Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1126–1130, February 1985
Cell Biology

Ultrastructural features of minute chromosomes in a methotrexate-resistant mouse 3T3 cell line

(electron microscopy/amplification/chromosome organization)

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Contributed by Robert T. Schimke, October 9, 1984

ABSTRACT **The Miller spreading procedure was applied to mouse metaphase spreads of methotrexate-resistant 3T3 cells that contain large numbers of minute chromosomes and dihydrofolate reductase genes. There is substantial variation in both size and numbers of minutes in individual cells, the smallest of which (estimated as 5×10^3 kilobase pairs) would be undetected by standard light microscopic analyses. Minute chromosomes are composed of nucleosomal chromatin, which is organized into typical higher order fibers that are folded to form rosette-like structures characteristic of normal chromosome organization. There is no evidence that the DNA in minutes is linear. Minutes exist singly and in pairs, and members of a pair are connected by higher order chromatin fibers, suggesting that they are topologically interlocked. They are often closely apposed to chromosomal telomeres or arms, a configuration that may be involved in their distribution at mitosis. In addition to typical minutes, which do not possess kinetochores, a small marker chromosome possessing all of the features of a centromere region is present in parental and resistant cells. An unusual feature of this cell line is the retention of resistance, minute chromosomes, and amplified dihydrofolate reductase genes; most methotrexate-resistant mouse cell lines with minute chromosomes lose these properties when grown in the absence of methotrexate.**

There are an increasing number of reports documenting amplification of a variety of DNA sequences in somatic mammalian cells either as a result of selection for drug resistances or as part of the malignant process (1). Amplification of DNA sequences results typically in two types of karyological consequences: in some cell lines, the amplified gene copies are present on one or more chromosomes (2–4), originally denoted homogeneously staining regions (HSRs) (5); in other cell lines, amplified genes are present on small extrachromosomal elements lacking centromeric regions, called minute chromosomes (6). The phenotype of drug resistance (e.g., to methotrexate) of cells containing chromosomally localized amplified genes is (relatively) stable when cells are grown in the absence of selection. Conversely, minute-containing methotrexate-resistant cells characteristically rapidly lose both the resistance phenotype and minutes during growth in the absence of the inhibitor (6, 7). Although one or the other of these karyotypic manifestations tends to predominate in a given cell line, both can coexist in a single cell (8, 9).

Our interest in details of the structure, distribution at mitosis, and instability of minute chromosomes has led us to study a mouse cell line, 3T3 R500, that is highly resistant to methotrexate and that possesses a large number of minute chromosomes that carry the gene for dihydrofolate reductase (*DHFR*). An unusual feature of this cell line is the retention of minute chromosomes and the relatively stable methotrexate-resistance phenotype when these cells are grown in the absence of selection. In these studies, we used the electron microscope to describe certain ultrastructural features of minutes in these cells, and we suggest a means for their distribution at mitosis such that they are not lost during growth of cells in the absence of methotrexate.

In addition to typical minutes, which do not possess kinetochores and, therefore, cannot segregate, we observed in all NIH 3T3 cells an armless microchromosome that is stable and capable of segregation. This small marker chromosome is about the size of the typical mouse centromere region and contains a large amount of mouse satellite DNA.

MATERIALS AND METHODS

Cell Culture. All studies were done with a mouse NIH 3T3 cell line developed for resistance to 500 μM methotrexate by Vera Morhenn (Department of Dermatology, Stanford University) (10). This cell line contains ≈200 copies of the *DHFR* gene, but it has not been described previously in any detail. The cells 3T3 R500 were grown in 500 μM methotrexate in T25 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin (GIBCO). Cells were arrested in mitosis by treatment with Nocodazole (final concentration, 100 ng/ml; Sigma) for 6 hr followed by selective detachment. The detached cells were pelleted, resuspended in 0.1 vol of medium, and lysed by 1:1 dilution with 1% Nonidet P-40 (pH 10).

Electron Microscopy. A 50-μl aliquot of the cell lysate was layered over a 1.0 M sucrose cushion (pH 8.5) in a Plexiglas microcentrifugation chamber (11), and chromosomes were deposited onto parlodion-carbon coated electron microscope grids according to Rattner and Hamkalo (12), except that centrifugation was at 600 rpm for 15 min in some experiments, to effect unfolding of chromatin without stretching of chromatin fibers. Grids were rinsed in 0.4% Photoflo (Kodak) solution, air-dried, stained with 1% alcoholic phosphotungstic acid for 30 sec, rinsed in 95% ethanol, and air-dried. All micrographs were taken on a JEOL 100C electron microscope operated at 80 kV.

Electron Microscope *in Situ* Hybridization. Chromosomes were deposited on gold electron microscope grids as described above and carried throughout the electron microscope *in situ* hybridization protocol described by Hutchison *et al.* (13) with minor modifications (unpublished data). Briefly, glutaraldehyde-fixed alkali-denatured chromosomes were hybridized to biotinylated mouse satellite DNA overnight at 30°C in the hybridization buffer described by Brahic and Haase (14) with the addition of 10% dextran sulfate. Af-

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Abbreviations: HSR, homogeneously staining region; DHFR, dihydrofolate reductase.

ter removal of unhybridized DNA sequences, hybrids were detected with a two-step antibody procedure involving reaction with affinity-purified rabbit anti-biotin and then goat anti-rabbit IgGs adsorbed to the surface of 20-nm collodial gold particles (Janssen Pharmaceutica, Beerse, Belgium).

RESULTS

Minute Chromosomes in Cells under Methotrexate-Selective Pressure. Fig. 1*a* shows a low magnification micrograph of chromosomes released upon lysis of a 3T3 R500 mitotic cell. In addition to normal acrocentric mouse chromosomes with paired kinetochore structures associated with centromeric heterochromatin, a number of small single and paired structures (single and double minutes) are easily distinguishable. The number of minutes varies (between 25 and 100) among individual cells in the population, as previously reported for other methotrexate-resistant mouse cell lines (6, 15). Parental 3T3 cells do not contain minutes, as determined by either light or electron microscopy.

When mitotic spreads are prepared under conditions that enhance chromosome unfolding, certain features of the higher-order structure of minutes can be observed (Fig. 1*b*). The chromatin is nucleosomal and is composed of loops of 20- to 30-nm chromatin fibers that exhibit nucleosome-packing patterns similar to those described previously for unamplified chromatin (12, 16). These loops are quite uniform in length for a given minute and are organized, in turn, into rosette-like structures.

Although the partners in a double minute are often very close together, occasionally they are stretched apart during specimen preparation. Fig. 1*c* shows such a pair of minutes in which multiple strands of higher-order chromatin fibers appear to connect the two minutes. We have observed numerous connected minutes, and a notable feature is the relative consistency in the size of the two partners, suggesting that they are products of an unresolved replicative intermediate (i.e., concatenated structures).

In addition to the numerical variability of minutes in a single cell, there is also variability in the size of minutes in a single cell, and the smallest minutes visible by electron microscopy undoubtedly would be overlooked in standard light

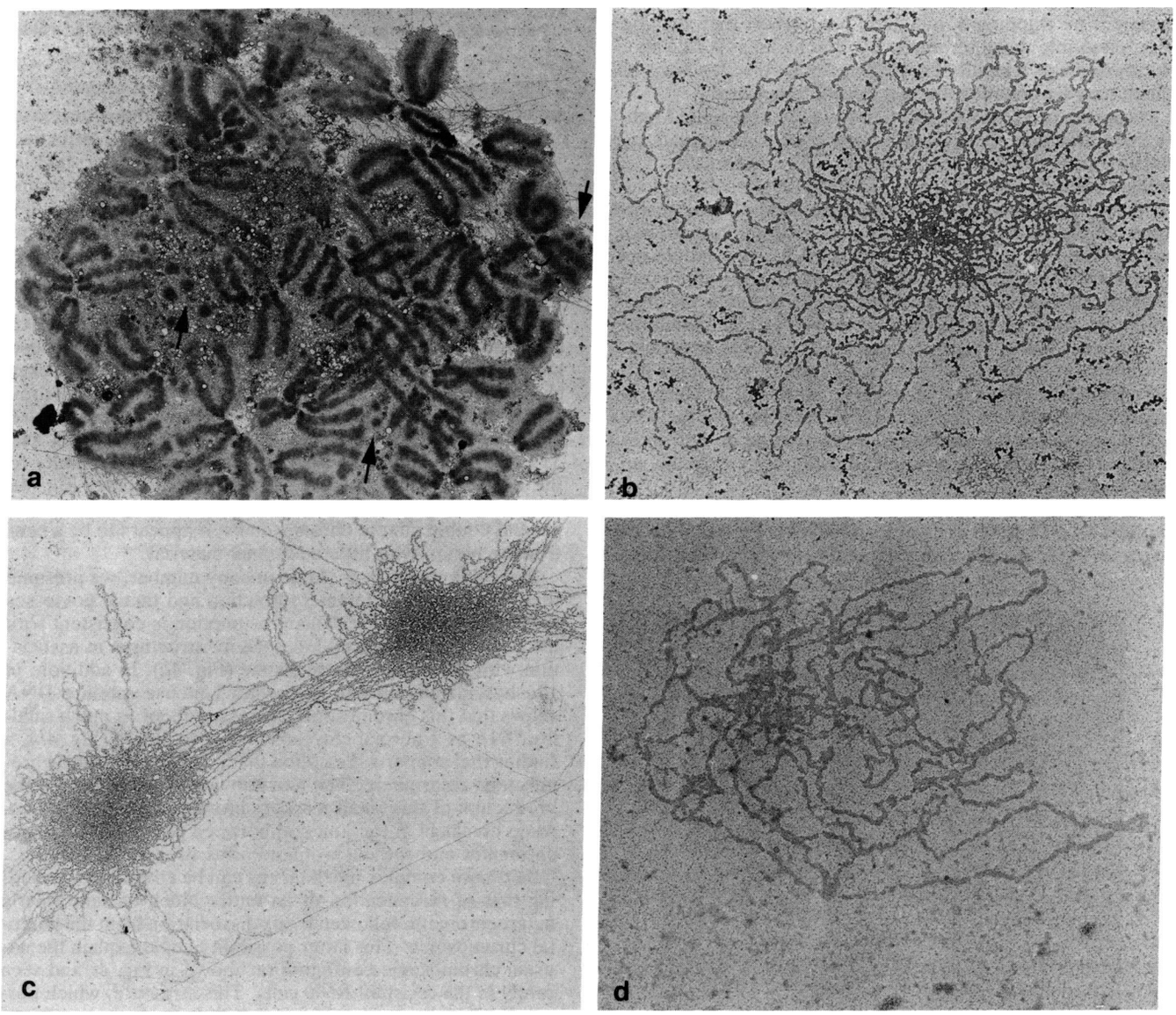

FIG. 1. Electron micrographs of mitotic chromosomes derived from NIH 3T3 R500 cells. (*a*) Low-magnification photograph illustrating numerous minute chromosomes (arrows) (×1150); (*b*) single minute chromosome showing typical higher-order organization (×24,000); (*c*) a pair of minute chromosomes connected by higher-order chromatin fibers (×8900); (*d*) smallest minute observed in these studies (×26,000).

microscopic analyses. Fig. 1*d* shows one of the smallest minutes observed in our studies. Although we cannot definitively prove that this and other minutes are circular structures, no free ends are visible. We determined the contour length of this minute to estimate its DNA content. Assuming a 40-fold packing ratio of DNA in a 20- to 30-nm chromatin fiber (17), we calculate that this minute contains $\approx 5 \times 10^3$ kilobase pairs of DNA. This DNA content is orders of magnitude greater than the size of the DHFR transcription unit, which is 35 kilobase pairs (18), and is in keeping with the large estimated sizes of amplification units in some cell lines (2, 3).

Minutes in this cell line are often seen in close proximity to normal chromosomes. Fig. 2 shows a double minute that is closely apposed to the telomeres of sister chromatids of an acrocentric chromosome. It has been proposed (19) that mitotic partitioning of the nuclear matrix in which minute chromosomes are embedded determines their distribution at mitosis. However, the persistence of minute-chromosome associations such as that shown in Fig. 2, even under conditions that solubilize the nucleoplasm, suggests to us that specific minute-chromosome associations may also be significant in the distribution of some minutes at mitosis. That is, minutes are not inevitably randomly associated with chromosomes by trapping in a matrix, but, rather, they may be segregated into daughter nuclei by "hitchhiking" a ride on a chromosome as a result of specific associations, the basis of which may be homologous sequence interactions.

Properties of 3T3 R500 Cells Grown in the Absence of Methotrexate Selection. Growth of a number of methotrexate-resistant mouse cell lines in the absence of methotrexate, most studied being 3T6 R50 (15) and 3T6 R400 (20), results typically in the rapid loss (in <100 cell doublings) of drug resistance, amplified *DHFR* genes, and double minute chromosomes. The 3T3 R500 cell line is unusual, because it does not revert to methotrexate sensitivity even after numerous (>100) cell doublings in methotrexate-free media. In other cell lines, Bostock and Clark (21) as well as Kaufman *et al.* (7) showed that this lack of rapid reversion was associated with integration of extrachromosomal DHFR sequences into chromosomes. However, analysis by both light and electron microscopy indicates that the 3T3 R500 cells grown for >100 cell doublings in the absence of methotrexate do not reveal visible HSRs, whereas minute chromosomes are readily apparent (Fig. 3).

The organization and sizes of minutes in methotrexate-withdrawn 3T3 R500 cells (3T3 R500W) are essentially the same as those in cells under selection. The mean number of minutes per cell decreases from 100 to 30, and this decrease is accompanied by an ≈50% decrease in the ED_{50} (effective 50% killing concentration) and a similar relative decrease in *DHFR* gene copy number (data not shown). These results indicate that a cell line containing minute chromosomes does not inevitably respond to removal of selection pressure by nearly complete loss of the resistance phenotype and minute chromosomes.

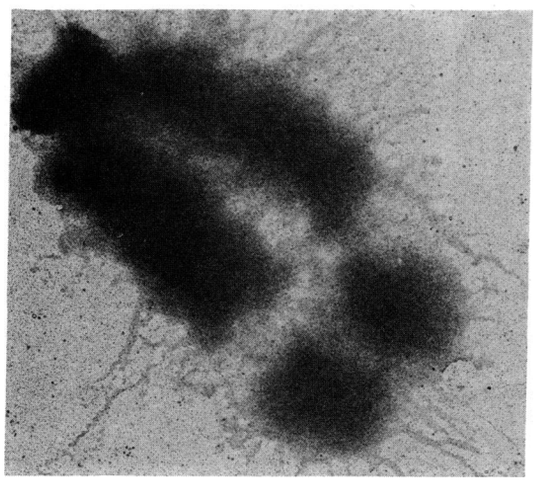

FIG. 2. Electron micrograph of a pair of minute chromosomes in close apposition to the telomeres of sister chromatids (×12,000).

FIG. 3. Low-magnification micrograph of the chromosomes in a 3T3 R500W cell after growth for 135 cell doublings in the absence of methotrexate. Numerous minute chromosomes are readily visible (arrows) (×1150).

An Unusual Microchromosome in 3T3 Cells. Despite the aneuploidy of 3T3 cells, the chromosomes are typical in size and centromere position when compared to a normal mouse karyotype. However, in this cell line there is a small marker chromosome that escaped detection by light microscopy (Fig. 4*a*). This microchromosome is present in two copies per metaphase in all cells studied (i.e., 3T3, 3T3 R500, and 3T3 R500 W), and it tends to be seen in close proximity to the centromere region of a normal chromosome. Fig. 4*a* shows this marker chromosome in comparison to typical single and double minute chromosomes; it appears to be a centromere region with little additional material.

Since this structure is stable in copy number, we presume that it has acquired telomere function and that it possesses the ability to segregate. This presumption is consistent with the occurrence of paired kinetochore structures in association with the marker chromosome (Fig. 4*a*). In addition, *in situ* hybridization using biotinylated mouse satellite DNA shows that this chromosome possesses about as much satellite DNA as a normal chromosomal centromere (Fig. 4*b*), a finding that supports the notion that it is derived from a chromosomal centromere. The most obvious mechanism for the production of this small marker chromosome is by chromosome breakage at the junction between centromeric heterochromatin and the euchromatic arms of sister chromatids. Other more complex mechanisms can be envisioned, including that of rereplication of an entire block of centromeric heterochromatin followed by its dissociation from the parental chromosome. This latter possibility could explain the unusual chromosome configuration shown in Fig. 4*c* and seen rarely in the resistant R500 cells. This structure, which possesses two chromosome arms but four centromere regions with associated kinetochores, could represent an intermediate in the formation of centromere minutes via rereplication rather than by simple chromosome breakage.

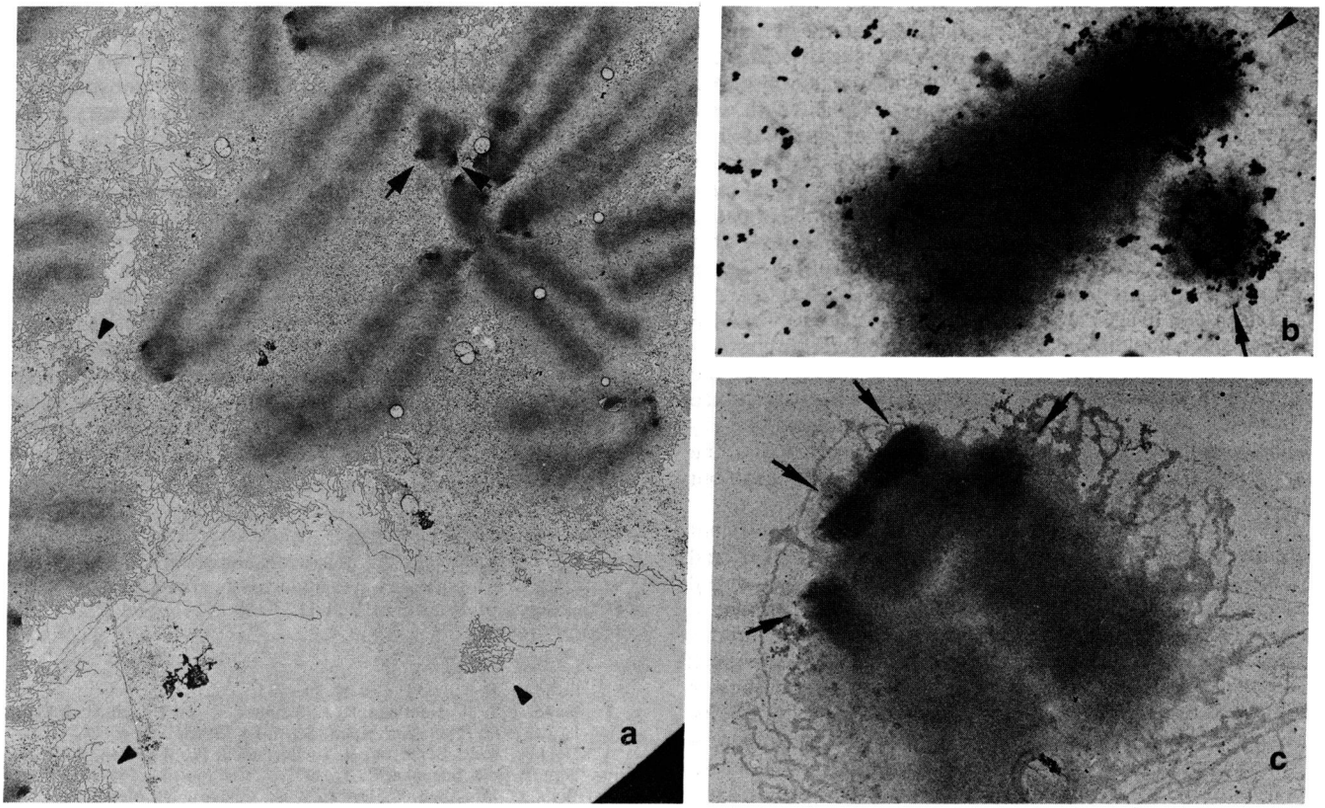

FIG. 4. Electron micrographs of a small marker chromosome seen in all 3T3 cells. (*a*) Marker chromosome with associated kinetochores (arrows) along with typical minute chromosomes (arrowheads) (×3200); (*b*) electron microscope *in situ* hybridization of biotinylated mouse satellite DNA followed by detection with colloidal gold particles according to Hutchinson *et al.* (13) shows equivalent labeling of the marker (arrow) and a normal chromosomal centromere region (arrowhead) (×15,500); (*c*) an unusual chromosome containing four kinetochores (arrows) that may be related to the marker in *a* and *b* (×11,800).

DISCUSSION

We have used electron microscopy of Miller spread preparations (11) to study the structure of minute chromosomes in a NIH 3T3 cell line highly resistant to methotrexate as a result of an ≈200-fold amplification of the *DHFR* gene. Certain basic organizational features were observed that are seen also in minutes of human carcinoma cells (22), features also typical of normal mitotic and meiotic chromosomes in diverse organisms. The existence of nucleosomal subunits on minute chromosomes was suggested in studies on isolated chromatin by Barker (23) and was confirmed by our studies and those of Rattner and Lin (22). Under the preparative conditions used, most of the chromatin retains a higher order organization of 20- to 30-nm fibers, which are composed of closely packed nucleosomes in packing patterns typical of eukaryotic nuclear chromatin (16). Superimposed on this level of folding is one in which the thick chromatin fibers are organized into loops that emanate from a central locus generating rosette-like structures. These structures are reminiscent of intermediates in the folding of *Bombyx mori* meiotic prophase chromosomes (24) and are consistent with the radial loop model proposed by Marsden and Laemmli (25) for folding of mitotic chromosomes.

Minute chromosomes also possess several unusual features. Although the classical observations describe these chromosomes as paired (i.e., double minutes), they are not inevitably paired. Single minutes have been observed by both light and electron microscopy of metaphase spreads (22). A striking feature of the partners of a double minute is their relative uniform size, suggesting that they are replicas of each other. Our observations on double minutes suggest that the partners are connected via chromatin strands, perhaps as a consequence of post-replicative topological linking of circular molecules that has not been resolved—i.e., they are concatenated circles. Since higher order minute structures (i.e., triplets and higher) typically are not seen, one may presume that double minutes must be resolved into single minutes prior to and/or during the following replication. Alternatively, such larger-sized concatenated structures may be subject to extensive micronucleation and loss from the nucleus. It should be noted that there is extensive micronucleation of DNA in the 3T3 R500 cell line (10). Although we have no direct evidence for the existence of circularity of the DNA in minutes, the smallest minutes observed have no free ends visible. In addition, there is precedence for circular extrachromosomal amplified genes in amphibian oocytes (26) and in the parasitic protozoan *Leishmania tropica* (27).

There is variability in both the number and the size of minute chromosomes and, obviously, their DNA contents within individual cells. The sizes of the smallest minutes observed in the electron microscope would probably render them undetectable by light microscopy. We have proposed (1) that amplified DNA results from overreplication of DNA sequences in a single cell cycle, followed by recombination of the overreplicated strands to generate circular (extrachromosomal) structures, or recombination into the chromatid to generate HSR structures. Theoretically, then, the unit size of initially amplified DNA could be highly variable. The size of the unit of amplified DNA in HSR structures can vary from 150 (28) to 500 kilobase pairs, in which case cytologically identifiable HSRs are generated. However, extensive

chromosomal amplification of the *DHFR* gene need not be associated with a *HSR* (29), suggesting that the length of DNA constituting the amplified DNA sequence in a chromosome can sometimes be very small. In contrast, all observed minute chromosomes are very large in total DNA length, and in the 3T3 R500 cell line the smallest minutes contain $\approx 5 \times 10^3$ kilobase pairs of DNA. Consistent with this, Barker and Stubblefield (30) estimated the DNA content of typical minutes in a human tumor cell line to be $\approx 10^3$ kilobase pairs, based on its size relative to normal human chromosome. It is unlikely that we are losing smaller molecules during sample preparation, because the conditions used to visualize this small molecule are nearly identical to those used to visualize extrachromosomal amplified ribosomal gene circles containing <100 kilobase pairs of DNA (26). We suggest that the large size of minutes reflects a minimum size for persistence of such self-replicating structures in the nucleus and that extrachromosomal structures of smaller size may be lost at the time of nuclear envelope reformation and/or by leakage out of nuclear pores.

The 3T3 R500 cell line is unusual in retaining significant methotrexate resistance, high *DHFR* gene copy number, and numbers of minute chromosomes when grown for prolonged times in the absence of methotrexate. This is in contrast to mouse 3T6 (15) and S-180 (6) methotrexate-resistant variants in which resistance, amplified genes, and extrachromosomal elements are lost when cells are grown in the absence of methotrexate. The minute chromosomes in the 3T3 R500 cells are frequently in apposition to normal chromosomes, as observed with the electron microscope (Fig. 2). An interesting hypothesis for the basis of the minute chromosome associations in the 3T3 R500 cells is that they reflect the presence of sequences of the amplified DNA in at least some of the minute chromosomes in this cell line that have sequence homology with chromosomes, similar to heterochromatin associations of normal chromosomes (31), and that such DNA sequences are not, or are rarely, present in minutes of the 3T6 R50 cell line. With appropriate probes, this possibility is testable using *in situ* hybridization.

The associations of minutes with chromosomes may explain in part the lack of complete reversion (loss of resistance, minute chromosomes, and *DHFR* genes) of the 3T3 R500 cell line in the absence of methotrexate, inasmuch as there would be a tendency of the minutes to segregate nonrandomly with chromosomes at mitosis, resulting in their retention. An alternative, and not mutually exclusive, hypothesis for the persistence of minute chromosomes and amplified *DHFR* genes is that the minutes are replicating more than once in a given cell cycle. Indeed, the R500 cells grown in the presence of methotrexate as well as the cells grown in the absence of methotrexate for 100 cell doublings contain many micronuclei (10), which contain amplified DHFR DNA sequences (unpublished results). Thus, the persistence of amplified *DHFR* genes may constitute a new steady-state balance between their generation at a high rate and a constant rate of loss (by micronucleation). It is interesting to note that a number of continuous cell lines of tumor origin contain multiple minute chromosomes, some of which contain amplified unknown sequences. One explanation for the persistence of such minutes in tumor cells is their requirement for growth. However, an alternative explanation is that such minutes may also be extensively overreplicated in each cell cycle.

The stable microchromosome described in this paper is of interest with regard to how it was generated and its sequence composition. It presumably arose from a normal chromosome, because it contains a typical complement of mouse satellite DNA and paired kinetochores. Yet, it does not have chromosomal arms. Its persistence in both parental and derived methotrexate-resistant cells argues that it has acquired telomere function. The rare and unusual chromosome configuration we have observed (Fig. 4*c*), which appears to possess four centromeric regions, may be related to this marker chromosome. The simplest explanation for its generation is rereplication of centromeric heterochromatin followed by incomplete segregation. Since the microchromosome is seen in unselected parental 3T3 cells, it clearly is not a result of the selection process; it may reflect this cell line's propensity toward rereplication of a variety of DNA sequences. The chromosome is approximately the same diameter as the largest minute chromosome, which raises the additional possibility, contrary to common belief, that a rare double minute may possess centromeres. Recombination between minute chromosomes and a segment of DNA that possesses centromere function would be expected to render the minute mitotically stable.

We acknowledge Dr. Peter C. Brown who in 1981 first observed that the 3T3 R500 cells did not revert completely to methotrexate sensitivity. These studies were supported by grants from the National Institutes of Health to B.A.H. (GM 23241) and R.T.S. (GM 14931).

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