

ULTRASTRUCTURE OF DOUBLE MINUTES FROM A HUMAN TUMOR CELL LINE

P. E. BARKER and E. STUBBLEFIELD. From the Department of Biology, Section of Cell Biology, The University of Texas System Cancer Center M. D. Anderson Hospital and Tumor Institute, and The University of Texas Graduate School of Biomedical Sciences, Houston, Texas 77030

ABSTRACT

Double minutes (dm) have been isolated from human tumor cells by zonal centrifugation and by differential pelleting of chromosome suspensions. These methods allowed collection of dm in sufficient quantity and purity for visualization with electron microscopy. Ultrastructurally, the chromatin fibers in dm resemble those in metaphase chromosomes. No evidence of attached membrane fragments was found. When the two isolation protocols were compared, differential pelleting was shown to increase purity twofold to 85% dm by mass. The differential pelleting procedure enables easy collection of dm in sufficient quantity and purity for chemical analysis.

KEY WORDS human tumor cells · double minutes · ultrastructure · chromosome isolation · differential centrifugation

Ascertainment of the chemical and biological nature of the nuclear structures from human (2–6, 10, 12–14) and murine (7–9, 11, 14) tumor cells known as double minutes (dm) has been hampered by their small size and by the lack of appropriate technology for their easy isolation. Recently, these DNA-containing structures seen at metaphase of the cell cycle have been implicated in the unstable amplification of cistrons mediating methotrexate resistance in cultured murine cells (5, 7). There is widespread speculation that dm may represent extrachromosomal genetic elements analogous to well-characterized episomes in prokaryotic systems, although supportive evidence has not been reported. Dm vary considerably in number among cells (2–14). There is evidence to suggest integration (8) and excision (2) of dm in a fashion reminiscent of the prokaryotic system. We report here the ultrastructural features of dm and a protocol for their isolation from human carcinoma cell line SW 613-S 18.

MATERIALS AND METHODS

Human carcinoma cell line SW 613 was cultured as previously reported (5). Initially, SW 613 showed a mean of 10 dm/cell. A variant of this line, S 18, was selected by sequential shakeoff and replating of mitotic cells for 18 rounds of selection. The variant showed a mean of 40 dm/cell and an 18-h cell cycle (3). Cells grown in 490 cm² roller bottles were synchronized by consecutive 2 mM thymidine blocks followed by incubation of cells in 10⁻⁷ M colcemid and mechanical shakeoff of mitotic cells. The procedure resulted in populations of cells which showed 85% mitosis. Control experiments were carried out with a karyotypically normal human fibroblast cell line derived from a foreskin biopsy. Culture conditions, synchronization methods, and chromosome isolation were identical to those used for cell line SW 613-S 18. The control fibroblast line showed no dm in the 1,000 metaphases screened.

Dm isolation followed published chromosome isolation procedures (15). This involved hypotonic swelling of mitotic cells followed by lysis of cells into neutral chromosome isolation buffer or 50% acetic acid (15). Resulting chromosome suspensions were fractionated on 20–40% linear sucrose gradients by zonal centrifugation as reported (15). Gradient fractions were assayed for dm percentage by comparison of the ethidium bromide-staining areas of chromosomes and dm in 20 microscope fields photographed at × 1,000 as previously reported (5).

The differential pelleting procedure involved collection of chromosome suspensions as above and fractionation as in Fig. 2. Assay of resulting fractions was as above or as indicated in figure legends.

Electron microscopy of particles in the 70,000 g pellet followed

published procedures (16). Dm were pelleted onto copper grids coated with Formvar film, critical point dried, and examined by transmission electron microscopy on a Philips 300 electron microscope (16). Uniform diameter 0.5 μ m-polystyrene microspheres (Coulter Electronics, Inc., Hialeah, Fla.) were included in the preparations as an internal size standard.

RESULTS

In the zonal centrifugation procedure, dm migrated to a region of 22-25% sucrose which corresponded to an approximate sedimentation coefficient of 5×10^4 s. The maximum enrichment of dm by zonal centrifugation was 43% dm by mass.

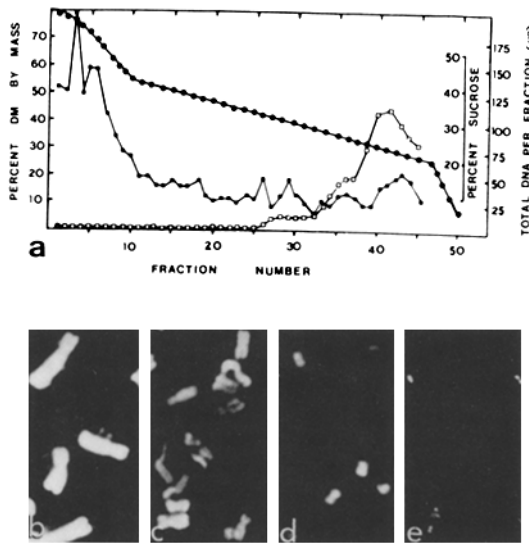


FIGURE 1 Sucrose gradient fractionation of mitotic cells containing dm. The 20-40% (wt/vol) linear sucrose gradient (1 M hexylene glycol, 0.5 mM CaCl_2 , 0.1 mM PIPES, pH 6.8) was loaded dynamically with mitotic lysate as reported (15). Centrifugation was performed for 1 h at 3,000 rpm in a Sorvall RC-2B ultracentrifuge equipped with zonal rate controller and a SZ-14 zonal rotor. The gradient was fractionated statically into 20-ml aliquots. Sucrose percentage (●) was monitored by refractive index. DNA (■) was assayed by the diamino-benzoic acid assay (17). Dm mass percentage (□) was assayed by photographic comparisons of 20 microscope fields enlarged to $\times 8,000$ final magnification. (A) Quantitation of a typical sucrose gradient. Dm appeared in fractions 34-45 with the highest proportion in fraction 41 (43% dm). (B-E) Micrographs of ethidium bromide-staining particles pelleted from indicated fractions onto round coverslips. B is an unfractionated metaphase showing chromosomes and dm. C is from gradient fraction 5 and shows C-group human chromosomes. D is from fraction 17 and shows smaller G- and F-group human chromosomes. E is from fraction 41 and shows dm. Bar, 10 μ m.

Contaminating ethidium bromide-staining particles were primarily F- and G-group chromosomes.

In the differential pelleting protocol in Fig. 2, the initial centrifugation at low speed removed interphase nuclei and intact mitotic figures. The second centrifugation step at 16,300 g pelleted chromosomes. The final high speed centrifugation at 70,000 g pelleted dm. The 70,000 g pellet was found to contain 85% dm by mass. Under these conditions, one third of the particles remained in typical double form and two thirds were present as single particles as in Fig. 3 (1,318 particles scored). Contamination of the 70,000 g pellet was monitored in 30 fields in a preparation containing an average of 1,000 dm/field when photographed at $\times 1,000$. The results were: two A-group chromosomes, 35 C-group chromosomes, two D-group chromosomes, two E-group chromosomes, 21 F-group chromosomes and 13 G-group chromosomes. Comparison of these results with unfractionated metaphase cells as in Fig. 3 indicated an enrichment for dm of 100-fold. In parallel control experiments with karyotypically normal human fibroblasts, the 70,000 g pellet was free of ethidium bromide-staining material.

The characteristically double particles observed in light micrographs showed ultrastructural features similar to mammalian chromosomes prepared by the same procedures (16). As expected

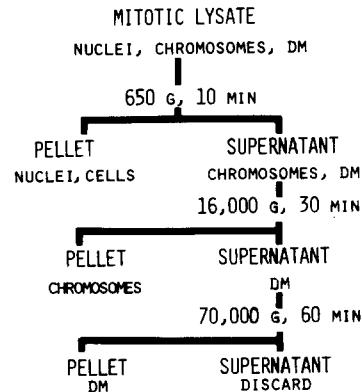


FIGURE 2 Differential pelleting protocol used for the isolation of dm from human carcinoma cell line SW 613-S 18. About 10^7 mitotic cells were lysed as reported into 5 ml of chromosome isolation buffer (15). Mitotic lysate was then layered onto 20% (wt/vol) sucrose for the first step which pellets nuclei and intact metaphase figures. The 650 g supernate was decanted, diluted 1:1 (vol/vol) with buffer and centrifuged as indicated. The first two centrifugation steps were performed in an HB-4 rotor in a RC-2B ultracentrifuge. The final pelleting was done with an SW 27 rotor in an L5-65 ultracentrifuge.

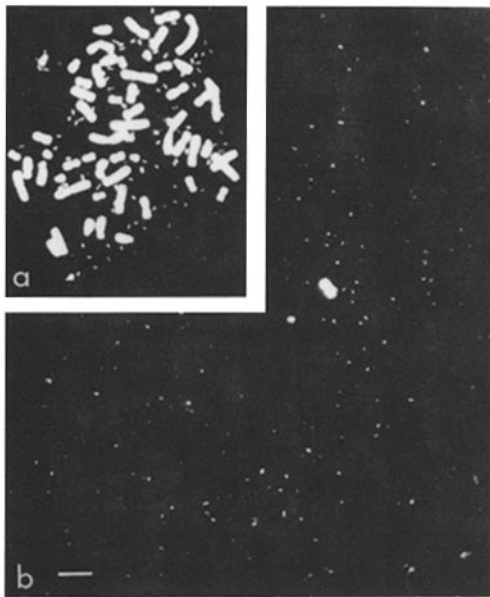


FIGURE 3 Micrograph of isolated dm from human carcinoma cell line SW 613-S 18. (A) shows an ethidium bromide-stained unfractionated metaphase cell. Particles from the 16,300 g supernate were pelleted onto copper grids coated with Formvar film and stained with ethidium bromide for light microscope evaluation (B). Similar grids were examined by transmission electron microscopy as in Fig. 4. The field shown contains numerous ethidium bromide-staining particles which are either double (dm) or single (sm). One human C-group chromosome is noted in this field. Bar, 10 μ m.

from previous anaphase cytology and cytochemical staining of dm in SW 613 (4), the dm showed no evidence of centromeric constrictions or membrane fragments often observed at chromosomal telomeres. Chromatin fibers similar to those of metaphase chromosomes are evident in dm as in Fig. 4. In some cases, the two single particle halves may be connected by similar fibers. The heterogeneity in size of dm in light micrographs is also noted in electron micrographs of isolated dm from cell line SW 613-S 18. It is presently unclear whether the sizes of dm represent a continuous distribution or whether dm are present in discreet multimers of some unit size. Dm are $\sim 0.5 \mu$ m in size (diameter of the single minute) by comparison with the microspheres included in the preparation.

DISCUSSION

Dm in this variant cell line have been shown to contain semi-conservatively replicating DNA (3,

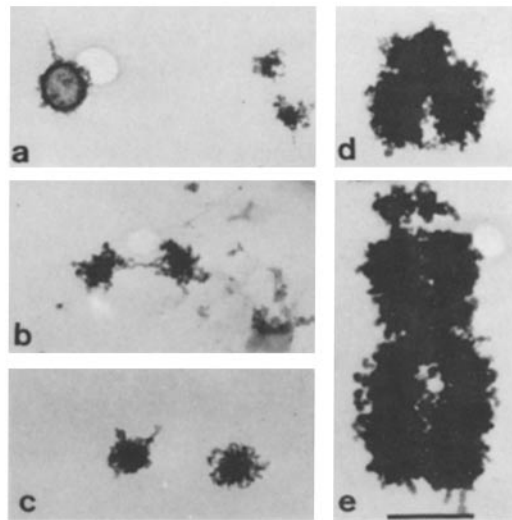


FIGURE 4 Transmission electron micrographs of unstained chromosomes and dm from human carcinoma cell line SW 613-S 18. (A) A 0.5- μ m polystyrene microsphere is noted on the left and a dm pair appears to the right. (B) Fibers of chromatin interconnect the singlet halves of a dm. (C) Larger dm appearing without interconnecting fibers. (D) A human G-group chromosome. (E) A human C-group chromosome adjacent to a dm. Bar, 1 μ m.

5). Dm-DNA from SW 613-S 18 replicates only at S phase of the cell cycle, not at G1, G2, or mitosis (3). In this line, dm-DNA replicates throughout the S phase in distinction from the simultaneous replication of all dm early in S phase in other systems (8). Although the ultrastructural similarities intimate the presence of proteins, no direct data are available. Photographic measurements of dm and normal human chromosomes indicate that dm are, on the average, $\sim 3\%$ the size of normal human G-group chromosomes, or $\sim 10^{-15}$ g DNA. This is on the order of size of an *Escherichia coli* genome and should contain $\sim 10^3$ kb DNA. At most, dm-DNA from SW 613-S 18 might encode $\sim 10^3$ genes/dm. In a murine system (7), there is evidence that dm-DNA may code for dihydrofolate reductase. This suggests dm-DNA contains structural genes in some systems. In SW 613-S 18, it is not known whether dm-DNA is transcribed or whether the dm-DNA base sequence is eventually represented in a translated mRNA as protein.

The unequal segregation of dm at anaphase leads to a random distribution of dm to daughter cells in SW 613 (3-5). Characteristically, the cells

contain dm in numbers ranging from zero to over 1,000 dm/cell in the same cell population (3). This cytological variability predicts a parallel variability of phenotype among cells in which dm-DNA codes for specific cell products. The variability in number of dm among cells of SW 613-S 18 is consistent with absence of centromeres (3-5). Clusters of dm contained in a matrix suspected to be RNA (4, 9) and adherence of these clusters as the chromosomes segregate at anaphase suggest a mechanism for the random distribution of dm to daughter cells at cell division (4). The relationship between dm clusters and the micronuclei seen at interphase in the cells of SW 613-S 18 is not clear.

Direct preparations of tumor cells from patients before chemotherapeutic intervention have shown dm (12), an indication that dm are not artifacts of cell culture or the direct result of therapeutic regimen. Although reports of tumors containing dm show a distinctive bias toward tumors of neurogenic origin, this association must be evaluated for possible ascertainment bias as dm have also been reported in diverse other tumor types and in leukemia (3, 5). The association of cellular resistance to methotrexate with dm (7) makes isolation and chemical investigation of dm of possible clinical interest. The isolation protocol reported here enabled the ultrastructural study of dm with the result that dm appear smaller in size, but ultrastructurally similar to chromosomes in the organization of fibers in each. In addition, the simple differential pelleting procedure resulting in 100-fold enrichment for dm should allow chemical work on dm which was previously not feasible for lack of an appropriate, high-yield isolation procedure.

We thank Dr. D. L. Robberson for use of his electron microscope facility.

This investigation was supported in part by National Science Foundation grant PCM 78-08926 and by National Cancer Institute grant CA 16672.

Received for publication 1 August 1979, and in revised form 24 September 1979.

REFERENCES

1. ALT, F. W., R. E. KELLEMS, J. R. BERTINO, and R. T. SCHIMKE. 1978. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.* **253**:1357-1370.
2. BALABAN-MALENBAUM, G., and F. GILBERT. 1977. Double minute chromosomes and homogeneously staining regions in chromosomes of a human neuroblastoma cell line. *Science (Wash. D. C.)* **196**:739-741.
3. BARKER, P. E. 1978. Double minutes in human tumor cells. M.S. Thesis. University of Texas Graduate School of Biomedical Sciences, Houston, Texas.
4. BARKER, P. E., and T. C. HSU. 1978. Are double minutes chromosomes? *Exp. Cell Res.* **113**:456-458.
5. BARKER, P. E., and T. C. HSU. 1979. Double minutes in human carcinoma cell lines with special reference to breast tumors. *J. Natl. Cancer Inst.* **62**:257-262.
6. COX, D., C. YUNCKEN, and A. I. SPRIGGS. 1965. Minute chromatin bodies in malignant tumors of childhood. *Lancet.* **1**:55-58.
7. KAUFMAN, R. J., P. C. BROWN, J. H. NUNBERG, and R. T. SCHIMKE. 1979. Karyotypic alterations associated with stable and unstable methotrexate resistance and dihydrofolate reductase gene amplification. *J. Supramol. Struct. Suppl.* **3**:79.
8. LEVAN, A., G. LEVAN, and N. MANDAHL. 1978. A new chromosome type replacing the double minutes in a mouse tumor. *Cytogenet. Cell Genet.* **20**:12-23.
9. LEVAN, G., N. MANDAHL, B. O. BENGTSOON, and A. LEVAN. 1977. Experimental elimination and recovery of double minute chromosomes in malignant cell populations. *Hereditas.* **86**:75-90.
10. LUBS, H. A., J. H. SALMON, and S. FLANIGAN. 1966. Studies of a glial tumor with multiple minute chromosomes. *Cancer (Phila.)* **19**:591-599.
11. MARK, J. 1967. Double minutes. A chromosomal aberration in Rous sarcomas in mice. *Hereditas.* **57**:1-22.
12. MARK, J. 1971. Chromosomal characteristics of neurogenic tumors in adults. *Hereditas.* **68**:61-100.
13. MOORE, G. E., L. K. WOODS, L. A. QUINN, R. T. MORGAN, and T. U. SEMPLER. 1979. Colon carcinoma cell lines from four patients. *In Vitro (Rockville)* **15**:197.
14. O'NEILL, F. J. 1974. Complex chromosome aberrations in continuous mammalian cell lines. *Chromosoma.* **47**:453-462.
15. PADGETT, T., E. STUBBLEFIELD, and H. E. VARMUS. 1977. Chicken macrochromosomes contain an endogenous provirus and microchromosomes contain sequences related to the transforming of ASV. *Cell.* **10**:649-657.
16. STUBBLEFIELD, E. 1971. Electron microscopy and autoradiography of chromosomes. *Methods Enzymol.* **40**:63-75.
17. THOMAS, P. S., and M. N. FARQUHAR. 1978. Specific measurement of DNA in nuclei and nucleic acids using diamino benzoic acid. *Anal. Biochem.* **89**:35-44.