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Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with double minute chromosomes

(gene amplification/tetrahydrofolate dehydrogenase/mouse chromosomes)

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ABSTRACT **Selection of mammalian cells in progressively increasing concentrations of methotrexate results in selective amplification of DNA sequences coding for dihydrofolate reductase (tetrahydrofolate dehydrogenase, 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3). In some cell variants the amplified genes are stable with growth in the absence of methotrexate, whereas in other variants the amplified genes are lost from the population. We have previously reported that in a stably amplified variant of Chinese hamster ovary cells, the genes are localized to a single chromosome. Herein we report that in mouse S-180 and L5178Y cell lines unstably amplified dihydrofolate reductase DNA sequences are associated with small, paired chromosomal elements denoted "double minute chromosomes," whereas in stably amplified cells of the same origin, the genes are associated with large chromosomes.**

Upon selection of mammalian cells in progressively increasing concentrations of methotrexate, a 4-amino analog of folic acid, resistant cells are obtained that have increased dihydrofolate reductase (tetrahydrofolate dehydrogenase, 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) synthesis (1, 2) proportional to amplification of DNA sequences containing the dihydrofolate reductase (*DHFR*) gene (3). This resistance and corresponding gene amplification occurs irrespective of whether cells are of hamster or of mouse origin or whether the cells are near diploid or are highly aneuploid (4). In some cell lines high methotrexate (MTX) resistance and *DHFR* gene copy number are stable when cells are grown in the absence of selection pressure (i.e., MTX) whereas in other cell variants, high resistance and *DHFR* genes are lost on continued growth of cells in the absence of MTX (3). We have previously reported, in two cell lines that are stably amplified, that the *DHFR* genes are largely, if not entirely, localized to expanded chromosome regions on single chromosomes called "homogeneous staining regions" (5, §).

In this report we describe studies that indicate that the amplified *DHFR* genes in unstably amplified cell variants are associated with small, paired chromosomal elements, denoted "double minute chromosomes," which are acentromeric and do not participate in equal segregation at mitosis. Thus we conclude that in stably amplified cell lines the genes are chromosomal and segregate equally at mitosis, whereas in unstably amplified cell variants we have studied the genes are largely, if not entirely, extrachromosomal and as a result of unequal distribution at mitosis can be lost from the cell population in subsequent generations.

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MATERIALS AND METHODS

Cell Lines. The generation and maintenance of unstably and stably MTX-resistant murine S-180 cell lines have been described (1, 6). The stably MTX-resistant murine L5178Y lymphoma was provided by J. Bertino.§ An unstably MTX-resistant L5178Y cell line has been generously provided by Courtenay and Robbins (7). The unstably MTX-resistant murine fibroblast 3T6 cell line was described by Kellems *et al.* (8). The stably MTX-resistant Chinese hamster ovary line has been described (5).

MTX-Fluorescein Labeling and Sterile Cell Sorting. S-180 MTX-resistant lines were prepared for fluorescence analysis and sterile cell sorting as described (9). After the cell sort, cells were propagated in the absence of MTX for five cell doublings and then karyotype analysis was performed.

Karyotype Analysis. Midlogarithmic phase cultures were treated with 0.06 μg of Colcemid per ml and metaphase spreads were prepared by a modification of the procedure of Deaven and Petersen (10). Cells were gently trypsinized, centrifuged at 800 × *g*, and resuspended with 0.5 ml of residual medium; 5 ml of 75 mM KCl (pH 7.0) at 37°C was added by drops and the mixture was incubated for 5–15 min at 37°C. Cells were again pelleted and resuspended in 0.5 ml of residual medium; 4 ml of ice-cold methanol/acetic acid, 3:1 (vol/vol), was added by drops and the mixture was placed on ice for 10 min. The cells were centrifuged again and fixation was repeated. Cells were finally centrifuged, resuspended in methanol/acetic acid, 3:1 (vol/vol), and dropped onto clean dry slides. Metaphases were stained for 10 min with 1 μg of ethidium bromide per ml in 50 mM potassium phosphate (pH 6.8) or for 30 min with 2% Gurr's Giesma in 50 mM potassium phosphate (pH 6.8). Chromosomes were quantitated by counting ethidium bromide-stained preparations. Because some double minute chromosomes did not contain recognizable double-staining regions, each staining region of the appropriate size, single or double, was scored as one double minute.

Chromosome Separation on Sucrose Gradients. Cells were prepared for metaphase chromosomes and separation was carried out by a modification of the procedure of Stubblefield *et al.* (11). Midlogarithmic phase cells from 20 T150 flasks (5×10^7 to 5×10^8 mitotic cells) were treated for 12 hr with 0.1 μg of Colcemid per ml and a mitotic shake-off was used to isolate metaphase cells (12). Cells were pelleted by centrifugation at 800 × *g* for 5 min and resuspended in 50 vol of 75 mM

Abbreviations: *DHFR*, dihydrofolate reductase gene; MTX, methotrexate.

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§ B. Dolnick, R. Berenson, J. R. Bertino, R. J. Kaufman, J. H. Nunberg, and R. T. Schimke, unpublished data.

KCl at 37°C for 15 min. After pelleting, the cells were resuspended in 5 ml of 75 mM KCl, and 20 ml of 50% acetic acid was slowly added at room temperature. In parallel, metaphase spreads were prepared for determination of the mitotic index, which routinely exceeded 50%.

Fixed cells were sheared by passage twice through a 23-gauge syringe needle with moderate pressure. An equal volume of 20% sucrose in 1 M hexylene glycol/0.5 mM 1,4-piperazinediethane sulfonic acid (Pipes), pH 6.8/0.1 mM $CaCl_2$ was added and the suspension was passed through the 23-gauge syringe once more, centrifuged at 400 × *g* for 10 min to remove interphase nuclei, and then kept on ice. The supernatant, containing metaphase chromosomes, was layered onto a 1-liter linear sucrose gradient (20–50% sucrose in hexylene glycol/Pipes/$CaCl_2$) and centrifuged (2500 rpm for 30 min at 20°C) in a reorienting Sorvall SZ-14 Zonal Rotor. Fractions (100 ml) were collected and chromosomes were pelleted (14,500 × *g* for 10 min in a HB-4 Sorvall rotor), washed with 10 mM Tris·HCl, pH 8.0/10 mM NaCl/1 mM EDTA, recentrifuged, resuspended in 200–400 μl of Tris·HCl/NaCl/EDTA with 0.5% sodium dodecyl sulfate and 50 μg of proteinase K per ml (13), and incubated for 12 hr at 37°C. Samples were extracted twice with an equal volume of phenol/$CHCl_3$, 1:1 (vol/vol), saturated with Tris·HCl/NaCl/EDTA and extracted twice with an equal volume of $CHCl_3$. The DNA was precipitated in 0.3 M NaCl with 2.5 vol of ethanol. Samples were centrifuged in an Eppendorf macrofuge (at 12,000 × *g*); pellets were washed with 70% ethanol/0.2 M NaCl and dried by lyophilization. DNA from each fraction was redissolved in equal volumes of Tris·HCl/NaCl/EDTA (usually 200 μl). DNA concentration was determined photographically from ethidium bromide fluorescence, with λ DNA as a standard (John Lis, personal communication).

***Eco*RI Digestion and Southern Blot Analysis.** DNA from constant percentage of each fraction of the gradient was digested by *Eco*RI restriction endonuclease.[¶] Reactions were monitored for completion by removing an aliquot of the main reaction and adding it to 0.4 μg of λ DNA. This analytic digestion was examined on a Tris borate/EDTA/0.5% agarose gel (14). The main reaction mixtures were stored for 6 hr at 4°C and then made 10 mM in EDTA and 0.25 M in NaCl; 2.5 vol of ethanol was added to precipitate the DNA. DNA was pelleted and washed with 70% ethanol/0.20 M NaCl. DNA pellets were lyophilized to dryness, rehydrated in sample buffer (20 mM Tris acetate/10 mM NaCl/1 mM Na_2EDTA/10% glycerol), incubated for 15 min at 37°C then for 5 min at 68°C, and finally put on ice. Samples were electrophoresed on a 0.5% agarose/Tris acetate/EDTA/NaCl gel system (15) and DNA was transferred to nitrocellulose paper (Schleicher and Schuell, type BA-85) as described by Southern (16) with modifications.[§]

Nitrocellulose filters were preincubated in 0.02% each polyvinylpyrrolidone, Ficoll, and bovine serum albumin, 0.5% sodium dodecyl sulfate, 1.25 mM sodium pyrophosphate, 40 mM sodium phosphate, and 0.75 M NaCl/0.075 M sodium citrate at pH 6.1 at 65°C for at least 12 hr (17). Filters were removed and hybridizations were carried out at 68°C in the same solution with the addition of 10 μg of denatured salmon sperm DNA per ml and 4 × 10^6 cpm of ^{32}P-labeled pDHFR11 (18) after heating at 106°C for 10 min. After at least 48 hr, the filters were washed three times for 2 hr each in the hybridization buffer, twice in 0.75 M NaCl/0.075 M sodium citrate at pH 6.1 at 68°C, and twice with 0.30 M NaCl/0.03 M sodium citrate at pH 7.0 at room temperature. Filters were prepared for autoradiography at −70°C as described elsewhere with a Dupont Lightning Plus intensifying screen and preflashed XR-5 Kodak film (19).

[¶] J. H. Nunberg, R. J. Kaufman, A. C. Y. Chang, S. N. Cohen, and R. T. Schimke, unpublished data.

Plasmid pDHFR11, which contains a 1600-base-pair insert derived from double-stranded cDNA to dihydrofolate reductase mRNA (18), was nick-translated (20) with [α-^{32}P]dCTP (350 Ci/mmol, Amersham; 1 Ci = 3.7 × 10^{10} becquerels) to a specific activity of 4 × 10^8 cpm/μg.

Microscopic Analysis of Gradient Fractions. For visual analysis of each fraction, diluted aliquots were layered over 20% sucrose in hexylene glycol/Pipes/$CaCl_2$ and centrifuged onto circular coverslips (14,500 × *g* for 10 min) (11). Coverslips were fixed in methanol/acetic acid, 3:1 (vol/vol), dried, stained, and photographed.

RESULTS

Unstably MTX-Resistant Cell Lines Contain Double Minute Chromosomes. Several parental and stably and unstably MTX-resistant mammalian cell lines were examined for the presence of double minute chromosomes. Table 1 shows the average number ±SD of double minutes per metaphase spread and the range for various populations. Only cell lines containing unstably amplified *DHFR* gene copies have large numbers of double minute chromosomes. Examples of metaphase spreads containing double minutes are shown in Fig. 1. Fig. 1 *upper* shows photomicrographs of S-180 unstably MTX-resistant and -sensitive cell metaphases prepared from logarithmically growing cells that were not hypotonically swollen or arrested with Colcemid prior to fixation and staining. Below are representative samples from the L5178Y unstably MTX-resistant and parental cells that were blocked with Colcemid and stained with ethidium bromide. Apparent in the upper photomicrographs are the large number of acentromeric double minute chromosomes present in the MTX-resistant S-180 cell lines that are peripheral to the chromosomal mass that is associated with the mitotic spindle apparatus (21).

Number of Double Minute Chromosomes Correlates with Number of Unstably Amplified *DHFR* Gene Copies. In order to correlate the presence of double minute chromosomes with the presence of unstably amplified *DHFR* gene copies, we used

Table 1. Unstably MTX-resistant murine cell lines contain minute chromosomes

Cell line*	*DHFR* gene copy no. relative to sensitive	Double minutes, mean ± SD	Mean chromosome no.
S-180:			
Stably resistant (R_1C)	60	5.5 ± 2.8	77
Unstably resistant (R_1A)	70	26 ± 35	68
Unstably resistant (R_2)	50	22 ± 17	83
Parental sensitive (S_3)	1	1 ± 0.8	74
L5178Y:			
Stably resistant†	400	0.1 ± 0.3	40
Unstably resistant (7)	ND§	41 ± 33	49
Parental sensitive‡	1	1.3 ± 1.4	42
3T6:			
Unstably resistant (8)	35	59 ± 23	75
Parental sensitive (8)	1	3 ± 3.0	82
CHO:			
Stably resistant (5)	150	0.5 ± 1.4	20

Each value represents the mean from counting 20 metaphase spreads.

* Letters in parentheses indicate the clone chosen for examination. Numbers in parentheses indicate the appropriate references.

† B. Dolnick, R. Berenson, J. R. Bertino, R. J. Kaufman, J. H. Nunberg, and R. T. Schimke, unpublished data.

‡ Parental cells from the stable or unstable L5178Y line gave similar results. However, the data presented here are from the L5178Y line described by Dolnick *et al.* (unpublished data).

§ ND, not determined.

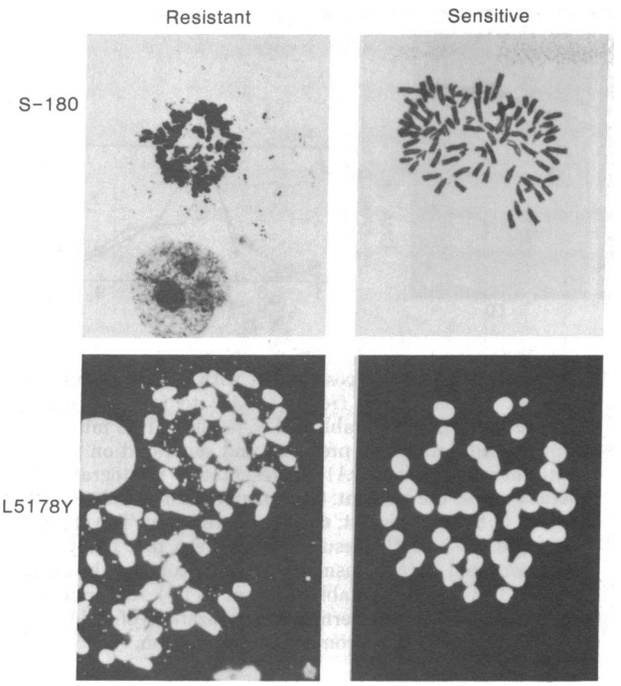

FIG. 1. Unstably MTX-resistant and parental metaphase spreads of S-180 and L5178Y cell lines.

a technique previously described that allows for direct sorting of cells from populations on the basis of dihydrofolate reductase content. First, a fluorescent derivative of MTX was used to saturate the cellular reductase such that the degree of fluorescence emission was proportional to the amount of reductase present (9). The fluorescence emission was also proportional to the degree of MTX resistance and the number of amplified *DHFR* genes (unpublished data). The fluorescence-activated cell sorter was used to isolate subpopulations of cells under sterile conditions, containing specified degrees of *DHFR* gene amplification.

Fig. 2*A* depicts the distribution of fluorescence in cells of the unstably MTX-resistant R_1A population. Two subpopulations containing high and low degrees of fluorescence and *DHFR* gene amplification were sorted under sterile conditions and Colcemid-arrested cells were examined for the presence of double minute chromosomes. The results indicate that the cells containing elevated *DHFR* gene copy numbers also contain elevated numbers of double minutes. Metaphases were found that contained over 300 double minute chromosomes. When the R_1A was propagated 120 cell doublings in the absence of MTX selection, it generated a stable, partial revertant line with approximately 7-fold the number of *DHFR* genes relative to parental MTX-sensitive cells (Fig. 2*C*). Coincident with the loss of amplified *DHFR* gene copies was a loss of double minutes (mean of 3 double minutes per metaphase). When the R_1A cell line was propagated for 3 years in the presence of MTX selection, it generated a stably MTX-resistant cell line (R_1C) with 50 stably amplified *DHFR* gene copies. Its fluorescence distribution is shown in Fig. 2*B*. Subpopulations of R_1C cells, sorted under sterile conditions, having the same degree of fluorescence as those sorted from the R_1A (Fig. 2*A*) contained very few double minutes (Fig. 2*B*). After 120 cell doublings in the absence of MTX, the fluorescence intensity of R_1C decreased very little (Fig. 2*D*), indicating the stability of this population. We have also sorted cells from another unstably MTX-resistant clone, R_2 (unpublished data), with similar results. Thus, double minute chromosomes are only abundant in cells containing amplified *DHFR* gene copies in an unstable state.

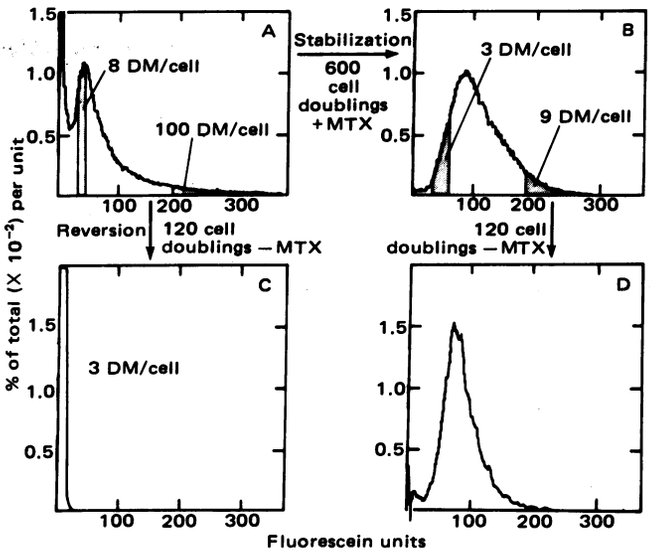

FIG. 2. Unstably resistant gene copies correlate with the double minute chromosome number. Cells were prepared for fluorescence analysis and sterile cell sorting after saturation of dihydrofolate reductase with a fluorescein derivative of MTX (9). Clone R_1A (*A*) was initially unstable and was propagated 120 cell doublings in the absence of MTX to generate a stable revertant (*C*) with 7-fold the reductase content of parental sensitive cells (9). R_1A was also propagated in the presence of MTX for 600 cell doublings and became stably resistant with a "fixation" of the *DHFR* genes (50 gene copies) (unpublished data). When this line was propagated for 120 cell doublings in the absence of MTX, it generated the fluorescence distribution of cells shown in *D*. Cells having the fluorescence values indicated (shaded areas) were sorted and metaphase spreads were prepared. The mean numbers of double minutes (DM) determined from 20 metaphases of the represented populations are shown.

In another set of experiments (data not shown) we found that the generation of unstably MTX-resistant lines is accompanied by the appearance of double minute chromosomes. A stable, moderately MTX-resistant S-180 clone containing approximately 10–15 *DHFR* gene copies was propagated in MTX for 100 cell doublings. After this time the mean fluorescence per cell increased from 10–15 fluorescein units per cell to 85 fluorescein units per cell (unpublished data). When metaphases were examined for the presence of double minute chromosomes, the original clone had a mean of 3 double minutes per cell and the line propagated in the presence of MTX contained a mean of 28 double minutes per cell. When the cell line that was propagated in the presence of MTX was examined for stability of elevated dihydrofolate reductase levels upon growth in the absence of MTX selection, the cells reverted to the 10–15 fluorescein units per cell level but not lower and no longer contained large numbers of double minutes (mean of 4 double minutes per cell).

Cosedimentation of *DHFR* Gene Copies with Double Minute Chromosomes. *In situ* hybridization and autoradiography by use of ^3H or ^{125}I to localize *DHFR* genes to double minute chromosomes is not presently feasible due to the insensitivity of the technique in localizing unique nucleotide sequences or sequences repeated only several times. In addition, the path length of the β particle is sufficiently long to obscure the association of any specific grains with double minutes. As an alternative approach, we have asked whether *DHFR* sequences will cosediment with double minutes upon separation of metaphase chromosomes on a sucrose gradient. The results of this experiment are shown in Fig 3 for the stably MTX-resistant R_1C and the unstably MTX-resistant R_2 S-180 clones. Fig 3*A* shows representative photographs taken from the indicated fractions of the gradient. There is evidence of a size

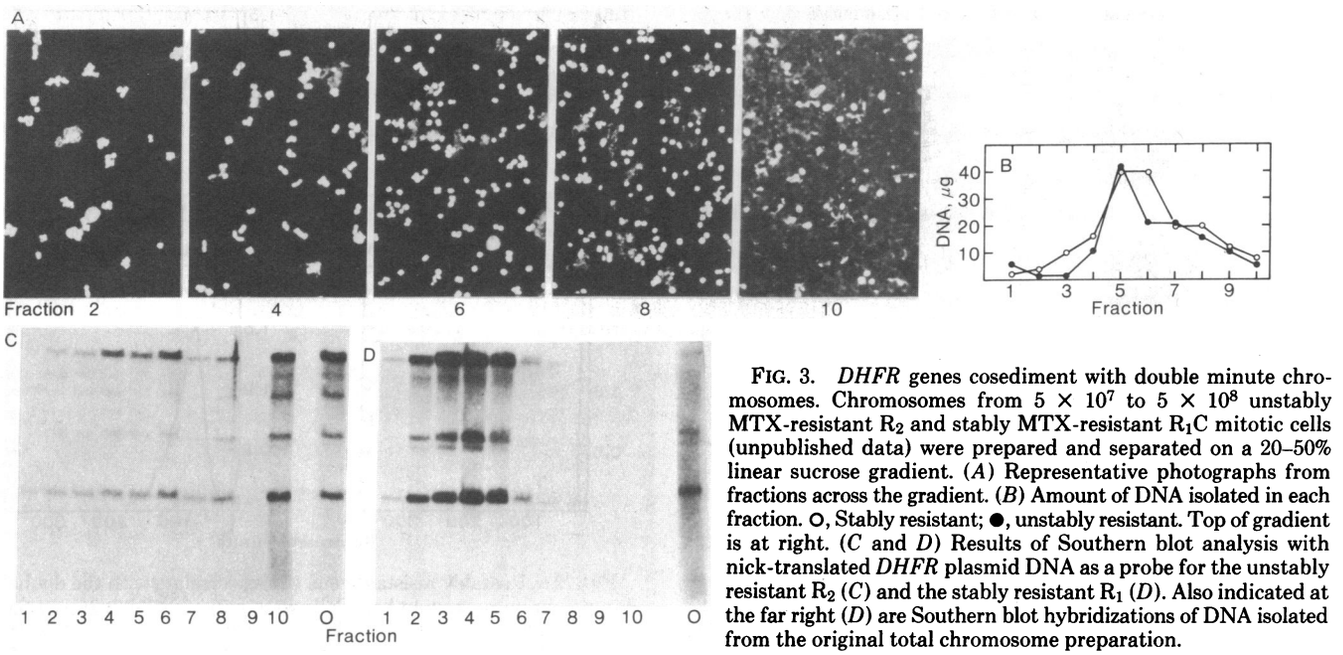

FIG. 3. *DHFR* genes cosediment with double minute chromosomes. Chromosomes from 5×10^7 to 5×10^8 unstably MTX-resistant R_2 and stably MTX-resistant R_1C mitotic cells (unpublished data) were prepared and separated on a 20–50% linear sucrose gradient. (*A*) Representative photographs from fractions across the gradient. (*B*) Amount of DNA isolated in each fraction. O, Stably resistant; ●, unstably resistant. Top of gradient is at right. (*C* and *D*) Results of Southern blot analysis with nick-translated *DHFR* plasmid DNA as a probe for the unstably resistant R_2 (*C*) and the stably resistant R_1 (*D*). Also indicated at the far right (*D*) are Southern blot hybridizations of DNA isolated from the original total chromosome preparation.

fractionation of the chromosomes with chromosomal aggregates appearing in fraction 2 and double minute chromosomes in fraction 10. Relative nucleic acid content from each fraction is shown in Fig. 3*B* and did not differ significantly between R_2 and R_1C. *Eco*RI endonuclease digestion and Southern blot analysis (16) with nick-translated *DHFR* plasmid DNA were performed on the DNA of fractionated chromosomes (Fig. 3 *C* and *D*). The unstably resistant R_2 clone showed the presence of *DHFR* sequences in the top fraction of the gradient (fraction 10), coincident with the sedimentation of double minute chromosomes as well as with chromosomes farther down the gradient. In contrast, R_1C showed no hybridization to fractions from the top of the gradient, and all *DHFR* sequences in this clone were associated with a class of rapidly sedimenting and presumably large chromosomes. We cannot exclude the possibility that, in the R_2 clone, *DHFR* sequences associated with sucrose gradient fractions containing larger chromosomes are a result of aggregation of double minutes having *DHFR* sequences or adhesion of these double minutes to chromosomes. Results similar to that of Fig. 3 have also been obtained with the unstably resistant R_1A and L5178Y cell lines.

DISCUSSION

In our attempt to understand the processes of amplification, loss, and fixation of *DHFR* genes that occurs in the generation, loss, and stabilization of MTX resistance, it has become important to localize the amplified *DHFR* gene copies. We have previously localized *DHFR* genes in stably MTX-resistant CHO (5) and murine L5178Y[§] lines to expanded homogeneously staining regions of specific chromosomes. Balaban-Malenbaum and Gilbert (22) have reported that several human neuroblastoma cell lines contain either homogeneously staining regions or double minute chromosomes. Because unstably MTX-resistant lines do not contain easily recognizable homogeneously staining regions, we undertook an investigation of whether these lines possess double minute chromosomes containing *DHFR* sequences. The results that indicate that unstably amplified *DHFR* genes are associated with double minutes are the following. (*i*) Double minutes are present in cell lines with unstably amplified *DHFR* genes and not in cell lines with stably amplified genes or parental MTX-sensitive cells. (*ii*) As unstably MTX-resistant cells are propagated in the absence of MTX, the amplified *DHFR* genes and the double minutes are lost. (*iii*) Subpopulations from an unstably MTX-resistant line contain numbers of amplified *DHFR* genes roughly proportional to the number of double minutes. (*iv*) Double minutes appear with the development of unstable MTX resistance. (*v*) When unstable MTX-resistant cells become stably resistant, double minutes disappear. (*vi*) *DHFR* sequences cosediment with double minutes in an unstably MTX-resistant line, whereas very few sequences are present in the comparable chromosome fraction from a stably MTX-resistant line.

The presence of double minute chromosomes associated with unstably amplified *DHFR* genes assists in explaining certain properties of unstably MTX-resistant cells. We have observed the heterogeneity in *DHFR* gene copy number in unstably resistant cell populations (unpublished data). Because it appears unlikely that double minutes contain centromeric DNA (23, 24) and because double minutes do not associate with the spindle apparatus at mitosis (see Fig. 1 and ref. 21), the double minutes would segregate randomly and unequally into daughter cells, thus generating heterogeneity in *DHFR* gene number as observed in cell populations (unpublished data). We have found that cells with low *DHFR* gene copy number have a slightly more rapid generation time and, hence, reversion (i.e., loss of gene copy number) could be accounted for by the overgrowth of cells containing few double minutes. Levan and Levan (21) report that double minutes tend to aggregate in mitosis with subsequent formation of micronuclei which may be expelled later. This process may account for our observations of single step losses of *DHFR* genes in CHO[‖] and S-180 (unpublished data) cell lines.

A number of questions arise concerning the properties of double minutes chromosomes and their relationship to *DHFR* gene amplification. Do these chromosomes replicate autonomously and, if so, is their replication synchronized in the cell

[‖] We have recently found that generation of stable MTX resistance in CHO cells occurs by a stepwise fixation of specified numbers of *DHFR* gene copies. Initially, however, cells undergoing MTX selection were partially unstable and exhibited stepwise losses in their *DHFR* gene copy number. The double minutes in those unstable cells (less than 5% of the total metaphases exhibited double minutes) were aggregated in the metaphase spreads prepared after hypotonic swelling and brief Colcemid arrest (unpublished data).

cycle? Levan *et al.* (25) have claimed that double minutes replicate early in S phase in SEWA mouse ascites tumor cells. On the other hand, Balaban-Malenbaum and Gilbert (22) have suggested that double minutes do not replicate at all and are only the result of fragmentation of homogeneously staining regions. If double minutes replicate autonomously and, in particular, if they are not regulated by events controlling normal chromosomal replication and segregation, amplification may occur by disproportionate *DHFR* gene replication and selective accumulation in resistant cells of double minutes containing *DHFR* gene sequences. Loss of resistance in the absence of selective pressure might occur by reversal of the events leading to the accumulation of double minutes containing *DHFR* genes because cells of reduced *DHFR* gene copy number and corresponding MTX resistance have an enhanced growth potential in MTX-free media. Levan *et al.* (26) have described the generation and loss of double minutes in cells when propagated under cell culture conditions *vis-a-vis* growth in an ascites form in the peritoneum of mice, although it is not clear what their observations mean in terms of growth and selective pressures related to MTX resistance in our studies.

How are double minute chromosomes generated? We have previously suggested, by analogy to gene duplications in prokaryotes, that the generation of amplified DNA sequences in cultured animal cells is a random process and that growth in MTX selects for those cells in which the amplified DNA sequences contain *DHFR* sequences (27, 28). The initial event may involve disproportionate chromosomal replication and subsequent excision-replication (29). Conversely, DNA may be taken up by lysis of cells and subsequent replication of DNA containing the *DHFR* sequences. We have found that the generation of stable MTX resistance from originally unstably resistant cells occurs by a stepwise fixation of a specified number of *DHFR* sequences and have discussed possible mechanisms involved in this process (unpublished data).

How many *DHFR* gene sequences are present on each double minute chromosome? We have found in mouse cell lines of various origins and in mouse liver DNA, that the 1600 *DHFR* mRNA nucleotides are represented by a minimum of 40,000 base pairs of DNA with five intervening sequences.[¶]

Based on the degree of *DHFR* gene amplification and the number of double minutes (Fig. 2 indicates 100 double minutes in cells sorted with approximately 100–200 *DHFR* gene copies), it is conceivable that each double minute chromosome contains between 1 and 5 *DHFR* sequences. Estimates comparing the gene copy number with the size of the chromosomal expansion (homogeneously staining region) in two stably amplified cell lines (ref. 5,[§]), suggest that the DNA sequence that is amplified is of the order of 500–1000 kilobases. Fig. 1 suggests size heterogeneity of different double minutes in a single metaphase spread. Although this may be attributed to different degrees of chromosome condensation, it may well indicate heterogeneity in DNA content among different double minutes. However, we do not know that each double minute, in fact, contains *DHFR* sequences or, indeed, whether these *DHFR* sequences are functional.

The loss of *DHFR* genes in unstably MTX-resistant S-180 and L5178Y cell lines associated with gene localization to double minute chromosomes may differ from the loss of MTX resistance described by Biedler and Spengler (30). These workers have associated a loss of MTX resistance in several Chinese hamster lung cell isolates with a progressive diminution in the size of homogeneously staining regions in chromosomes. This latter process may involve a progressive excision of DNA segments from regions of tandemly repeated DNA sequences containing *DHFR* genes and their subsequent loss without extrachromosomal replication. The reasons why some homogeneously staining regions are stable over long periods of time whereas others are unstable is not readily apparent. We can only conclude from our studies and those of others that individual cells and different cell lines vary in properties of stable and unstable resistance.

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