MOLECULAR AND CELLULAR BIOLOGY, Dec. 1981, p. 1084-1093
0270-7306/81/121084-10$02.00/0

Vol. 1, No. 12

Loss and Stabilization of Amplified Dihydrofolate Reductase Genes in Mouse Sarcoma S-180 Cell Lines

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Received 25 March 1981/Accepted 27 July 1981

We studied the loss and stabilization of dihydrofolate reductase genes in clones of a methotrexate-resistant murine S-180 cell line. These cells contained multiple copies of the dihydrofolate reductase gene which were associated with double minute chromosomes. The growth rate of these cells in the absence of methotrexate was inversely related to the degree of gene amplification (number of double minute chromosomes). Cells could both gain and lose genes as a result of an unequal distribution of double minute chromosomes into daughter cells at mitosis. The loss of amplified dihydrofolate reductase genes during growth in the absence of methotrexate resulted from the continual generation of cells containing lower numbers of double minute chromosomes. Because of the growth advantage of these cells, they became dominant in the population. We also studied an unstably resistant S-180 cell line (clone) that, after 3 years of continuous growth in methotrexate, generated cells containing stably amplified dihydrofolate reductase genes. These genes were present on one or more chromosomes, and they were retained in a stable state.

Previously, workers from our laboratory have shown that amplified dihydrofolate reductase (DHFR) genes in methotrexate (MTX)-resistant Chinese hamster ovary (21) and murine L5178Y (12) cell lines are stable when the cells are grown in the absence of MTX. The DHFR genes are associated with a characteristic expansion of a specific chromosome, a homogeneously staining region (HSR) (5). In contrast, murine S-180 (17) and 3T6 (7) cells which contain unstably amplified DHFR genes are associated with extrachromosomal, self-replicating elements called double minute chromosomes (DMs). In this work we studied the properties of murine S-180 cells and various subclones during the process of reversion from MTX resistance to MTX sensitivity when cells were grown in the absence of MTX. Our results were consistent with the hypothesis that because DMs contain no centromeric regions (4, 19), they can be distributed unequally into daughter cells. We found that S-180 cell growth rates were inversely related to the number of DMs; hence, over a large number of cell doublings, cells with progressively fewer DMs became dominant within the population.

In addition, we studied subclones of S-180 cells which originally contained amplified DHFR genes that were associated with DMs, but after prolonged growth in medium containing MTX generated cell populations with stably amplified genes that were associated with one or more chromosomes.

MATERIALS AND METHODS

Cell and culture conditions. The parental S_3 and MTX-resistant R_1 and R_2 murine S-180 clones have been described previously (2, 13). Cells were maintained as monolayers in Eagle minimal essential medium supplemented with 10% fetal calf serum. MTX-resistant lines were maintained in medium containing 5×10^{-5} M MTX, 3×10^{-5} M thymidine, and 3×10^{-5} M glycine. The R_1 line was divided into three sublines (designated R_1A, R_1B, and R_1C), and each of these sublines was propagated continuously for different lengths of time in selected media containing MTX, thymidine, and glycine. Subclones were obtained by dilution plating (9) or by fluorescence-activated cell sorting.

Fluorescence-activated cell sorting. The method used to analyze overproduction of DHFR in resistant cells is described in one of the accompanying papers (18).

Determination of MTX resistance and cell growth. Relative growth rates were determined by inoculating 10^5 cells into multiple T-25 flasks containing media with or without MTX, thymidine, and glycine. A Coulter Counter (Coulter Electronics) was used for all cell counts. On day 4 or 5, the cells were subcultured in an identical fashion, and this procedure was repeated thereafter such that cumulative growth curves could be constructed and compared after several weeks of continuous growth in log phase that was

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interrupted only by intermittent passaging of the cells. It is important to note that this determination of growth potential of cells was a composite of the effects of cell doubling time and plating efficiency.

Measurement of DHFR specific activity and relative rates of DHFR synthesis. DHFR specific activity in late-log-phase cells was determined as described in one of the accompanying papers (18). The relative rate of DHFR synthesis was measured as described previously (2) by direct immunoprecipitation of DHFR from extracts of early-log-phase cells which had been labeled for 45 min with 70 μCi of [^{3}H]leucine (New England Nuclear Corp.) per 4 ml of leucine-free medium in T-25 flasks. Results are expressed as percentages of immunoprecipitable counts compared with total trichloroacetic acid-precipitable counts of protein in a 100,000-×-*g* supernatant fraction.

Quantitation of DHFR gene sequences. Deoxyribonucleic acid (DNA) isolation and DHFR gene quantitation were performed essentially as described previously (1).

Karyotype analysis and in situ hybridizations. Measurements of DM chromosomes in fixed metaphase chromosome preparations were performed as described in one of the accompanying papers (7). In situ hybridizations were performed on metaphase chromosomes prepared by standard procedures (7), using a procedure provided by Richard Padgett, Stanford University. Slides were treated sequentially with 100 μg of ribonuclease per ml previously heated at 80°C for 10 min in 2× SSC (pH 7.0) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine (pH 8.0) (15), 50% (vol/vol) formamide (deionized formamide and 2× SSC, pH 7.0, at 70°C for 10 min); then the slides were dehydrated in an ethanol series (70, 80, 95, and 100% ethanol) and air dried. Hybridization with approximately 10^5 cpm of ^{125}I-labeled DHFR complementary DNA (8) that was prepared by nick-translation (25) with ^{125}I-labeled deoxycytidine triphosphate (New England Nuclear Corp.) and denatured at 100°C for 10 min was performed under a sealed cover slip at 42°C overnight in 50% deionized formamide containing 2× SSC (pH 7.0), 1× Denhardt solution (11), 25 mM Na_2HPO_4, 1.5 mM $Na_4P_2O_7$, 10% dextran sulfate (28), 10 μg of single-stranded, sheared salmon sperm DNA per ml, and 1 mM unlabeled 5'-iododeoxycytidine triphosphate. After hybridization, the slides were washed twice for 5 min each at 45°C in 5× SSC, 2× SSC, and 1× SSC and finally dehydrated in an ethanol series, as described above. The dried slides were then dipped in emulsion (Kodak NTB-2 diluted 1:1 with 2% glycerol), exposed for 3 days, and developed in Dektol. Slides were stained with Giemsa stain and photographed.

RESULTS

Loss of MTX resistance by S-180 cells when they were grown in the absence of MTX. The time course of loss of high rates of DHFR synthesis in the S-180 cell line AT-3000 (14) and in various clones derived from this cell line (2) has been described previously. In this work we characterized the loss in one particular clone, clone R_2. Figure 1 shows the loss of elevated rates of DHFR synthesis as a function of time of growth in the absence of MTX, and these data resembled data published previously (2). MTX resistance and elevated rates of DHFR synthesis were due to the acquisition of multiple copies of the DHFR gene, and the loss of MTX resistance was accompanied by a loss of the

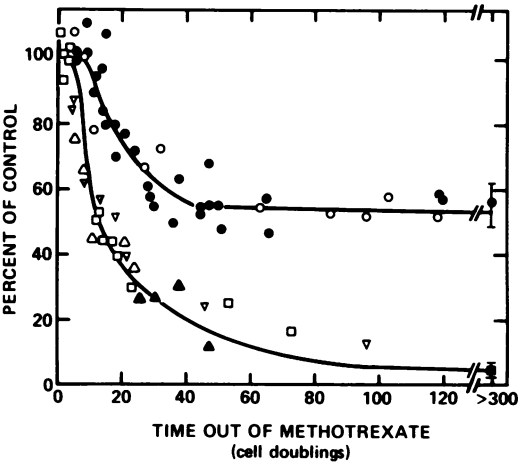

FIG. 1. *Loss of elevated DHFR levels after growth of R_2, R_1A, and R_1B cells in the absence of MTX. The R_2, R_1A, and R_1B cell lines were grown for increasing numbers of cell doublings in the absence of MTX. Periodically, samples were taken to determine DHFR specific activity (closed symbols) and relative rates of DHFR synthesis (open symbols). Results are expressed as percentages of the control values determined with a culture grown in the presence of MTX. Symbols: □ and ■, R_1A cells studied more than 3 years previously (2); △ and ▲, R_1A cells frozen for 2 years before this experiment; ○ and ●, R_1B cells derived from the original R_1A line by continuous growth in the presence of MTX for 2 years. The values representing growth in the absence of MTX, thymidine, and glycine for more than 300 cell doublings represent the averages of multiple determinations, and the brackets indicate the averages of these values. The respective average values for DHFR specific activity and relative rate of DHFR synthesis were 1,580 U/mg of soluble protein and 4.3% of the soluble protein for the R_1B cell line and 1,360 U/mg of soluble protein and 8.9% of the soluble protein for the recently thawed R_1A cell line. The R_1A and R_1B cells had comparable DHFR specific activities. After short- and long-term labeling with [^{3}H]leucine, the relative rates of synthesis and the steady-state amounts of DHFR were determined by direct immunoprecipitation (2), and both were twofold greater in R_1A cells than in R_1B cells. Since this indicated that the turnover of DHFR in the two lines was similar, we ascribed the activity-immunoprecipitability discrepancy to the presence of an altered DHFR in the R_1A cell line (i.e., cross-reactive material).*

extra DHFR gene copies. We analyzed populations of MTX-resistant S-180 cells during the process of loss of amplified DHFR genes by using the fluorescence-activated cell sorter technique (17). Figure 2a shows the distribution of fluorescence in R_2 cells grown in medium containing 50 μM MTX. Although the population had a mean fluorescence intensity of 90 fluorescein units (FU) per cell, the population was skewed, with some cells having as many as 1,400 FU/cell. Figure 2b shows the R_2 population which was grown in the absence of MTX for 50 cell doublings; the mean DHFR content of this population decreased to 30% of the original value. Sensitive cells contained a mean of 0.8 FU/cell, and essentially no cells contained more than 1.5 FU/cell. During long-term growth in the absence of MTX, the R_2 cells generated a population of cells containing a peak of 2 FU/cell (data not shown). This peak did not overlap the fluorescence distribution of sensitive cells, and no cells with parental DHFR levels were detected.

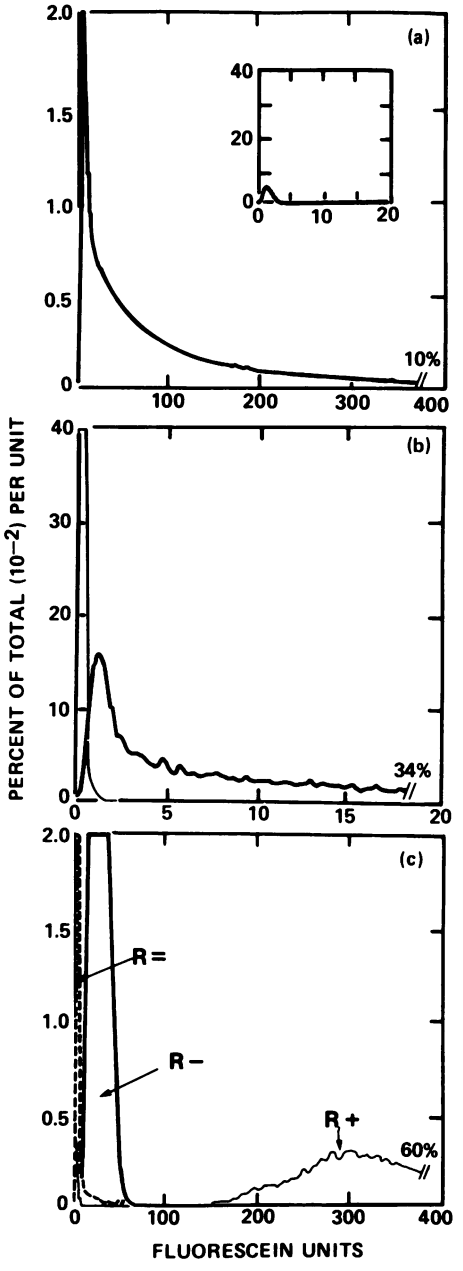

FIG. 2. *Distribution of DHFR levels in cells of the R_2 clone. Cellular DHFR in mid-log phase cells was saturated with the fluorescein conjugate of MTX, and the cells were prepared and analyzed for fluorescence intensity by using the fluorescence-activated cell sorter technique, as described in the text. (a) Original R_2 clone grown in the absence of MTX for 4 days in order to permit saturation of DHFR with the fluorescein conjugate of MTX. The inset shows the lack of cells with fluorescence between 0 and 20 FU/cell. A total of 10% of the cells had more than 400 FU/cell, and some cells had up to 1,500 FU/cell. (b) Fluorescence distribution in R_2 partial revertant cells (thick line) which were grown for approximately 50 cell doublings in the absence of MTX. Some cells in this population had up to 100 FU/cell. Note the change in the ordinate and abscissa. Also shown is the fluorescence in the parental MTX-sensitive cloned line, S_3 (thin line). (c) The cells in (a) were sorted sterilely to obtain three subpopulations, which were analyzed immediately after the cell sorting. These three sorted subpopulations contained high degrees of fluorescence (R_2+ cells), moderate degrees of fluorescence (R_2− cells), and very low levels of fluorescence (R_2= cells).*

Growth of S-180 cells as a function of DHFR gene copy number. An important question related to the mechanism of the loss of MTX resistance in the unstably amplified R_2 cell line was whether there was a selective growth advantage for cells with lower degrees of DHFR gene amplification. We examined this question by measuring the growth rates of R_2 cells, as well as subpopulations derived from the MTX-resistant R_2 population (Fig. 2c). Figure 3a shows that the DHFR level was inversely related to the growth rate when cells were grown in the absence of MTX. Figure 3b compares growth potential in the absence of MTX with the mean number of FU per cell. Figure 3b also shows the results obtained with the MTX-sensitive S_3 cell line, the three sorted subpopulations of the R_2 cell line, and the two populations of cells that underwent different degrees of partial reversion toward sensitivity as a result of growth in the absence of MTX for different times. Although the relationship was not simple

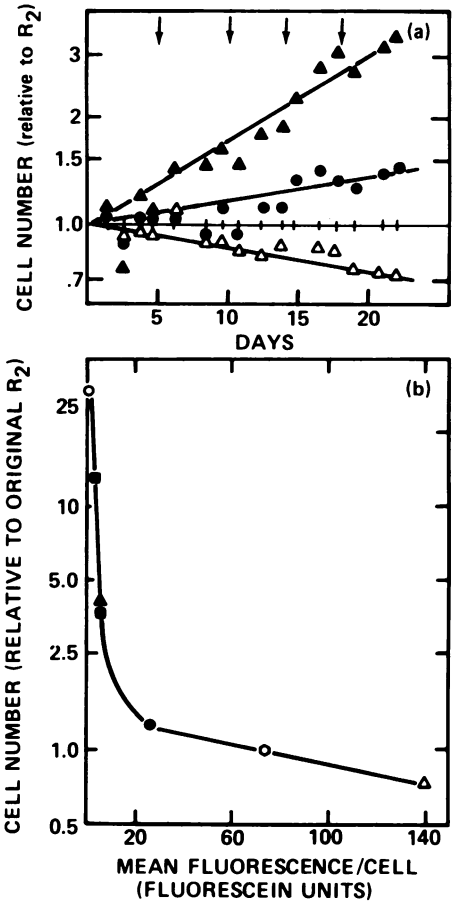

FIG. 3. *Growth rate in the absence of MTX as a function of the mean number of FU per cell. A total of 10^5 cells were plated into multiple 25-cm^2 flasks containing medium lacking MTX, and after different times the cell number was determined as described in the text. To maintain the cells in the logarithmic phase of growth, the cells were subcultured every 4 days (arrows), and the cells from two flasks were pooled and inoculated into new 25-cm^2 flasks (10^5 cells per flask). Cumulative cell numbers were determined from duplicate flasks, averaged, and divided by the initial cell number. (a) Log of the cell number measured from duplicate flasks as a function of time for the three sorted subpopulations shown in Fig. 2c. All values were normalized to the original R_2 cells, which had a doubling time of 23 h in the absence of MTX. (b) Relative growth rates determined from (a) related to the numbers of FU per cell. To determine the relative growth rates, the slopes of the lines in (a) were measured. To determine mean fluorescence values, binding of the fluorescein conjugate of MTX and fluorescence analysis were performed before and after the growth experiment, and the two sets of values were averaged to give the values shown here. In addition to the four lines shown in (a), two partial revertant populations were also studied. Symbols: ▲, R=; ●, R−; △, R_2+; ○, R_2; ○, S_3; ■, partial revertants.*

or strictly linear, there was an inverse relationship between growth potential in the absence of MTX and the mean number of FU per cell. We concluded for the R_2 cells that DHFR gene amplification affected growth potential. Thus, the loss of MTX resistance involved, in part, a growth selection for those cells with lower degrees of DHFR gene amplification on the basis of different growth rates.

Loss and gain of DHFR in sorted subpopulations and individual clones. We studied changes in DHFR levels in individual cells within the sorted R_2 subpopulations during growth in the absence and presence of MTX to determine the dynamics of loss and gain of DHFR genes. Figure 4 shows representative results for the R+, R−, and R= subpopulations of R_2 analyzed after 2, 7, and 25 cell doublings in MTX-free medium. Figure 2c shows the original distribution of these subpopulations. These subpopulations contained the following mean numbers of DHFR genes compared with S_3 cells: R+, 150; R−, 15; R=, 3. In the cells with high DHFR levels (Fig. 4a through c, thin lines), the DHFR content per cell declined rapidly, and after 25 cell doublings the majority of cells had low DHFR contents; however, a small number of cells had very high DHFR contents. Cells sorted from the middle of the R_2 population (Fig. 4a through c, thick lines) showed an initial randomization with respect to DHFR content per cell, with cells having higher and lower DHFR contents than the original sorted subpopulations. After additional growth the population displayed a progressively skewed population similar to the cells sorted with high DHFR levels. Cells sorted with low DHFR contents showed very little change in distribution (data not shown).

These populations were also analyzed after growth in the presence of 50 μM MTX (the concentration in which they were maintained before sorting). Figure 4d shows the fluorescein graphs of the R+ cells (thin line) and the R− cells (thick line) after 25 cell doublings in medium containing 50 μM MTX. The fluorescence of the R+ cells decreased somewhat (compare with Fig. 2c), and these cells constitute a population similar to the original R_2 population (Fig. 2a). The R− subpopulation contained many more cells with higher DHFR levels than were present in the initial subpopulation and was indistinguishable from the population of R+ cells. Taken together, the data in Fig. 4 suggested that the daughter cells of individual cells could both gain and lose DHFR genes.

In experiments not shown here, we also cloned individual cells with varying degrees of fluorescence and found that upon growth in the absence

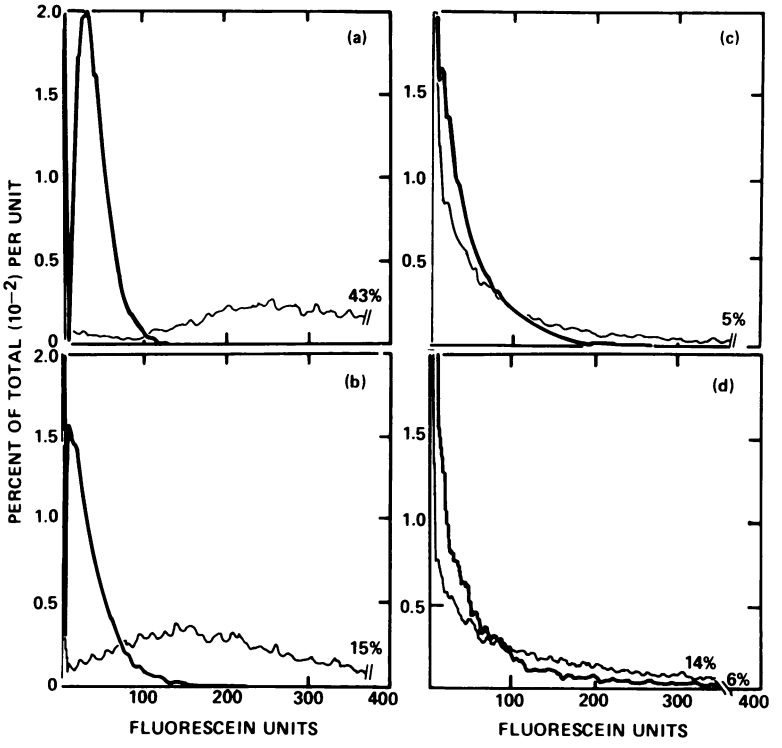

FIG. 4. *Changes in the distribution of DHFR content in unstably resistant R_2 subpopulations when they were grown in the absence and presence of MTX. Cells sorted from the original R_2 population (Fig. 2c) were grown in the absence of MTX, thymidine, and glycine for increasing times. These cells were analyzed for DHFR contents after 2 (a), 7 (b), and 25 (c) cell doublings in the absence of MTX and after 25 cell doublings in the presence of MTX, thymidine, and glycine (d). The thin and thick lines represent cells sorted for high (R+) and intermediate (R−) DHFR contents, respectively (see Fig. 2c).*

of MTX for short time periods, the progeny behaved as the populations studied in the experiments shown in Fig. 4 (R. J. Kaufman, Ph.D. thesis, Stanford University, Stanford, Calif., 1981). Thus, cloned cells also became heterogeneous rapidly, with some progeny gaining fluorescence and some losing fluorescence.

We concluded from the subpopulation sorting experiments and the clonal analyses that individual cells could give rise to progeny that could either lose or gain DHFR genes and that the initial "randomization" of genes could occur extremely rapidly in terms of cell doublings.

Stabilization of DHFR genes in an S-180 cell line. Figure 1 shows the time course of loss of high DHFR specific activity and the rates of DHFR synthesis in cloned cell line R_1 during growth in the absence of MTX. This cell line was subcloned from the original AT-3000 cells obtained from Hakala (14). This result was first reported in 1976 and was reproduced in these experiments (2). The reversion of this cell line followed the same time course as the reversion of the R_2 cell line. When the original R_1 cell line (designated R_1A) was propagated in the presence of 50 μM MTX continuously for approximately 2 years (now designated R_1B) and then examined for kinetics of loss of DHFR specific activity in the absence of MTX, it had become relatively stable. As Fig. 1 shows, this cell line eventually stabilized at a point where the DHFR specific activity was 50-fold greater than the DHFR specific activity of the parental MTX-sensitive cell line. Figure 1 also shows that when highly unstable R_1 cells which had been frozen for the previous 2 years were examined for instability, they showed the original rapid and extensive reversion. Thus, the change from instability to partial stability for elevated DHFR levels in the R_1 clone was due to a change in some property of the cells during the period of prolonged growth in the presence of MTX.

Figure 5 shows fluorescence graphs of the R_1 cells as they appeared originally (R_1A cells) (Fig. 5a), after propagation in MTX for 2 years (R_1B cells) (Fig. 5b), and after propagation in medium containing MTX for a total of 3 years (R_1C cells) (Fig. 5c). Approximately 10% of the highly un-

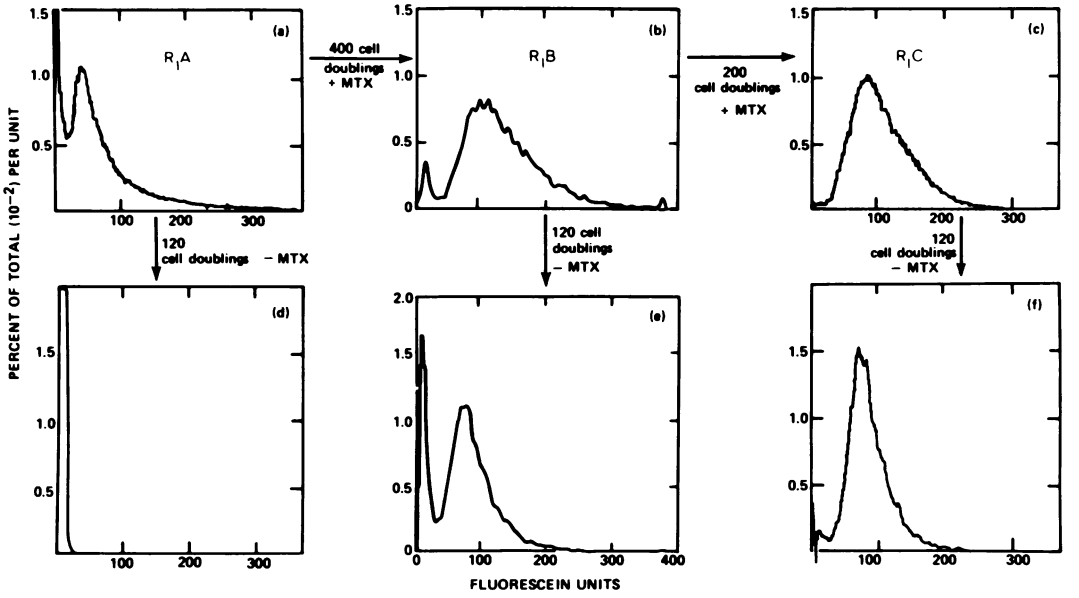

FIG. 5. *Distribution of DHFR in cells of the R_1 line that became stably MTX resistant. Cells were saturated with the fluorescein conjugate of MTX and were analyzed by the fluorescence-activated cell sorter technique. (a) Distribution of fluorescence in the R_1A population which was thawed recently. (b) Fluorescence distribution in R_1A cells after 2 years of growth in the presence of MTX, at which time the population was partially stably resistant and was designated R_1B. After 1 year of growth in the presence of MTX, this line had the fluorescence distribution shown in (c) and was designated R_1C. Stable partial revertants of these lines were generated by growth in the absence of MTX for 120 cell doublings. The fluorescence distributions of these populations are shown in (d) through (f).*

stable R_1A cells contained a mean of 5 FU/cell, and the remaining cells were heterogeneous, with a mean fluorescence of approximately 50 FU/cell. When this population was grown for 120 cell doublings in the absence of MTX, it gave rise to a population in which all of the cells contained approximately 5 FU/cell (Fig. 5A); this was discretely different from the results with sensitive cells. Further growth of these cells in MTX-free medium produced no further loss of fluorescence.

The partially stable MTX-resistant R_1B population (Fig. 1 and 5b) produced a complex fluorescence graph. Compared with the R_1A cells, the average number of FU per cell was higher (140 Fu/cell), and there was a smaller percentage of cells (5%) in the peak with 5 FU/cell. During growth in the absence of MTX, two peaks of cells containing 15 and 80 FU/cell appeared; these peaks comprised 25 and 75% of the population, respectively. After further growth (300 cell doublings) there was no change in the percentages of cells in the two peaks. This suggested that one population of cells did not have a selective growth advantage over the other when they were grown in the absence of MTX. Indeed, in studies similar to those shown in Fig. 3, we found that both of these cell populations grew as rapidly as sensitive cells despite the large number of DHFR genes per cell.

After 3 years of growth in the presence of 50 μM MTX, the R_1C line showed a cell distribution with a mean of 80 FU/cell, and this line lacked the lower peak of cells (Fig. 5c). The R_1C cells showed a minimal loss of fluorescence intensity when they were grown in the absence of MTX, and they gave rise to very few cells containing 15 FU/cell. Thus, after a longer period of growth in the presence of MTX, the population stabilized its elevated levels of DHFR. It was important to determine whether the R_1B population (Fig. 5b) contained two subpopulations with high DHFR levels, one able to revert to the low-level peak (15 FU/cell) and one having the capability of stabilization at the high level (80 FU/cell). This was examined by cloning cells containing more than 280 FU/cell from the original R_1B population (data not shown). A total of 16 individual clones were grown in the absence of MTX and analyzed by the fluorescence-activated cell sorter technique 20 and 80 cell doublings later. All clones progressively gave rise to the two populations with high and intermediate DHFR contents. However, the percentage of cells containing 15 FU/cell varied from 0.5 to 80% of the cells in different clones. Thus, indi-

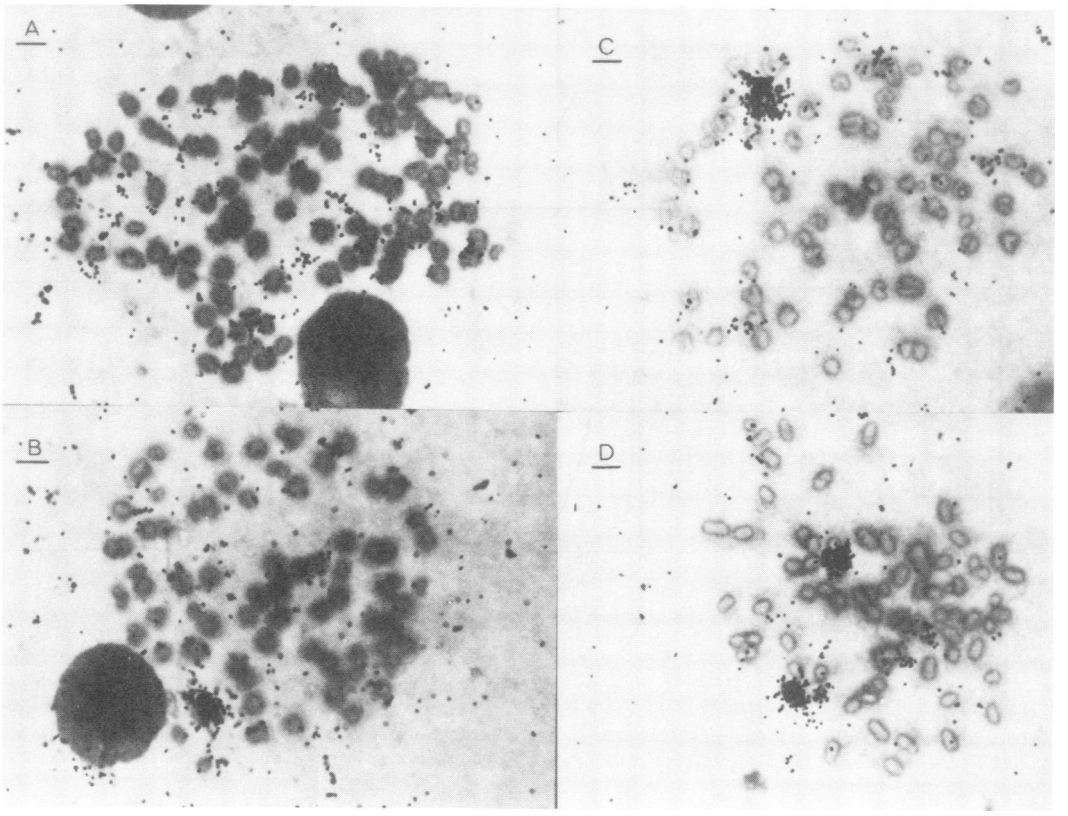

FIG. 6. *Chromosomal locations of amplified DHFR sequences in unstable (R_1A) and stable (R_1C) cells. In situ hybridizations with ^{125}I-labeled, cloned DHFR complementary DNA were performed with R_1A and R_1C metaphase chromosome preparations. Slides were scored for the presence and number of demonstrable grain clusters in metaphase chromosome spreads. The numbers of metaphase spreads examined were 95 and 138 for R_1A and R_1C cells, respectively. DM counts were made from ethidium bromide-stained slides which were prepared in parallel with the slides used for the in situ hybridizations, and at least 60 metaphase spreads of each cell type were examined. (A) and (B) R_1A, scored as having zero and one cluster, respectively. (C) and (D) R_1C, scored as having one and two grain clusters; respectively.*

vidual cells lost some DHFR genes and could give rise to cells containing either 15 or 80 FU/cell. However, cells containing 15 or 80 FU/cell were stable and did not give rise to the other cell populations.

Localization of DHFR genes in stably amplified S-180 cells. Using nucleic acid hybridization techniques, we estimated that cells containing 15 FU/cell had approximately 10 to 15 copies of the DHFR gene and that cells containing 80 FU/cell had approximately 50 copies of the DHFR gene compared with sensitive cells. Previously, we reported that in the R_1C cell line (i.e., in cells containing 50 DHFR genes in a stable state) the DHFR genes are present on chromosomes. These studies involved sedimenting metaphase chromosomes on a sucrose gradient and showing that in R_1C cells the DHFR genes were present in a fraction containing relatively large chromosomes, whereas unstable R_2 cells contained DHFR genes in a sedimentation fraction enriched in DMs (17).

Next, we examined the R_1C cell line for the localization of DHFR genes by in situ hybridization, using a complementary DNA clone (8). As Fig. 6 shows, in this stably amplified cell line the DHFR genes were clustered on one or several chromosomes. In contrast, in situ hybridization with R_1A cells produced few metaphase spreads with evidence of clustering of silver grains. Because of the nature of the background in these in situ hybridizations, as well as the fact that any individual DM contains at most a few DHFR genes (7), we were not able to determine the localization of DHFR genes on DMs by this method. Table 1 provides data concerning the distribution of DMs and shows the localization of DHFR genes in R_1A and R_1C cells. In the unstably amplified R_1A cell line, DMs were present in virtually all cells, and as discussed previ-

TABLE 1. *Distribution of DMs and DHFR gene clusters in S-180 R_1A and R_1C cell lines*[a]

Cells	% of cells with no. of DMs per cell:						% of cells with no. of grain clusters per cell:			
	0	1-10	11-20	21-30	31-40	>40	0	1	2	3
R_1A	29	33	10	8	3	16	61	30	8	0
R_1C	75	17	3	3	0	0	7	35	51	5

[a] R_1A and R_1C cells were examined for the presence of DMs and the number of DHFR gene clusters. For the examination of DMs, we used metaphase spreads from 91 R_1A cells and 62 R_1C cells. For the gene cluster analyses we examined metaphase spreads from 138 R_1A cells and 95 R_1C cells.

ously (17), the number of DMs was related to the degree of DHFR gene amplification. In an occasional metaphase spread, it appeared that there was a small cluster of DHFR genes as determined by in situ hybridization; when these cells reverted, the amount of fluorescence per cell was approximately three to five times that of sensitive cells (cell line S_3), which indicated a low level of amplified (stable) DHFR genes. In contrast, the percentage of R_1C cells that had detectable DMs was small. What was most interesting was that metaphase spreads of these stably resistant cells contained from one to three chromosomes with clustered DHFR genes.

DISCUSSION

Unstable MTX resistance and DMs. We have presented evidence that unstably amplified DHFR genes in 3T6-R50 (7) and S-180 R_2 (17) cells are localized to DMs. DMs are self-replicating, extrachromosomal elements (4, 23) which lack centromeric regions (4, 19) and thus are incapable of interaction with the mitotic apparatus. As a result, DMs can be partitioned randomly into daughter cells at mitosis. This process results in the generation of populations of cells containing heterogeneous numbers of DHFR genes and DMs (Fig. 2) (7), as well as the rapid generation of heterogeneous cell populations from cells sorted for a given amount of fluorescence per cell (Fig. 4) and individual cloned cells (Kaufman, Ph.D. thesis). Thus, during growth in media containing increasing MTX concentrations, daughter cells which receive greater numbers of DMs and associated DHFR genes acquire a selective growth advantage. Once DMs are generated initially, it is apparent why subsequent selections for higher MTX resistance can be accomplished rapidly. When cells are grown in the absence of MTX, random segregation of DMs also occurs, but the growth advantage due to the presence of DMs is reversed, and for reasons about which we can only speculate, cells containing fewer DHFR genes proliferate more rapidly (Fig. 3) and eventually predominate in the cell population. The growth differential of resistant and revertant populations apparently does not result from an imbalance in intracellular metabolites resulting from elevated DHFR levels, inasmuch as the stable derivative of cell line S-180 (R_1C) grows at a rate similar to the rate of sensitive cells (data not shown). The effect of DMs on the growth rate is perhaps analogous to the retardation of the growth rate in rye (3, 16) and maize (21) that results from increased numbers of B-chromosomes in these plant species. Levan et al. (20) have described a mouse ascites tumor line (SEWA cells) which contains DMs when the cells are grown in a mouse but loses DMs when the cells are grown in culture medium. This is analogous to the growth of S-180 cells with and without MTX and suggests that there is a selective advantage due to certain DNA sequence amplifications when the SEWA cells are grown in an animal. It remains to be determined what, if any, gene product is present in such cells that might impart a growth advantage in an animal vis a vis cell culture medium.

Rapid and sometimes single-step losses of DHFR genes might result from the generation of so-called micronuclei at mitosis, where multiple numbers of extrachromosomal elements may aggregate and nuclear membranes may reassemble about such aggregates (20). Indeed, numerous micronuclei have been observed in our unstably resistant cell lines, and preliminary data in cell line 3T3-R500 (7) have indicated that DNA isolated from such micronuclei is enriched in DHFR sequences (J. Deschatrette, unpublished data). Thus, if such micronuclei failed to undergo replication, fused with lysosomes, or were extruded from cells, large step losses of amplified genes could occur.

Biedler et al. (4a) have found that certain Chinese hamster lung cell lines with HSRs progressively lose MTX resistance when they are grown in the absence of MTX and that the cells which emerge have HSRs that are reduced in length, suggesting the possibility that genes in an HSR configuration (i.e., on a chromosome) may be excised and lost. However, the kinetics of loss of MTX resistance in these cell lines are much slower than the 50% loss in 20 cell doublings which we have observed in unstably amplified cell lines, including newly selected Chinese hamster ovary cells. These results, as well as our own with S-180 R_1C (Fig. 5c), show that the property of stability appears to be a relative term which is defined by the rate at which cells revert towards drug sensitivity.

Stabilization of DHFR genes with continued MTX selection. Cells which have been

selected recently for resistance to MTX are unstable, and amplified DHFR genes are lost during growth in MTX-free medium. This was observed in Chinese hamster ovary cells (18). As cells are grown further at a single concentration of MTX, cell populations with stable resistance emerge. Chinese hamster cells maintained for as short a time as 100 additional cell doublings after the initial selection no longer revert when they are grown in the absence of MTX (18). In contrast, S-180 cells have maintained the unstable phenotype over many years. Alt et al. (2) subcloned resistant S-180 cells which were developed by Hakala et al. (14). These clones (R_1 and R_2) were grown for 2 additional years, which resulted in the emergence of the stable R_1C population and the continuation of instability in the R_2 subclone. Our studies with S-180 and Chinese hamster ovary cells indicated that there were considerable differences both in the propensity and in the timing of stabilization of amplified DHFR genes.

The emergence of stably amplified DHFR genes after long periods of growth in MTX was illustrated by the S-180 R_1 clone described here. When first studied, these cells were unstable and contained large numbers of DMs. After 3 years of continuous growth in medium containing MTX, they became stably resistant and lost most, if not all, DMs. Instead, they acquired DHFR genes which were clustered on one, two, or three chromosomes (Fig. 6). It is not known whether such clustering of DHFR genes on multiple chromosomes in S-180 cells is similar to the clustering of DHFR genes on a HSRs of specific chromosomes in Chinese hamster ovary cells (21) and mouse L5178Y cells (12). HSRs in MTX-resistant cell lines were first reported by Biedler and Spengler (5), who suggested such regions were sites of DHFR gene amplification in Chinese hamster lung cells. In the Chinese hamster cell line the HSR is characteristically on the long arm of chromosome 2 (4a, 21), is a region that lacks normal trypsin-Giemsa bands, and is flanked by banding patterns of the normal chromosome both proximal and distal to the HSR. In preliminary studies, Roberts et al. (M. Roberts, K. M. Huttner, R. T. Schimke, and F. H. Ruddle, J. Cell Biol. **87**:288a, 1980) localized the unamplified DHFR gene in the K_1 Chinese hamster ovary cell line from which the MTX-resistant MK_{42} cell line was derived to the long arm of chromosome 2. This suggests that the amplified genes are localized to the site of the resident gene in this cell line.

In contrast to such HSRs, the chromosomal abnormality reported by Bostock et al. (6) in an MTX-resistant mouse melanoma cell line has as many as three to five large, abnormal chromosomes containing regions of alternating satellite and nonsatellite DNAs. These structures bear no relationship to chromosomes with normal banding patterns. Levan et al. (20) have observed in the mouse SEWA cell line the occasional appearance of a chromosome structure which they designated "C-minus chromosome" because it lacks centromeric staining regions. These workers, as well as Cowell (10), have suggested that DMs may occasionally assemble to form such chromosome-like structures. This may explain the generation of stable DHFR genes in the S-180 cells which we studied (in particular, if assembled DMs were associated with one or more chromosomes bearing a centromer). Bostock et al. (6) suggested that the presence of satellite DNA in their MTX-resistant melanoma cell line can facilitate unequal crossing over to generate amplified DNA sequences. The ubiquitous changing nature of repeated DNA sequences in cultured cells (24) suggests the possibility that these highly repetitious elements may also facilitate the association of DMs into chromosome-like structures. The presence of either DMs or abnormal chromosomal structures in human tumor cell lines (4) may be another example of this phenomenon.

Possible mechanisms for the amplification process have been discussed elsewhere (26, 27).

ACKNOWLEDGMENTS

We thank Len Herzenberg and Gene Filson for the use of and assistance in the use of a FACS II instrument, Phillip Hanawalt for the use of a Coulter Counter, Debra Pittman and Brian Mariani for the synthesis of the fluorescein conjugate of MTX, and Sharon Dana for assistance in cell culturing.

This research was supported by Public Health Service research grant CA 16318 from the National Cancer Institute. P.C.B. is a recipient of a postdoctoral fellowship from the Damon Ruyon-Walter Winchell Cancer Fund.

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