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Relationship of Amplified Dihydrofolate Reductase Genes to Double Minute Chromosomes in Unstably Resistant Mouse Fibroblast Cell Lines

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Murine 3T6 cells selected in increasing concentrations of methotrexate were unstable with respect to dihydrofolate reductase overproduction and methotrexate resistance when they are cultured in the absence of methotrexate. An analysis of the karyotypes of these resistant cells revealed the presence of numerous double minute chromosomes. We observed essentially identical kinetics of loss of dihydrofolate reductase gene sequences in total deoxyribonucleic acid and in deoxyribonucleic acid from fractions enriched in double minute chromosomes and in the numbers of double minute chromosomes per cell during reversion to methotrexate sensitivity, and this suggested that unstably amplified gene sequences were localized on double minute chromosomes. This conclusion was also supported by an analysis of cell populations sorted according to dihydrofolate reductase enzyme contents, in which relative gene amplification and double minute chromosome content were related proportionally.

Double minute chromosomes (DMs) are small, often paired, extrachromosomal elements which have been found in a wide variety of permanent cell lines of human (1, 2, 24) and mouse (8, 18) origin, as well as in biopsied tumor tissue of human origin (2, 19, 25). Although no functions were assigned to DMs in the above-cited studies, the persistence of these chromosomes in permanent cell lines and tumors suggests that they confer a selective advantage to cells. Previously, we reported the occurrence of DMs in cells which were selected for high levels of resistance to methotrexate (MTX). In mouse cell lines S-180 and L5178Y, the existence of DMs is associated with unstable MTX resistance, and we have found preliminary evidence which suggests that the DMs contain amplified dihydrofolate reductase (DHFR) genes (13).

In this work we extended our studies to two MTX-resistant murine fibroblast cell lines derived from 3T6 and 3T3 cells. We present additional data which substantiate the localization of unstably amplified DHFR genes to DMs.

MATERIALS AND METHODS

Cell and culture conditions. Parental NIH Swiss 3T3 and 3T6 cells and derivative lines resistant to MTX were generous gifts from Vera Morhenn. The resistant cells were obtained by sequential selections in media containing increasing concentrations of MTX (16). Cells were grown in Dulbecco modified Eagle medium supplemented with either 5% (3T6 cells) or 10% (3T3 cells) dialyzed fetal calf serum and, for resistant cells, 50 μM (3T6-R50 cells) or 500 μM (3T3-R500 cells) MTX. The resistant cells were propagated continuously for no more than 6 months at the final concentration of MTX before the experiments described below were initiated. Clones of 3T6-R50 cells were obtained by dilution plating in Dulbecco modified Eagle medium supplemented with 10% dialyzed fetal calf serum, 2 mM glutamine, and 1 mM sodium pyruvate. The doubling times of the 3T6, 3T6-R50, and 3T3-R500 cells were approximately 16, 18, and 24 h, respectively. Cells were counted with a hemacytometer. Periodic examinations for the presence of mycoplasma (7) were consistently negative.

Labeling of cells with the fluorescein conjugate of MTX. Labeling of cells with the fluoresceinated derivative of MTX and analysis by the fluorescence-activated cell sorting technique were performed as previously described (12).

DNA extraction and quantitation by fluorimetric assay. To extract total deoxyribonucleic acid (DNA), whole cells or nuclei (21) were lysed in TENS [10 mM tris(hydroxymethyl)aminomethane chloride, pH 8.0, 10 mM NaCl, 1 mM ethylenediaminetetraacetate, 0.5% sodium dodecyl sulfate], digested with 50 μg of proteinase K (Beckman) per ml for 2 to 4 h at 37°C, made 1 M in $NaClO_4$ with 50 mM tris(hydroxymethyl)aminomethane chloride (pH 7.5), extracted twice with redistilled phenol-chloroform [1:1; saturated with 50 mM tris(hydroxmethyl)aminomethane, pH 7.1] and twice with chloroform alone, and precipitated with 2.5 volumes of ethanol. The DNA was rehydrated in TEN (TENS without sodium dodecyl sulfate), and 20 μg of ribonuclease A per ml previously heated to 80°C for 10 min was added; then

the preparation was incubated for 1 h at 37°C, digested with proteinase K, and extracted as described above, except that 100 mM NaCl was substituted for 1 M $NaClO_4$.

DNA was extracted from the pelleted DM fraction in 0.4 ml of TENS by digestion in proteinase K, extracted, and ethanol precipitated as described above. The DNA was quantitated by a fluorimetric assay in which Hoechst 33258 stain (5) was substituted for 4',6-diamidino-2-phenylindole·2HCl (DAPI) stain (11). DNA (2 to 50 ng) was added with blending in a Vortex mixer to TEN containing 5 ng of Hoechst 33258 stain per ml (total volume, 2 ml). Fluorescence was determined by using a Perkins-Elmer model 512 spectrophotometer (excitation wavelength 360 nm; emission wavelength 454 nm). A standard curve was constructed by using purified cell line 3T6 DNA that had been quantitated previously spectrophotometrically (1 U of absorbance at 260 nm = 50 μg/ml), and this curve was used to estimate the DNA concentrations in experimental samples. This method was extremely economical, was not affected by any contaminating ribonucleic acid, and was accurate to within 20% of the DNA concentrations estimated by standard spectrophotometric determinations.

Gel blotting and quantitation of DHFR genes. Gel electrophoresis, Southern blots, and hybridization with ^{32}P-labeled pDHFR-11 were performed essentially as described by Kaufman and Schimke (15), except that we included 10% dextran sulfate (Sigma Chemical Co.) (27), 10 μg of polyuridylic acid per ml, and 10 μg of polycytidylic acid per ml (Collaborative Research, Inc.). DNA filters were prepared as described by McGrogan et al. (20). Filter disks were counted by liquid scintillation spectrometry. The data for filter disk hybridizations are expressed as counts per minute per microgram of filter-bound DNA, and these values have been corrected for hybridization to equivalent amounts of sensitive cell DNA. Each value, except where indicated, represents the average from three filter disks, each of which contained a different amount of DNA.

Karyotype analysis and visualization of chromosomes. Metaphase cells were accumulated by brief colcemid arrest (0.06 μg/ml) and were prepared by standard procedures. Determinations of DMs were made on ethidium bromide-stained metaphase spreads (1 μg of ethidium bromide per ml in 1× SSC [0.15 M NaCl plus 0.015 sodium citrate], mounted in 50% glycerol in 1× SSC), and these preparations were viewed under rhodamine excitation conditions (excitation wavelength, 545 nm) with an Olympus series BH microscope with vertical fluorescence illumination and a 60× objective lens. Hoechst 33258-stained preparations were prepared as described previously (17) and were viewed under ultraviolet excitation conditions (excitation wavelength, 365 nm). Photography was with Kodak Tri-X film, which was developed with Accufine at a total magnification of 360.

RESULTS

Instability of DHFR overproduction in 3T6 cells. We used the fluorescence-activated cell sorter technique and the fluorescein conjugate of MTX to determine the stability of DHFR overproduction in a mouse 3T6 fibroblast cell line selected for resistance to 50 μM MTX (cell line 3T6-R50). Initially, the resistant population contained a small percentage of cells with a fluorescence distribution that was characteristic of sensitive cells (1 to 2 fluorescein units [FU] per cell), whereas the remaining cells showed a heterogeneous pattern of fluorescence, with 91% of cells containing more FU than sensitive cells (Fig. 1A). During growth in MTX-free medium, the percentages of cells with high DHFR levels decreased to 71% (Fig. 1B), 45% (Fig. 1C), and 22% (Fig. 1D) after 17, 34, and 47 cell doublings, respectively. The mean fluorescence value of the cells with fluorescence values greater than sensitive-cell fluorescence values decreased only slightly during growth in the absence of MTX, decreasing from 16.4 FU/cell (Fig. 1A) to 13.2 FU/cell after 47 cell doublings in medium without MTX (Fig. 1D). An examination of 10 independently selected 3T6-R50 clones showed essentially identical kinetics of loss of DHFR levels (data not shown). That reduced fluorescence in reverting populations reflected decreasing resistance to MTX was shown by the reduced growth rates of revertant populations when they were plated and grown in medium containing 50 μM MTX (data not shown).

DMs in resistant cell lines. Figure 2 shows metaphase chromosome spreads of 3T6-R50 and 3T3-R500 cells. DMs were recognizable in both types of spreads as small (0.5-μm), paired, extrachromosomal elements. No DMs were detected in parental 3T6 or 3T3 cell lines which were sensitive to MTX. Figure 2 shows the size heterogeneity of DMs both within a cell and between cell lines. Occasionally, single unpaired elements were observed, and these "single minutes" in 3T6-R50 cells may have represented up to 25% of the extrachromosomal elements. The number of DMs in the cells of each line varied from several to more than 100 per cell. The mean number of DMs in 3T6-R50 cells was 28 DMs per cell, and in 3T3-R500 cells this value was more than 100 DMs per cell.

Based on the relative size of DMs compared cytologically with *Drosophila* chromosome IV from Schneider cell metaphase spreads, 3T3-R500 DMs appeared to contain approximately 10^3 kilobase pairs of DNA (data not shown). This estimate compared favorably with the DNA content estimated in DMs from a human breast cell line (3). By extrapolation from cytological examinations, 3T6-R50 DMs appeared to contain approximately 200 to 500 kilobase pairs of DNA.

Instability of DHFR gene amplification and DMs. To determine the extent of DHFR gene amplification, we compared serial dilutions

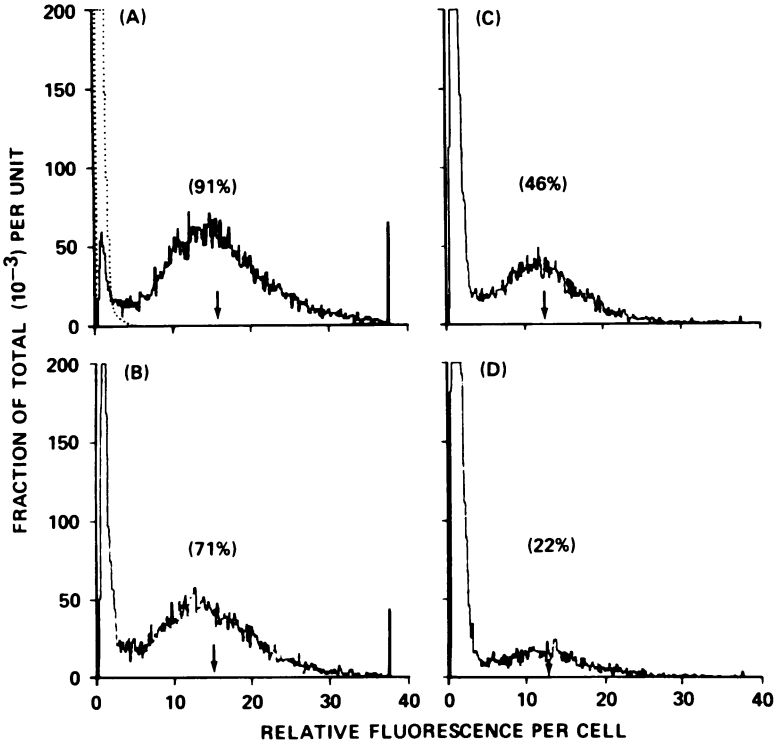

FIG. 1. *Loss of DHFR enzyme overproduction during growth in MTX-free media. Murine 3T6 cells resistant to 50 μM MTX were grown for different times in MTX-free medium, labeled with the fluorescein derivative of MTX, and analyzed by fluorescence-activated cell sorter technique (model FACSII). (A) Dotted line, 3T6 sensitive parental cells; solid line, resistant cells. (B) Cells grown for 17 generations without MTX. (C) Cells grown for 34 generations without MTX. (D) Cells grown for 47 generations without MTX. The values in parentheses are the percentages of cells with fluorescence levels greater than sensitive cell levels (4 to 40 FU), and the arrows indicate the mean of this group of cells.*

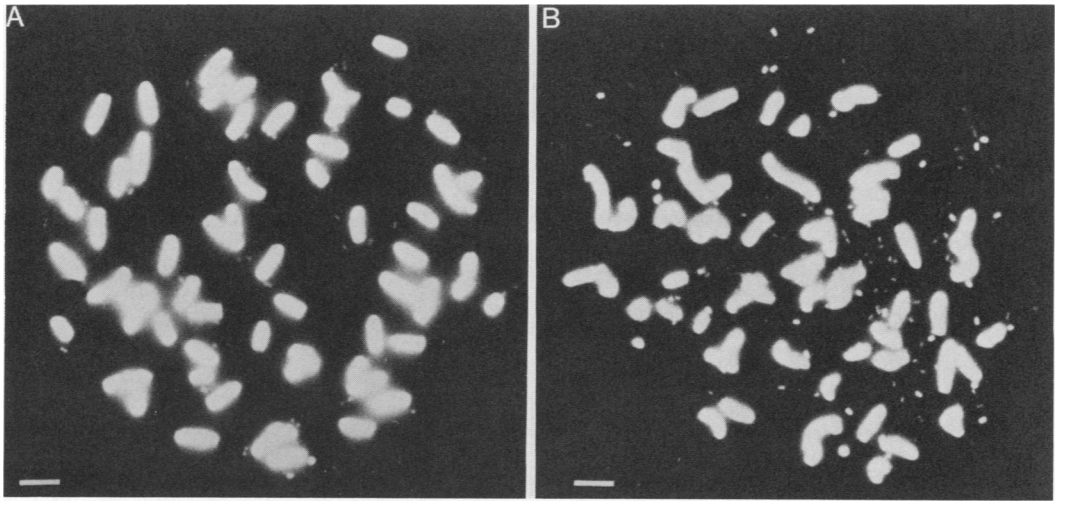

FIG. 2. *Metaphase chromosome spreads of MTX-resistant 3T6 and 3T3 cells. Metaphase spreads of 3T6-R50 and 3T3-R500 cells were stained with Hoechst 33258 stain and photographed. (A) 3T6-R50 cells. (B) 3T3-R500 cells. The chromosomes were overexposed intentionally to show the DMs more clearly. Bar = 5 μm.*

of 3T6-R50 DNA digested with *Eco*RI (Fig. 3, lanes 1 through 7) with MTX-sensitive 3T6 cellular DNA (lane 8) by the Southern technique (26). Each lane of the autoradiograph was scanned, and the grain density of the 3.5-kilobase fragment was used for comparison. From this analysis, 3T6-R50 cells appeared to have 40 to 50 DHFR gene copies, as compared with sensitive cells. The patterns of restriction fragments were similar in sensitive and resistant cells, and thus no gross rearrangement of the DHFR genes occurred with amplification; this was in keeping with previous findings with mouse S-180 cells (22). Figure 3 also shows the results with DNA samples from 3T6 cells that underwent reversion as described in the legends to Fig. 1 and 2 (lanes 9 through 11); and these data indicated that DHFR gene sequences were lost during the reversion process.

Figure 4 shows that there was a correlation between the loss of DHFR genes in total DNA and the number of DMs per cell in 3T6-R50 cells grown for different times in the absence of MTX.

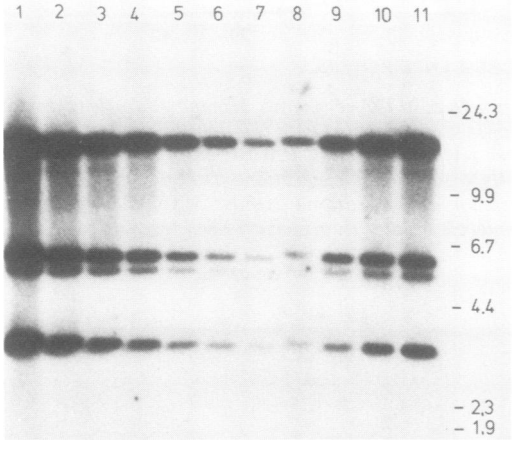

FIG. 3. *DHFR gene quantitation by Southern blot analysis. DNA was digested to completion with restriction endonuclease EcoRI, electrophoresed through a 0.5% agarose gel, transferred to a nitrocellulose filter, hybridized with cloned ^{32}P-labeled plasmid-bearing DHFR complementary DNA (6) (specific activity, 1.6×10^9 cpm/μg), and exposed to X-ray film. Lanes 1 through 7 contained the following serial dilutions of 3T6-R50 DNA: lane 1, 10 μg; lane 2, 5 μg; lane 3, 2.5 μg; lane 4, 1.3 μg; lane 5, 0.6 μg; lane 6, 0.3 μg; lane 7, 0.15 μg. Lane 8 contained 10 μg of parental sensitive 3T6 cellular DNA. Lanes 9 through 11 contained DNAs (10 μg/lane) from cells grown for different times in MTX-free medium (see Fig. 1 and 2). Lane 9, cells grown for 47 generations; lane 10, cells grown for 34 generations; lane 11, cells grown for 17 generations. The marker fragments used were HindIII digestion fragments of bacteriophage λ.*

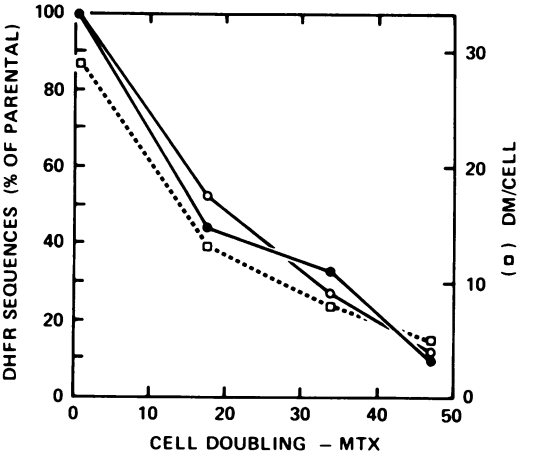

FIG. 4. *Losses of total and DM-associated DHFR sequences and DMs during growth in MTX-free medium. Relative DHFR gene abundances were determined by filter hybridization of total cellular DNA (○) and DNA from a filtrate of metaphase chromosomes passed through a 1-μm membrane filter (Nuclepore Corp.) (●) after growth for different times in the absence of MTX. Data are expressed as percentages of the appropriate parental resistant cell DNA samples (zero cell doublings without MTX). For total DNA this value was 490 cpm/μg and for DM-enriched DNA it was 9,500 cpm/μg (sensitive cell DNA control, 75 cpm/μg). DMs were counted in at least 75 randomly selected metaphase chromosome spreads at each point, and average values are shown.*

We also performed studies to attempt to isolate DMs by filtration (10) from preparations of metaphase chromosomes (4) during reversion and attained 15- to 27-fold enrichment of DHFR sequences compared with total DNA from each sample (25a). Furthermore, such preparations were enriched in DMs cytologically. Somewhat less enrichment (10- to 15-fold) of DHFR sequences was achieved when we used differential centrifugation of metaphase chromosomes (3). Although these DM preparations clearly were not pure (our estimates of purity would have required more than a 100-fold enrichment of DHFR genes), they showed that amplified DHFR sequences were enriched in chromsome fractions containing DMs. Figure 4 also shows data for DM-associated DHFR sequences from filtered DM fractions during reversion, and these data showed good correlation with the loss of DHFR sequences in total DNA and with the decrease in numbers of DMs per cell.

Analysis of resistant cell populations sorted for DHFR enzyme content. To establish the relationship between DM content and DHFR gene amplification further, we used a second approach. 3T6-R50 cells were labeled with the fluorescein conjugate of MTX and

sorted according to fluorescence into the four fractions shown in Fig. 5A. Cells from each fraction were grown for an additional two cell doublings, and DM determinations were made from metaphase spreads. In an independent experiment 3T6-R50 cells were grown for 3 months in the absence of MTX, and the cells with 0 to 4 FU/cell were sorted from this population. No DMs were detected in this population, whereas several DMs (more than 20) were present in the cells containing between 15 and 40 FU/cell (data not shown).

DNA was isolated from the four sorted cell populations (Fig. 5) and analyzed for relative DHFR sequence abundance by filter hybridization. These data, along with the average DM determinations and mean fluorescence values, are plotted in Figure 5B for each fraction of sorted cells. There was a high correlation among relative DHFR gene copy number, the DM content per cell, and the DHFR enzyme content (i.e., fluorescence per cell).

DISCUSSION

Our previous evidence that suggested an association of DHFR genes with DMs came primarily from studies with murine sarcoma S-180 cell lines (13). This evidence was as follows: (i) DMs were found only in unstably MTX-resistant cell lines (in contrast, in stably resistant Chinese hamster ovary cells [23] and mouse L5178Y cells [9], the amplified DHFR genes are associated with specific chromosomes); (ii) DHFR gene sequences cosedimented with fractions enriched in DMs during sucrose gradient sedimentation of isolated metaphase chromosomes; and (iii) when unstably resistant cells were grown in the absence of MTX, both DMs and DHFR genes were lost. In this study, we extended these findings in a more quantitative fashion.

We allowed the unstably resistant 3T6-R50 cell line to revert to the sensitive phenotype and quantitated in relative terms the loss of DHFR sequence abundance in both total cellular DNA and DM-enriched fractions at different points in the reversion. In both instances the kinetics of loss of DHFR sequences were essentially identical and compared favorably with the kinetics of loss of DMs. Because the DHFR sequences in total and DM-enriched samples declined in an identical fashion to less than 10% of the original values from parental resistant cells, we suggest that at least 90% of the amplified DHFR sequences and probably all such sequences are localized to DMs. This conclusion was supported by the observation that 3T6-R50 cells that reverted to sensitive-cell DHFR enzyme levels

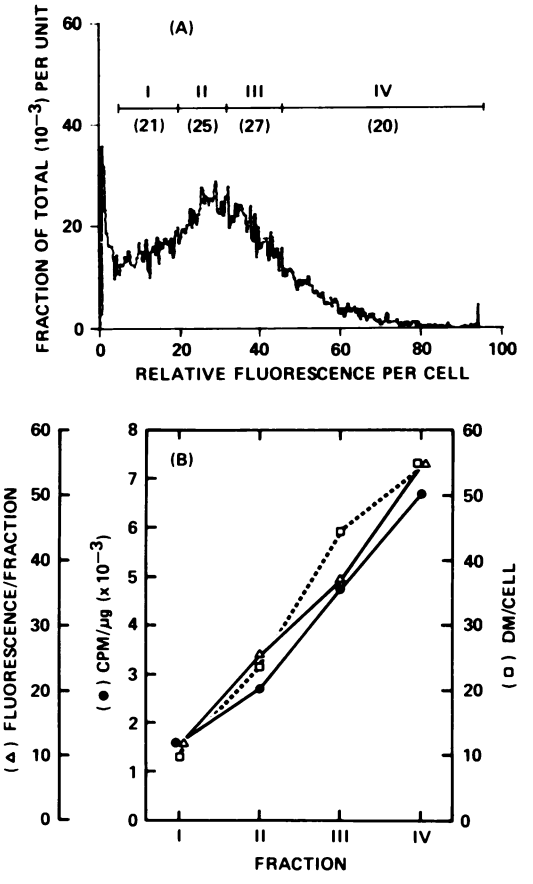

FIG. 5. *Comparison of DHFR gene amplification and DMs in sorted cell populations labeled with the fluorescein conjugate of MTX. (A) 3T6-R50 cells were labeled with the fluorescein conjugate of MTX and sorted according to fluorescence into four fractions (fractions I through IV). The numbers in parentheses are the percentages of total cells that comprised each fraction. From each fraction, samples were prepared for immediate DNA isolation (2.5 × 10^5 cells) and for karyotype analysis (1 × 10^5 cells) after growth for 2 additional days in MTX-free medium. (B) Relative DHFR sequence amounts were determined by filter hybridization (specific activity of probe, 1.6 × 10^9 cpm/μg) of DNA (0.6 to 0.7 μg/filter) from cells of each fraction. Although only one filter from each DNA sample was analyzed due to limitations of material, the hybridization data are quantitative because linear hybridization values were obtained with increasing amounts of filter-bound standard 3T6-R50 DNA included separately in the hybridization. Hybridization values were corrected for hybridization to equivalent amounts of filter-bound sensitive cell DNA plus nonspecific binding to filters (145 cpm/μg). DMs were counted in at least 25 metaphase spreads from each fraction, and average values are shown. The mean fluorescence values of all fractions are also shown.*

completely lacked DMs (data not shown). In addition, in situ hybridization of plasmid DNA containing the DHFR complementary DNA sequence to metaphase chromosome spreads of 3T6-R50 cells revealed no clustering of genes in these unstably resistant cell chromosomes under conditions in which chromosomal clustering of DHFR genes has been found in mouse S-180 cells containing a similar number of amplified DHFR genes (14). We also used the fluorescence-activated cell sorter technique to fractionate the 3T6-R50 cell population into subpopulations with different levels of DHFR. In these cell populations there was a high correlation among fluorescence, which was indicative of DHFR enzyme levels (12), the number of amplified DHFR genes, and the number of DMs (Fig. 5).

From these results (i.e., that there were 40 to 50 times as many DHFR genes in 3T6-R50 cells as in sensitive cells and that the mean number of DMs per cell was 28), we estimate that there are between three and four copies of the DHFR gene per DM (assuming one DHFR gene per haploid genome). Since the structural DHFR gene is approximately 35 kilobases long (22) (C. Simonsen, G. Crouse, and J. Schilling, manuscript in preparation) and since DMs contain at least 200 kilobases and perhaps as much as 1,000 kilobases of DNA, a significant amount of DNA of unknown function coexists with the DHFR structural gene on DMs. DHFR appeared to be the only protein that was overproduced in all of the amplified cell lines that we examined (including Chinese hamster ovary 3T3-R500, and 3T6-R400 cells); therefore, the function of this excess DNA remains unknown, and characterization of it will be important in fully understanding the biology of DMs.

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