings in MTX-free medium (2). We have reported previously that stably amplified DHFR genes in an MTX-resistant Chinese hamster ovary (CHO) cell line (20) and in a mouse lymphoma cell line (8) are localized to a so-called homogeneously staining region (4) on a single, long, distinctive marker chromosome. In contrast, we have presented preliminary evidence that in unstably resistant mouse cell lines the amplified DHFR genes reside on self-replicating extrachromosomal elements, called double minute chromosomes (12). Double minute chromosomes lack centromeric regions (3, 14), and hence there is no mechanism to insure that they segregate equally into daughter cells at mitosis; because of this, DHFR genes can be lost from cells.

All previous studies of cell lines with elevated DHFR levels have been performed with cells that have been selected in a stepwise fashion and grown for relatively long periods of time at a specified MTX concentration. Characteristically, in hamster cell lines the amplified DHFR genes are stably amplified. Consequently, we studied the characteristics of CHO cells undergoing the first steps in the selection process. The cell line used was cloned derivative K_1B_{11} from the parental CHO cell line K_1, which after prolonged selection produced MTX-resistant cell line MK_{42} containing 150 copies of the DHFR gene localized to a homogeneously staining region (20). We used the fluorescence-activated cell sorter technique and a fluorescein conjugate of MTX to study individual cells in a cell population that underwent MTX selection. We have shown previously that this technique quantitates DHFR levels (11); in this study we found that the relative DHFR gene copy number and the DHFR levels as determined by the fluorescence-activated cell sorter technique are proportional in CHO cells. Hence, this simple technique could be used to determine gene copy number in cell populations undergoing amplification, as well as to study the loss of DHFR genes when cells were grown in the absence of MTX.

MATERIALS AND METHODS

Cell culture. The CHO K_1 cell line was provided by L. Chasin, Department of Biological Sciences, Co-

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lumbia University. Subclone K_1B_{11} was obtained by dilution plating (7) from this line and was used in all experiments, unless otherwise noted. All cell lines were grown in Ham F-12 medium lacking thymidine, glycine, and hypoxanthine but supplemented with 10% fetal calf serum. MTX-resistant K_1B_{11}CHO cells were selected by progressively increasing the concentration of MTX in the growth medium containing dialyzed fetal calf serum. Selections were made sequentially in media containing 0.02, 0.1, 0.5, and 2 μM MTX. The 0.02 μM concentration reduced the relative plating efficiency to 50% of the plating efficiency of the original cells. All lines were subcultured twice weekly at similar densities.

MTX fluorescence labeling and cell sorting. Cells were labeled with the fluorescein conjugate of MTX, as described previously (11). To remove MTX bound to DHFR, cells were grown for 4 days in the absence of MTX before labeling with the fluorescein conjugate of MTX. Then cells were labeled with the fluorescein conjugate of MTX (30 μM) for 20 to 24 h in medium supplemented with 30 μM thymidine, 30 μM glycine, and 30 μM hypoxanthine in order to prevent cell death due to tetrahydrofolate deficiency. The cells were washed with medium, incubated for 15 min at 37°C in fresh medium, trypsinized, and suspended in medium. Then the cells were centrifuged through 5 ml of 0.3 M sucrose for 5 min at 1,000 rpm in a model Sorvall GLC centrifuge. The resulting cell pellets were suspended in medium and kept on ice until they were analyzed for fluorescence with the fluorescence-activated cell sorter.

Cells were analyzed by using a model FACS II fluorescence-activated cell sorter (laser power, 400 mW; photomultiplier voltage, 420 V; Becton, Dickinson & Co.). All cultures were more than 95% viable, as determined by light scattering (16). Cells were analyzed and sorted for scatter-gated fluorescence in order to prevent scoring of dead cells and debris. The data presented below represent either the fluorescence distributions of the cell populations or the fluorescence of individual cells as a function of size. Cells were simultaneously sorted and cloned as described previously (21). Individual cells were sorted into microtiter test dishes containing 200 μl of Ham F-12 medium supplemented with 10% fetal calf serum. When clones contained 2×10^6 cells, they were analyzed for DHFR content as described above.

Measurement of DHFR activity. DHFR specific activities in 100,000 × *g* supernatant fractions of extracts of late-log-phase cells were determined by the [^{3}H]folate assay described previously (11); 1 U of activity was defined as the amount of protein required to reduce 1 nmol of folic acid in 15 min at 37°C. Protein was determined by the method of Lowry et al. (17).

Southern blot hybridizations. High-molecular-weight deoxyribonucleic acid (DNA) from stationary-phase cells was isolated (12) and used in Southern blot hybridization experiments (24), as described previously (20). *Eco*RI-digested total genomic DNA samples (10 μg) were subjected to electrophoresis on a 0.5% agarose gel in tris(hydroxymethyl)aminomethane-acetate-ethylenediaminetetraacetate-sodium chloride buffer (9) and then transferred to nitrocellulose filter paper (type BA-85; Schleicher & Schuell) by the method of Southern (24), as modified by Nunberg et al. (19). Filters were prehybridized for 12 h at 65°C in a solution containing 0.02% polyvinylpyrrolidine, 0.02% bovine serum albumin, 0.02% Ficoll, 0.5% sodium dodecyl sulfate, 10 μg of salmon sperm DNA per ml, 25 mM sodium phosphate, and 1.25 mM sodium pyrophosphate in 5× SSC (1× SSC is 150 mM sodium chloride plus 15 mM sodium citrate). Plasmid pDHFR11 DNA (19), which contains an insertion that represents approximately 1,600 nucleotides of the murine DHFR messenger ribonucleic acid, was nick-translated, (22) to a specific activity of 2×10^7 cpm/μg by using DNA polymerase I (New England Bio-Labs) and α-^{32}P-labeled deoxycytidine triphosphate; (350 Ci/mmol; Amersham Corp.), and 3×10^6 cpm was added to 3.5 ml of the pre-hybridization mixture. After the solution was heated for 10 min at 104°C, hybridizations were carried out in sealed plastic bags for 48 h at 68°C. Filters were washed and prepared for autoradiography as described previously (19). The bands in the autoradiograms were scanned by using a Joyce Loebl microdensitometer, and they were quantitated by determining the area under each peak. Completion of *Eco*RI digestions was monitored as described previously (19).

RESULTS

Stepwise selection for MTX resistance. Figure 1 shows the fluorescence distributions for various populations of CHO cells selected for MTX resistance by different stepwise treatments. Figure 1A shows that all of the cells of the original K_1B_{11} clone had negligible fluorescence. This clone was grown in medium containing 0.02 μM MTX and was examined by fluorescence analysis approximately every 2 weeks. The first detectable change was observed after 50 cell doublings, with some cells showing elevated DHFR levels. After 85 cell doublings in medium containing 0.02 μM MTX (Fig. 1B), 70% of the cells in the population had parental DHFR levels (0.4 fluorescein unit [FU] per cell) and 30% of the cells had approximately 2 FU/cell. Another sensitive cell population was grown for only 25 cell doublings in medium containing 0.02 μM MTX, followed by growth in medium containing 0.1 μM MTX for 50 cell doublings. In this cell population (Fig. 1C) the percentage of cells with parental DHFR levels was 3%, the number of cells in the 2-FU/cell peak increased, and a number of cells with higher DHFR levels were present. A third sensitive cell population was grown for 25 cell doublings in medium containing 0.02 μM MTX, followed by 15 cell doublings in medium containing 0.1 μM MTX and then 12 cell doublings in medium containing 0.5 μM MTX. This population (Fig. 1D) contained few cells with low DHFR levels and constituted a continuous spectrum of cells with elevated DHFR levels. When cells selected by the third

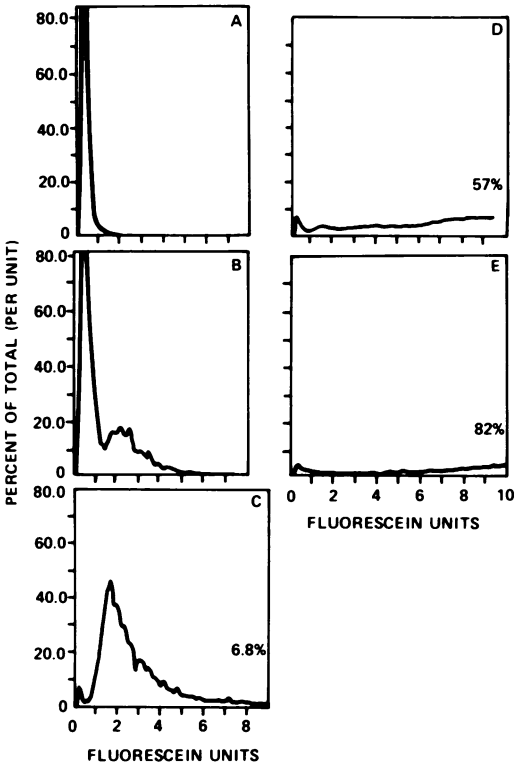

FIG. 1. *Analysis of cells selected for MTX resistance with the fluorescein conjugate of MTX. Cells were labeled with the fluorescein conjugate of MTX and prepared for fluorescence analysis as described in the text. Analyses of all lines were carried out at the same time. Each graph represents the results from an analysis of 10,000 cells. (A) K_1B_{11} parental MTX-sensitive cloned line. (B) Cloned line K_1B_{11} after growth in medium containing 0.02 μM MTX for 85 cell doublings. (C) Original K_1B_{11} line grown in medium containing 0.02 μM MTX for 25 cell doublings and then in medium containing 0.1 μM MTX for 50 cell doublings. (D) K_1B_{11} line grown in medium containing 0.02 μM MTX for 25 cell doublings, in medium containing 0.1 μM MTX for 15 cell doublings, and then in medium containing 0.5 μM MTX for 12 cell doublings. (E) K_1B_{11} line grown in medium containing 0.02 μM MTX for 25 cell doublings, in medium containing 0.1 μM MTX for 15 cell doublings, in medium containing 0.5 μM MTX for 5 cell doublings, and then in medium containing 2 μM MTX for 6 cell doublings. The percentages on the right sides of the graphs indicate the numbers of cells that contained more than 9.5 FU/cell.*

treatment were grown for only 5 cell doublings (compared with the 12 cell doublings of Fig. 1D) and then for an additional 6 cell doublings in medium containing 2 μM MTX (Fig. 1E), the majority of cells contained more than 10 FU. These results showed that once a certain degree of MTX resistance was generated (i.e., after 25 to 50 cell doublings at the initial MTX selection concentration [0.02 μM]), increasing resistance could be generated rapidly. It should be noted that if cells were first selected at a MTX concentration of 0.05 μM (i.e., 25 times the concentration used in these experiments), no MTX-resistant cells were obtained.

Because DHFR content was based on a per cell determination, the distributions shown in Fig. 1 tended to obscure discrete subpopulations, because cells inherently are different sizes (10) and because DHFR content varies in the cell cycle (25; B. D. Mariani, D. L. Slate, and R. T. Schimke, Proc. Natl. Acad. Sci. U.S.A., in press). Figure 2 shows the fluorescence analysis data as scatter plots, in which the amount of fluorescence per cell was plotted against the size of each cell (scatter). Each cell constituted a single datum point, and subpopulations appeared as clouds of individual points. Figure 2A shows the population of cells (15,000 counted) shown in Fig. 1D. We found at least three subpopulations with 0.4, 2.0, and 4.0 FU/cell, as well as large numbers of cells with higher numbers of FU per cell, which constituted a heterogeneous popula-

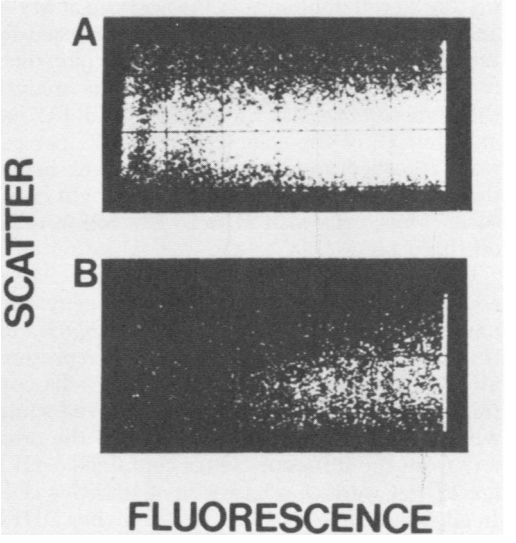

FIG. 2. *Light scattering and fluorescence analysis of cells grown in medium containing 0.5 μM MTX. Cells were prepared for analysis with the fluorescein conjugate of MTX as described in the text and were analyzed for MTX fluorescence (X axis), which was linear with DHFR level, and light scattering (Y axis), which was linear with cell size. Each dot represents the coordinates of a single cell for the two parameters. (A) Analysis of 15,000 cells from the population shown in Fig. 1D. (B) Analysis of 5,000 cells from the same cell line after growth in medium containing 0.5 μM MTX for another 100 cell doublings. The cells at the far right contained more than 9.5 FU/cell.*

tion. Figure 2B shows the same population of cells as Fig. 2A (5,000 cells counted), except that these cells were grown for an additional 100 cell doublings in medium containing 0.5 μM MTX (the cells shown in Fig. 2A were grown in medium containing 0.5 μM MTX for only 12 cell doublings); Fig. 2B shows that only cells with high DHFR levels were present when the selection pressure was maintained for longer time periods.

Clonal analysis of stability of increased DHFR content. Since prolonged selection of parental CHO cell line K_1 (from which cell line K_1B_{11} was subcloned) generated cell line MK_{42} containing stable amplification of DHFR genes (20), we wished to determine whether the CHO cells recently selected for MTX resistance were stable or unstable in terms of DHFR enzyme levels. To do this, we sorted and cloned individual cells from the cell population shown in Fig. 1D (25 cell doublings in medium containing 0.02 μM MTX, 13 cell doublings in medium containing 0.1 μM MTX, and 12 cell doublings in medium containing 0.5 μM MTX). We cloned cells that contained 0.5, 1.8, and 21 to 25 FU/cell. The resulting clones were grown for approximately 20 cell doublings in the absence of MTX, and then all of the progeny were analyzed for DHFR content by using the scatter plot technique. It should be noted that in this analysis the fluorescence scale stopped at 9.5 FU/cell and that all of the cells with more FU per cell were recorded as a group at that level; hence, there was a pile-up of cells at the far right of the scale. Thus, cells with 21 to 25 FU/cell were far off this scale.

Cloned cells obtained from the population containing 0.5 FU/cell produced progeny with parental levels of DHFR (Fig. 3A). Figures 3B through E show the progeny of four representative clones that were isolated from cells containing 1.8 FU/cell. Each clone behaved somewhat differently; 56, 13, 3, and 0.1% of the progeny from the different clones contained DHFR levels that were characteristic of sensitive cells. In all cases, some progeny had far higher DHFR levels than the cells from which the populations were derived. In particular, the progeny of clone B were heterogeneous for DHFR content. In general (Fig. 3C through E), the majority of the progeny appeared as discrete populations containing approximately 2 FU/cell. The clones that contained a greater percentage of cells with parental DHFR levels also gave rise to cells with higher DHFR levels (Fig. 3B and C). The reciprocity of loss and gain of unstably amplified DHFR genes is also evident with mouse S-180 cells (13).

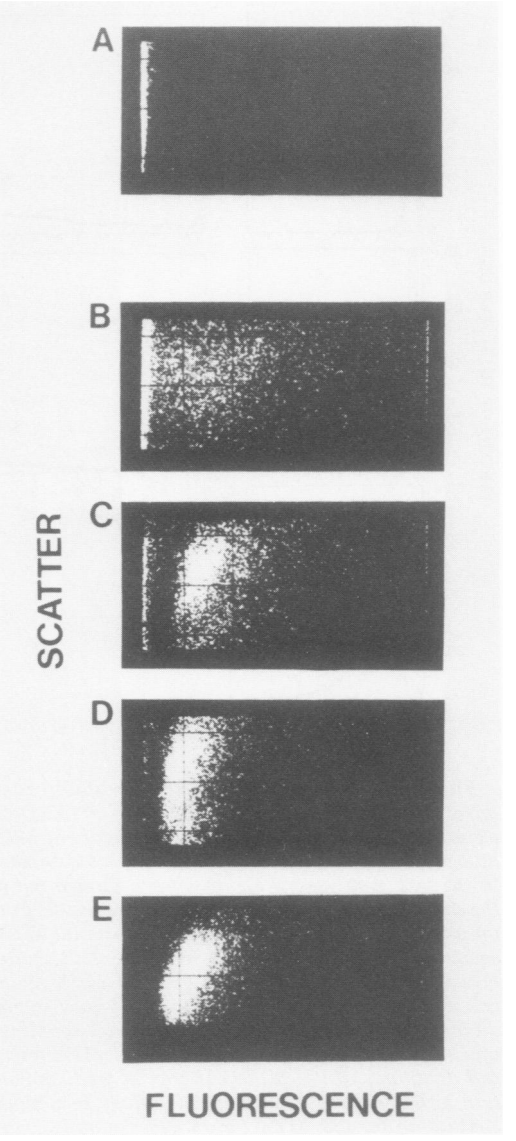

FIG. 3. *Clonal analysis of cells sorted for low and intermediate levels of DHFR. The K_1B_{11} line was grown in medium containing 0.5 μM MTX for 18 cell doublings and was prepared for single-cell sorting as described in the text. After this line had a distribution similar to that shown in Fig. 2A. We isolated five clones that contained 0.5 FU/cell. (A) Analysis of one representative clone after 20 cell doublings in the absence of MTX. The cells of this clone contained a mean of 0.33 FU/cell. We also isolated seven clones that contained 1.8 FU/cell. (B through E) Analyses of four representative clones after 20 cell doublings in the absence of MTX. In each case 5,000 cells were analyzed. The cells at the far right contained more than 9.5 FU/cell.*

Most interesting were the clones derived from cells containing 21 to 25 FU/cell. Figure 4 shows seven representative clones. Figure 4A shows the progeny of one cell; virtually all of this population completely lost the elevated DHFR content (but note that there were a few cells with very high DHFR levels). Some cells produced progeny that had sensitive DHFR levels (Fig. 4B, C, E, and F), whereas other populations (Fig. 4E through G) contained predominately cells with high enzyme levels. The progeny shown in Fig. 4B through D were predominately cells containing 2 FU/cell. In all cases, however, the progeny lost genes, inasmuch as the number of FU per cell was within the fluorescence scale (the upper limit was 9.5 FU/cell), whereas they were selected from a population containing 21 to 25 FU/cell.

We concluded that CHO cells newly selected for MTX resistance were unstable with respect to DHFR levels. However, the loss of elevated DHFR levels was variable in the progeny of

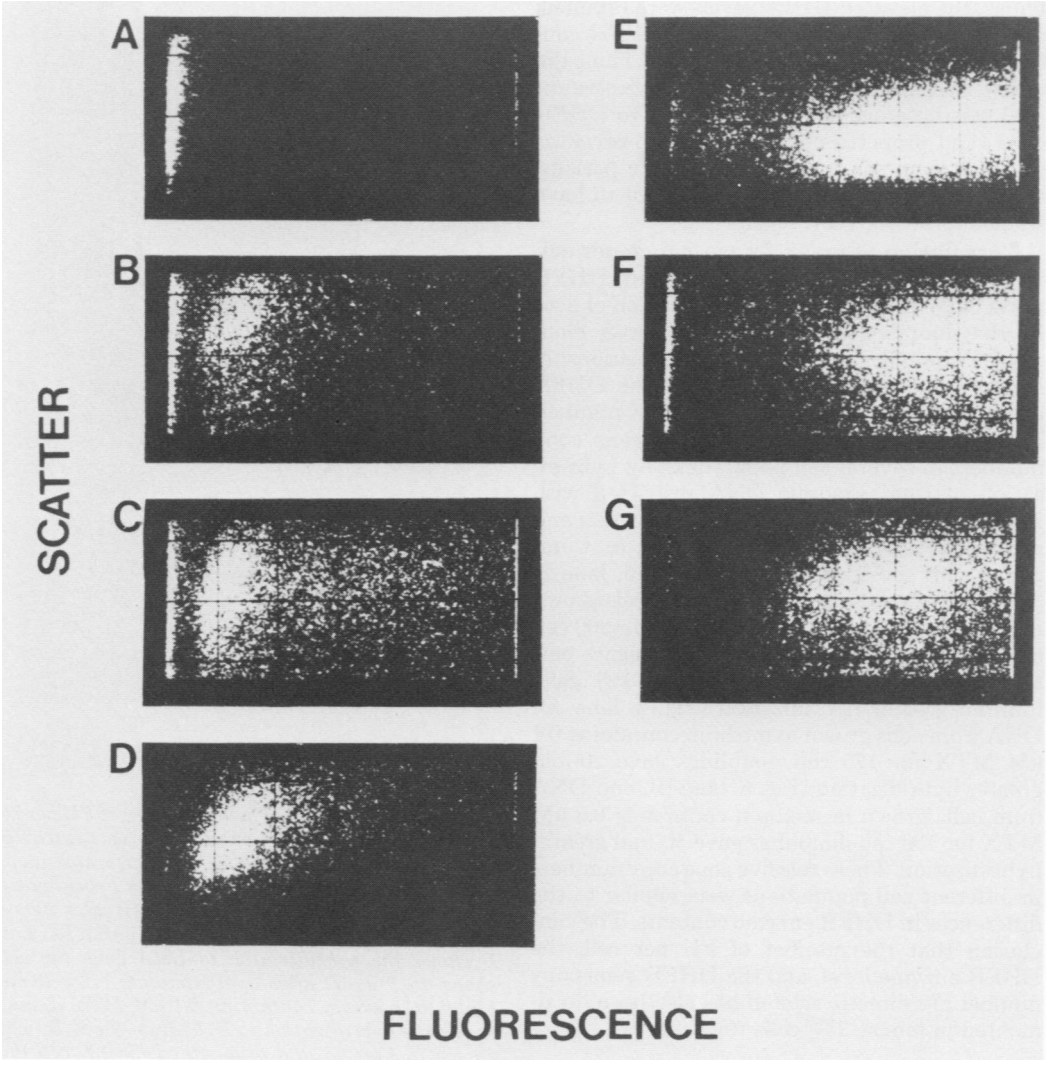

FIG. 4. *Clonal analysis of cells sorted for high levels of DHFR. Cells containing 21 to 25 FU/cell were sorted and cloned from the K_1B_{11} line grown in medium containing 0.5 μM MTX for 18 cell doublings (see legend to Fig. 3 and text). Seven of the clones obtained were analyzed after approximately 20 cell doublings in the absence of MTX. Each analysis represents approximately 5,000 cells, except (D), which represents 15,000 cells. The cells at the far right contained more than 9.5 FU/cell.*

different cloned cells. Some progeny reverted essentially to sensitive enzyme levels, some progeny had high enzyme levels, and some progeny constituted a heterogeneous population with respect to DHFR content. Of particular interest were the progeny shown in Fig. 3D and G, which formed relative discrete, non-heterogeneous populations with different mean levels of DHFR per cell.

When cells were grown in medium containing 0.5 μM MTX for more than 100 cell doublings (the population shown in Fig. 2B) and then grown in the absence of MTX for 40 cell doublings, the elevated DHFR levels were retained, and no subpopulations with lower DHFR contents were detected (data not shown). Thus, the newly selected population of MTX-resistant cells was heterogeneous with respect to DHFR levels and properties of reversion. However, as the cells were selected for longer time periods, the population that emerged appeared to have stable elevated DHFR levels.

Correlation of mean fluorescence per cell with DHFR specific activity and DHFR gene copy number. The interpretation of data by the fluorescence-activated cell sorter technique required that there was a relationship among the number of FU per cell, the DHFR enzyme level, and the DHFR gene copy number. We determined the relative DHFR gene copy numbers in several cell populations by hybridization of total genomic DNA restricted with *Eco*RI, using the Southern blot method (22) and mouse DHFR plasmid DNA (5) (Fig. 5). Compared with sensitive cell levels (Fig. 5, lane D [not easily detectable]), DNA from cells grown in medium containing 0.02 μM MTX for 200 cell doublings (a population with a fluorescence pattern similar to that shown in Fig. 1C) gave fourfold greater hybridization (Fig. 5, lane A). DNA from cells grown in medium containing 0.1 μM MTX for 175 cell doublings gave 20-fold greater hybridization (Fig. 5, lane B), and DNA from cells grown in medium containing 0.5 μM MTX for 130 cell doublings gave 50-fold greater hybridization. These relative gene copy numbers in different cell populations were similar to the differences in DHFR enzyme contents. The conclusion that the number of FU per cell, the DHFR enzyme level, and the DHFR gene copy number are directly related has also been documented in mouse 3T3 cells (5).

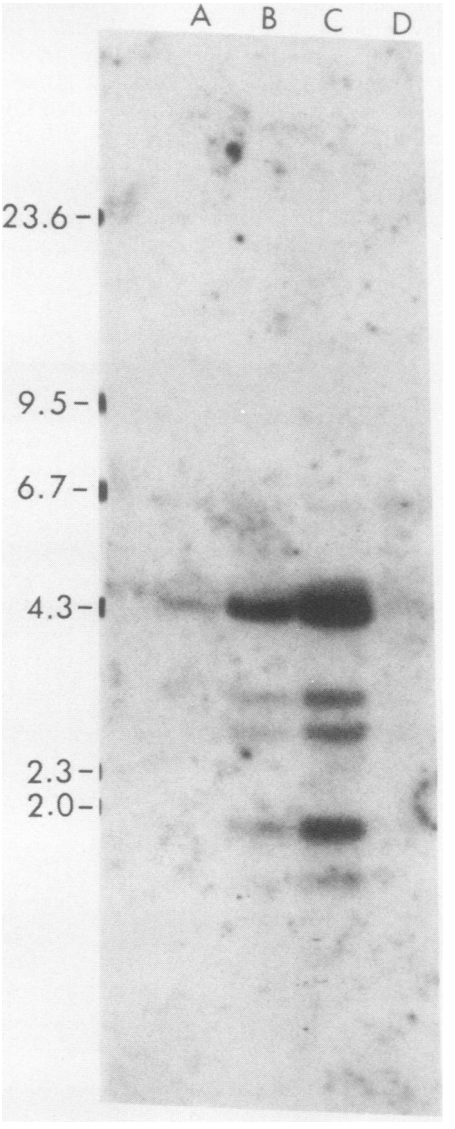

FIG. 5. *Correlation of mean number of FU per cell with DHFR gene copy number. DNA was extracted, and a Southern blot analysis was performed as described in the text. Lane A shows the hybridization to DNA from the cells shown in Fig. 1B after 200 cell doublings in medium containing 0.02 μM MTX. Lane B shows the hybridization to DNA from the cells shown in Fig. 1D after approximately 175 cell doublings in medium containing 0.1 μM MTX. Lane C shows the hybridization to DNA from the K_1B_{11} cells shown in Fig. 1E and propagated for 130 cell doublings in medium containing 2 μM MTX. Lane D shows the hybridization to DNA from the original K_1B_{11} clone. The numbers on the left are the sizes (in kilobases) of HindIII-digested λ marker DNA fragments.*

DISCUSSION

In this work we used the fluorescence-activated cell sorter technique and a fluorescein derivative of MTX to characterize populations

of cells during the acquisition and loss of MTX resistance. This technique showed that there are vast differences in the DHFR contents in the cells in a population and that there are differences in the behavior of individual cells during the loss of MTX resistance. We found that the fluorescence of individual cells is a measure of DHFR enzyme content and is a function of the DHFR gene copy number. Consequently, we interpreted our cell sorting data in terms of gain and loss of DHFR genes.

We found that stepwise selection in increasing MTX concentrations generates CHO cells with differences in DHFR levels and DHFR gene copy numbers. The first step in the selection (0.02 μM MTX) appears to generate a population with DHFR enzyme levels that are three to five times greater than the levels in the original population. Whether this difference constitutes the first possible degree of gene amplification or is simply the result of the MTX concentration chosen is not known. During subsequent increases in the MTX concentration in the medium, the population becomes progressively more heterogeneous with respect to gene copy number. Although an initial subpopulation with elevated DHFR levels requires approximately 50 cell doublings in order to be detected, the subsequent generation of highly MTX-resistant populations occurs more rapidly. When cells are grown under MTX selection for short time periods (up to 50 cell doublings), the elevated DHFR levels are characteristically unstable in CHO cells, and the progeny of individual, highly MTX-resistant cells may lose essentially all amplified genes or may constitute various populations with discrete or heterogeneous numbers of DHFR genes.

When cells are grown for longer time periods under a fixed selection pressure (i.e., at a constant MTX concentration), the population of cells that emerges contains stably amplified DHFR genes. We interpret the variable properties of loss of unstably amplified genes within the context that such genes occur as extrachromosomal elements which can be segregated unequally into daughter cells to generate heterogeneity within the population. Such extrachromosomal elements can also be lost from cells in variable numbers (including total loss) as a consequence of micronucleation (14). Cells with extrachromosomal elements which can be distributed unequally at mitosis or can be lost in large numbers are at a survival disadvantage over prolonged times of selection compared with occasional cells in which the requisite numbers of DHFR genes required for survival at a given MTX concentration are present on a chromosome that can segregate at mitosis. Thus, we propose that occasional cells in which DHFR genes are located on a chromosome in time become dominant in the cell population. If such a postulated event is rare, prolonged selection is required to obtain the stably resistant cells.

The instability of the amplified DHFR genes in emerging, resistant CHO cells is consistent with the hypothesis that they are present on extrachromosomal elements (i.e., double minute chromosomes) (12). However, we detected extrachromosomal elements in no more than 5 to 10% of the MTX-resistant CHO cells. Some of these extrachromosomal elements appear as packets (data not shown) and may be likely candidates for micronucleation. Others appear as discrete elements that may segregate unequally at mitosis. Since extrachromosomal elements tend to stick to chromosomes (3, 15) and since these elements stain poorly with Giemsa stain (or with other DNA or chromosome-staining dyes), we do not know whether we were simply unable to detect them or whether in some instances the amplified DHFR genes existed in a chromosome but in an unstable state. It is also possible that in CHO cells the process(es) that results in stable amplification of DHFR genes occurs more frequently than in the mouse cell lines, in which double minute chromosomes are characteristically present in all metaphase spreads of unstably MTX-resistant cell lines. This possibility is in keeping with the fact that the CHO cell lines became stably resistant when they were selected for as little as 100 cell doublings, whereas we have maintained mouse cell lines for 2 to 3 years with only occasional generation of stable cell lines (13). Whether this difference relates to the relative stability of the chromosome structure in CHO cell lines compared with the structure in mouse cell lines is unknown.

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