The Number of Mitochondrial Deoxyribonucleic Acid Genomes in Mouse L and Human HeLa Cells

QUANTITATIVE ISOLATION OF MITOCHONDRIAL DEOXYRIBONUCLEIC ACID*

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SUMMARY

Mitochondrial DNA can be isotopically labeled to the virtual exclusion of nuclear DNA labeling in cell lines lacking the major soluble thymidine kinase (EC 2.7.1.21) but retaining a mitochondrial thymidine kinase. This characteristic provides a means for the selective assay of mitochondrial DNA during isolation and a determination of the cellular content of mitochondrial DNA. The simplest of the three isolation procedures compared in this study is shown to yield approximately two-thirds of the total mitochondrial DNA in the tissue culture cells examined. Mouse L cells containing predominantly a 10^5 dalton closed circular mitochondrial DNA species have 1100 ± 250 molecules per cell. A second line of mouse L cells which has mitochondrial DNA of molecular weight 2 × 10^5 contains approximately 900 molecules per cell. HeLa cells have at least four times the mitochondrial DNA mass per mitochondrial volume as L cells.

Closed circular animal mtDNA possesses a number of distinctive characteristics which have stimulated a wide range of studies (1–3). The majority of such studies of mtDNA structure and function have examined properties of that fraction of mtDNA which can be purified from isolated mitochondria. Therefore, the proper interpretation of much of this work relies upon knowledge of the quantity of mtDNA in a cell and the efficiency with which it can be isolated. Reported attempts at mtDNA quantitation have related mtDNA content to the number of mitochondria or the mass of mitochondrial protein isolated. The studies reviewed by Nass (3) and by Borst and Kroon (4) indicate a yield of 0.2 to 1.8 µg of mtDNA per mg of mitochondrial protein, or a content of roughly 2 to 10 mtDNA molecules per organelle, depending upon the animal cell or tissue examined. However, in all of these studies it was impossible to selectively assay mtDNA during the often lengthy purification from a several hundred-fold excess of nuclear DNA. We report here an analysis of the isolation of mtDNA from animal cells in which mtDNA is isotopically labeled to a much higher specific activity than nuclear DNA. This analysis has enabled us to estimate both the total cellular mtDNA content and the yields of several types of mtDNA purification procedures.

The L and HeLa thymidine kinase minus cell lines used in this study were derived by growth in increasing quantities of BrdUrd1 (5), a procedure which selects for cells lacking the ability to incorporate exogenous thymidine into nuclear DNA due to the loss of the major cellular thymidine kinase (EC 2.7.1.21). Such cell lines retain a mitochondrial-specific activity (6, 7) which allows mtDNA labeling with exogenous radioactive thymidine. Two L cell lines were compared in this study. The LMTK− cell line contains predominantly monomer length (approximately 5 µm) mtDNA molecules, while the mtDNA from LDTK− cells is almost exclusively in the form of dimer length molecules (8). The radioactive labeling technique has also been used to quantitate the mtDNA content of HeLaTK− cells.

EXPERIMENTAL PROCEDURE

Materials

[methyl-³H]Thymidine from New England Nuclear (31.5 Ci per mmol) was repurified by descending paper chromatography on Whatman No. 3MM paper in a solvent of water-saturated 1-butanol-concentrated NH₄OH (100:1). Chromatographically purified thymidine was >99% pure in our assay and was recovered in 85% yield.

Cell Growth and Labeling Conditions

LDTK−, LMTK−, and C2-1 cells, a subclone of LMTK− (9), were grown in suspension cultures. Data obtained from C2-1 and LMTK− cells were combined since these cell lines showed no differences relevant to this report. A few experiments were performed with a cell line, LA9, which expresses the major cellular thymidine kinase. LA9 cells were also propagated in suspension culture. All spinner-adapted cell lines had doubling times of 18 to 22 hours at 37°, and reached stationary phase at 1.2 × 10⁶ cells per ml. HeLaTK− cells were grown in monolayer culture. All cells were grown in Eagle's minimum essential medium supplemented with 10% calf serum (Flow Laboratories or Pacific Biological Co.), 10⁻³ M glutamine, 100 i.u. per ml of penicillin, and 100 µg of streptomycin per ml. The abbreviations used are: BrdUrd, 5-bromodeoxyuridine; SDS, sodium dodecyl sulfate.

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cillin, and 50 µg per ml of streptomycin. Chase medium contained only 2% calf serum. All cell lines tested free of mycoplasma. Cells were counted either in a Coulter model B cell counter or with a hemocytometer. Cell counts using the hemocytometer were judged accurate when cell densities counted on two grids varied by less than 10%. Cell counts obtained both before and after incubation with isotope were averaged for the expression of data on a per cell basis. Isotopic labeling was achieved by addition of 1 µCi per ml of [methyl-3H]thymidine at a total concentration of 0.02 µCi to cells grown to 5 to 7 x 10^6 per ml. After 5 hours of incubation in the presence of isotope, cells were pelleted at 37°C, resuspended in 37°C medium made 0.33 µM in nonradioactive thymidine, and cultured for 1 hour before final harvesting by centrifugation.

Isolation of Mitochondria

Three different mitochondrial isolation procedures were used which will be referred to according to the type of sucrose gradient employed. Indicated reagent volumes refer to the handling of 1 ml of packed cells (approximately 4 x 10^6 mouse L cells). All steps were performed at 0-4°C except as indicated.

"Two Step" Procedure-The 1-ml pellet of T ills was washed in 10 to 20 ml of TD buffer (134 mM NaCl, 5 mM KC1, 0.7 mM Na_2HP0_4, 2.5 mM Tris, pH 7.5) and centrifuged for 5 min at 2.5 krpm in a Sorvall GLC-1 centrifuge. Cells were resuspended in 10 to 12 ml of MgrSB (reticulocyte standard buffer; 10 mM NaCl, 1.5 mM MgCl_2, 10 mM Tris, pH 7.5) and incubated for 10 min. The replacement of MgCl_2 with CaCl_2 in this reticulocyte standard buffer was tested as an experimental variable (see Results). Swollen cells were disrupted in a glass Dounce homogenizer to yield approximately 95% free nuclei as judged by phase contrast microscopy. Concentrated (2.5 times) mammalian-sucrose buffer was added immediately to a final concentration of 0.21 M mannitol, 0.07 M sucrose, 5 mM Tris, 5 mM EDTA, pH 7.5. Nuclei were removed by two successive sedimentations of 4 min each at 2.5 krpm in the Beckman JA-29 rotor. The resulting supernatant was designated the cytoplasmic fraction. Mitochondria were pelleted from this supernatant in a Beckman JA-20 rotor at 15 krpm for 20 min in the J-21 centrifuge. This pellet was resuspended in 5 ml of mannitol-sucrose buffer and layered over a discontinuous sucrose gradient consisting of 15 ml of 1.0 M sucrose, 5 mM EDTA, 10 mM Tris, pH 7.5, on top of 15 ml of 1.5 M sucrose, 5 mM EDTA, 10 mM Tris, pH 7.5. The tubes were centrifuged for 30 min at 22 krpm in a Beckman SW 27 rotor. The mitochondria were collected from the 1.0 to 1.5 M sucrose interface by pipetting, diluted with 2 volumes of mannitol-sucrose buffer, and pelleted by centrifugation as above.

"One Step" Procedure-Cells were homogenized in MgrSB as above. One-tenth volume of 2.0 M sucrose, 35 mM EDTA, 50 mM Tris, pH 7.5 (instead of concentrated mannitol-sucrose buffer), was added immediately to stabilize mitochondria against osmotic rupture. Nuclei were pelleted from the homogenate as in the "two step" procedure. The supernatant was layered over 15 ml of 1.5 M sucrose, 5 mM EDTA, 10 mM Tris, pH 7.5. The tubes were centrifuged at 22 krpm in an SW 27 rotor for 30 min. Mitochondria were collected from the interface of the one step sucrose gradient and pelleted as in the "two step" procedure.

"No Gradient" Procedure-The most direct mtDNA purification procedure used mitochondria isolated without a sucrose gradient step. This procedure represents a modification of the mitochondrial isolation method of Schneider (30). In this "no sucrose" procedure cells were homogenized and nuclei pelleted as in the "one step" procedure. Mitochondria were pelleted from the supernatant of the nuclear pellet at 15 krpm for 20 min in the Beckman JA-20 rotor. The mitochondrial pellet was resuspended in 20 ml of mannitol-sucrose buffer and mitochondria and nuclei were repelleted as above.

Isolation of DNA

The final mitochondrial pellet was dispersed in 2 ml of 10 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5. Lysis was achieved by the addition of 60 µl of 25% SDS and incubation for 3 to 5 min at 37°C. Then 0.5 ml of 7 M CaCl_2 was added before incubation of the tubes on ice for 10 min. Chilling precipitates cesteus dodecyl sulfate and some protein, which were removed by centrifugation for 10 min at 10 krpm in the JA-20 rotor. The effect of omitting this cesteus dodecyl sulfate removal is discussed under "Results."

Analytical Methods

The distribution of acid-insoluble radioactivity among fractions obtained in mtDNA isolation was studied by withdrawing and counting triplicate samples at each stage of purification. Samples were dried onto 2.4-mm Whatman GF/A filters, washed with 5% CCl_4/COOH, rinsed with 95% ethanol, and dried. Radioactivity was measured by counting in 5 ml of toluene-based cocktail containing either 4.0 g per liter of omnifluor or 4.95 g per liter of 2,5-diphenoxazole and 0.51 g per liter of 1,4-his (2,5-phenyloxazolyl)benzene in a Beckman LS290 liquid scintillation counter. Tritum counting efficiency under these conditions was 35 ± 2%.

For determination of specific activities, DNA samples were freed of ethidium bromide by dialysis for 10 to 24 hours against 1 M NaCl, 1 mM EDTA, 5 ml Tris, pH 7.5, containing Dowex 50 cation exchange resin. Dialysis continued for at least 60 hours against at least four changes of SSC buffer (0.13 M NaCl, 0.005 M sodium citrate, pH 8.3). Optical densities were determined in either a Gilford 240-S or Zeiss PMQ-II spectrophotometer. Concentration was calculated using the relation 1.0 A unit at 260 nm equals 50 µg per ml of DNA. Absorbance readings were considered accurate if the A_260/A_280 ratio exceeded 1.9. The degree of apparent DNA contamination of mtDNA preparations was determined using analytical alkaline CsCl buoyant density centrifugation. DNA samples were pelleted from SSC buffer in polyallomer tubes in the SW 50.1 rotor at 30 krpm for 20 hours and were then brought to pH 12.8 and density 1.752 g per ml and centrifuged for 30 hours at 44 krpm at 25°C in the Beckman model E ultracentrifuge equipped with photoelectric scanner. Preparative alkaline CsCl buoyant density gradients were prepared with the same solution conditions and were centrifuged for 48 hours at 30 krpm at 20°C in the SW 50.1 rotor.

Cell sections were prepared by a modification of the method of Karnovsky (11) and were observed in the Philips EM201 electron microscope. Negatives were obtained at a magnification of X 2300 on 35-mm film and were enlarged to 18 cm for analysis by the method of Long (12). Mitochondrial and nuclear fractions of the total cell volume were calculated from photographs of 20 randomly selected fields for each cell type analyzed.

RESULTS

Use of Thymidine Kinase Minus Cells-We have utilized the specific mtDNA labeling property of thymidine kinase minus cells in the studies described below. Since mtDNA in such cells is labeled to an approximately 70-fold greater specific activity than is nuclear DNA (6), one can assay for incorporated radioactive thymidine at any step in a purification procedure and quantitatively measure the recovery of mtDNA at that point. It is also possible to quantitate the total amount of mtDNA in these cells by determining the specific activity of mtDNA and the total amount of incorporated isotope per cell. However, even in TK- cell lines the majority of [H]thymidine incorporated is contained in the nuclear fraction. To demonstrate that this

2 When LMTK- cells were labeled with commercially obtained [methyl-3H]thymidine without additional purification, two-thirds of the radioactivity incorporated into the cytoplasmic fraction was alkali-labile and RNase-sensitive. [H]Thymidine obtained from either Schwarz-Manu or New England Nuclear was found to contain a radioactive contaminant with chromatographic mobilities characteristic of uridine. After correction for alkali-labile Incorporation, the data of experiments using impure [H]thymidine were consistent with experiments reported here in which purified [H]thymidine was used.
Table I

Specific activities of pulse-chase labeled DNA from TK− cells

Nuclear DNA and mtDNA were purified from LMTK−, LDTK−, and HeLaTK− cells in 2-to-8-μg quantities to allow specific activity determinations. All experimental procedures were as described for a 5-hour radioactive pulse and 1-hour chase. Data are used to allow estimation of the total cellular mtDNA content, which is also expressed as mtDNA genomes per cell.

| Cell and DNA | Specific activity | mtDNA |
|--------------|------------------|-------|
|              | 14C cpm/μg DNA   | μg/10⁶ cells | genomes/cell |
| LMTK− mtDNA | 2300 ± 400       | 1.8 ± 0.4 | 1100 ± 250 |
| Nuclear DNA  | 28 ± 4           | 140    |
| LDTK− mtDNA | 1200             | 3.0    | 1800    |
| Nuclear DNA  | 59               | 1090   |
| HeLaTK− mtDNA | 1640             | 15     | 8800    |
| Nuclear DNA  | 120-250          |        |

represents labeling of nuclear DNA rather than a loss of mtDNA, an aliquot of the washed nuclear pellet was lysed with SDS and centrifuged in a buoyant alkaline CsCl density gradient. Approximately 75% of the original radioactivity was recovered in the alkaline CsCl gradient (Fig. 1A). At least 95% of the radioactivity in the nuclear fraction did not show the differential buoyant density characteristic of mtDNA in alkaline CsCl (Fig. 1B) (5, 13). This observation was reproduced in two additional preparative alkaline CsCl gradients with nuclear DNA from HeLaTK− cells and a second preparation of LMTK− nuclei (data not shown). The nuclear labeling observed in LMTK− cells resulted from an average nuclear DNA specific activity of 28 ± 4 cpm per μg (Table I). Similar nuclear DNA labeling was observed in LDTK− and HeLaTK− cells.

mtDNA Isolation Procedure—One of the objectives of this study was to devise a procedure for purification of mtDNA in high yield. The following considerations are relevant to this objective. (a) The data of Robberson and Clayton (14) indicated that some mtDNA loss was caused by a DNase treatment of isolated mitochondria or by simple incubation of mitochondria at 37° in DNase buffer without added nuclease. (b) The discontinuation of this enzyme treatment, which had been a standard step in mtDNA purification procedures published by several groups (8, 15-17), led to an unavoidable contamination of mtDNA with nuclear DNA. (c) In the case of TK− cells, in which nuclear DNA is labeled to a low level relative to mtDNA, this significant contamination by nuclear DNA mass provided only a slight radioactive contamination in the upper band of the ethidium bromide-CsCl gradient.

When this study was begun, two sucrose gradient procedures (see “Experimental Procedure”) for purification of mitochondria were in use in this laboratory (6, 18). Early mtDNA labeling experiments with LMTK− cells (data not reported) revealed that the “two step” procedure isolated roughly 65% of the yield of mtDNA radioactivity obtained with the “one step” procedure or 36% of that obtained with the “no gradient” procedure (Fig. 2). This presumably resulted from loss of mtDNA due to mitochondrial damage or aggregation during the first mitochondrial pelleting of the “two step” procedure. The use of the “two step” isolation method was discontinued in further experiments. In addition, experimentation utilizing the “one step” procedure provided the following data. (a) No loss of mtDNA was noted when LMTK− cells were homogenized in 10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris, pH 7.0, containing Ca²⁺ (CaRSB) in place of Mg²⁺ (MgRSB). The use of CaRSB is preferred because it will not stimulate activity of any Mg²⁺ dependent endogenous DNase during mitochondrial isolation (19). Furthermore, the level of nuclear DNA contamination of mtDNA was consistently lower when L cells were homogenized in CaRSB instead of MgRSB, as judged by the comparison of upper band fluorescence intensity in ethidium bromide-CsCl gradients. (b) The conditions of mitochondrial lysis were thoroughly tested in order to maximize the yield of mtDNA. Lysis at 25°, instead of 37°, resulted in a 15% reduction in the yield of mtDNA. In all cases, SDS was used at a 0.6% concentration. Varying the duration of SDS treatment between 0.5 and 30 min had no effect in two trials. (c) Collection of a cesium dodecyl sulfate pellet appeared to cause a slight (<10%) loss of mtDNA during isolation from LMTK− or LDTK− cells. However, since this step removes a large amount of protein, its omission may result in extensive loss of free ethidium bromide from solution due to binding by protein, resulting in poor control over the dye concentration in the ethidium bromide-CsCl gradient.

Quantitation of mtDNA—The radioactivity in the cytoplasmic fraction of LMTK− and LDTK− cells represents mtDNA with a slight nuclear DNA contamination. The maximum level of this...
nuclear DNA contamination has been estimated with experiments using thymidine kinase plus LA9 cells in which only 4% of the total [3H]thymidine incorporation is in the cytoplasmic fraction. Then, assuming a maximum mtDNA loss of 5% in the nuclear fraction, our data show that LMTK- and LDTK- incorporate 4300 and 3600 cpm per 10⁶ cells, respectively, into mtDNA (Table II). The 5-hour pulse-1-hour chase labeling protocol used results in mtDNA specific activities of 2300 cpm per pg in LMTK- and 1200 cpm per pg in LDTK- cells. These data estimate the cellular content of mtDNA as 1.9 pg per 10⁶ cells. For the purposes of this report, we have not attempted to accurately determine the loss of mtDNA during purification from HeLaTK- cells. Instead, the minimum cell content of mtDNA has been estimated as the amount isolated in ethidium bromide-CsCl gradients. HeLaTK- cells grown in monolayer culture contained at least 15 μg of mtDNA per 10⁶ cells labeled to a specific activity of 1640 cpm per μg by the 5-hour pulse-1-hour chase labeling procedure (Table I). Even after considering the larger mitochondrial content of HeLaTK- (7% of cell volume), the mtDNA content of HeLaTK- cells is at least four times as large as that of either TK- L cell line. This result is consistent with our qualitative observation that the mitochondria of HeLaTK- cells are uniformly more well developed (i.e. contain more cristae per unit volume) than the mitochondria of L cells.

**TABLE II**

| Incorporation into mtDNA, cpm/10⁶ cells | LMTK- | LDTK- |
|---------------------------------------|-------|-------|
| mtDNA retained at purification stage, % |       |       |
| 1. Homogenization and nuclear pelleting | 4300 ± 1100 (100%) | 3600 ± 600 (100%) |
| 2. Final mitochondrial pelleting       | 95 ± 4 | 94 ± 4 |
| 3. Mitochondrial lysis and ethidium bromide-CsCl banding | 72 ± 15 | 68 ± 9 |

**FIG. 2.** Radioactivity profiles of ethidium bromide-CsCl buoyant density gradients of mtDNA obtained from LMTK- cells treated identically through the first mitochondrial pelleting and divided into equal parts for further purification by the "no gradient" procedure (●—●) and by the "two step" procedure (O—0). In this experiment the yield of the "no gradient" procedure was 72% of total mtDNA; that of the "two step" procedure was 28% of total mtDNA. Both gradients contained approximately 88% of the total radioactivity in seven lower band fractions.

Although this difference could reflect a different cellular mitochondrial content. Stereological analysis (12) of thin sections of LMTK-, LDTK-, and LA9 cells revealed that all contained 5 ± 1% of the total cell volume within mitochondria. However, the LDTK- cell was approximately 15% larger than the LMTK- cell by comparison of mean cell sizes in spinner cultures at comparable cell densities with the use of the Coulter model B cell counter. Thus, the mtDNA content per unit mitochondrial volume is approximately 40% greater in the LDTK- cell than in the LMTK- cell.

**DISCUSSION**

This study has attempted to quantitate the mtDNA contents of L and HeLa cells grown in tissue culture. This measurement has been complicated in earlier studies (10-11) by the large ratio of nuclear DNA to mtDNA. To compensate for this difficulty these previous studies have resorted to lengthy mtDNA isolation procedures which have involved the use of DNase to remove contaminating nuclear DNA. We have circumvented this problem with the use of cultured cells which, due to the lack of the major cellular thymidine kinase activity (6), label mtDNA to a high specific activity relative to nuclear DNA. Thus, by labeling thymidine kinase minus L and HeLa cell lines with [3H]-thymidine, we have selectively assayed mtDNA during a brief isolation from cultured cells in order to measure the cellular content of mtDNA and the efficiency with which it can be isolated by our methods.

This study has estimated the mtDNA content of LMTK-
cell as 1.9 ± 0.4 μg per 10⁶ cells. This amount is the equivalent of 1100 ± 260 mtDNA molecules per cell, which is in excellent agreement with the analysis by Nass of 1300 mtDNA genomes per wild type L cell (17). This agreement between our data and those of Nass may be fortuitous in light of the lengthy mtDNA purification procedure used in her study. We have further shown that the yield of mtDNA from LMTK⁻ cells may be as high as 75% with the “no gradient” isolation procedure or 35% to 50% with the “one step” sucrose procedure. These relatively high yields add credence to those studies of mtDNA structure and replication which have used procedures which isolate the majority of the mtDNA population. The “no gradient” procedure has the advantage of requiring only 90 min for the entire mtDNA isolation process. However, this method involves considerable nuclear DNA contamination of the upper band of the ethidium bromide-CsCl gradient, and is not suitable for mtDNA isolation at high purity without rebanding of closed circular DNA in ethidium bromide-CsCl. With one rebanding step we have consistently obtained mtDNA preparations containing only a 4% nuclear DNA contaminant (Fig. 1B).

Our experiments with LDTK⁻ and HeLaTK⁻ cells have revealed two significant relationships in comparison with LMTK⁻ cells. First, the two L cell lines contain comparable masses of mtDNA per unit of mitochondrial volume. The somewhat larger mtDNA mass of the LDTK⁻ cell may reflect a physiological balance between optimal gene dosage and a requirement for a minimal concentration of mtDNA molecular units. Secondly, the HeLaTK⁻ cell line was found to contain at least four times the mtDNA mass per mitochondrial volume as either L cell line.

Throughout this study we presented measurements of mtDNA content on a per cell basis. This method is convenient for measurements using tissue culture cells. Moreover, Hoffmann and Avers (20) have suggested that the mitochondrial volume of an animal cell may be organized in only one or a few giant branched organelles, as appears to be the case in yeast. However, our data do indicate that the most reasonable procedure by which to compare the mtDNA contents of different cell lines may be at the level of cellular mtDNA mass per mitochondrial volume.

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