DEVELOPMENT AND CHARACTERIZATION OF CONTINUOUS AVIAN CELL LINES DEPLETED OF MITOCHONDRIAL DNA

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SUMMARY

Populations of quail and chicken cells were treated with ethidium bromide, an inhibitor of mitochondrial DNA replication. After long-term exposure to the drug, the cell populations were transferred to ethidium bromide (EtBr)-free medium, and cloned. Clones HCF7 (quail) and DUS-3 (chicken) were propagated for more than a year, and then characterized. Analysis of total cellular DNA extracted from these cells revealed no characteristic mitochondrial DNA molecule by Southern blot hybridization of HindIII- or AvaI-digested total cellular DNA probed with cloned mitochondrial DNA fragments. Reconstruction experiments, where a small number of parental cells was mixed with HCF7 cells and DUS-3 cells before extraction of total cellular DNA, further strengthen the notion that the drug-treated cells are devoid of mitochondrial DNA molecules. The cell populations were found to proliferate at a moderately reduced growth rate as compared to their respective parents, to be auxotrophic for uridine, and to be stably resistant to the growth inhibitory effect of EtBr and chloramphenicol. At the ultrastructural level, mitochondria were considerably enlarged and there was a severe reduction in the number of cristae within the organelles and loss of cristae orientation. Morphometric analysis revealed a fourfold increase of the mitochondrial profile area along with a twofold decrease of the numerical mitochondrial profiles. Analysis of biochemical parameters indicated that the cells grew with mitochondria devoid of a functional respiratory chain. The activity of the mitochondrial enzyme dihydroorotate dehydrogenase was decreased by 95% and presumably accounted for uridine auxotrophy.

Key words: birds; mitochondrial DNA.

INTRODUCTION

We have previously reported that primary chicken embryo fibroblast (CEF) populations are inherently resistant to the growth inhibitory effect of the phenanthridine dye ethidium bromide (EtBr) when supplied with exogenous pyrimidines (21,22). The dye, an inhibitor of mitochondrial DNA (mtDNA) replication (25) and also an extremely efficient cytoplasmic mutagen in facultative anaerobic yeast (29), was found to render long-term EtBr-treated CEF populations respiration deficient (21). When transferred to EtBr-free medium, CEF populations were found to remain respiration deficient and auxotrophic for pyrimidines until senescence. Quantitation of mtDNA by DNA-DNA reassociation kinetics with a probe of chicken liver mtDNA revealed very little or no mtDNA after long exposure of CEF populations to EtBr (5). These results were confirmed by Southern blot hybridization of restriction endonuclease-digested total cellular DNA. These and other observations (5) were interpreted to indicate that EtBr induces loss of mtDNA in CEF populations. These cells resemble Saccharomyces cerevisiae of the [rho0] phenotype (9,24). Recently, we reported that EtBr can also induce the loss of mtDNA from immortalized fibroblastoid-like avian cells (6). The present paper deals with the development and characterization of this cell line maintained in the absence of EtBr for over a year as well as those of a mtDNA-depleted, continuous epithelioid-like cell line derived from another subspecies of galliform birds.

MATERIALS AND METHODS

Cell lines and culture conditions. LSCC-H32 cells, a continuous quail cell line (6), were a gift from O.R. Kaaden, Institute for Virology and Clinic for Poultry, Hannover School of Veterinary Medicine, Hannover, FRG. The immortalized cells produced avian lymphoid leukemia viruses of subgroups A and B (13). DU249 cells, a transplantable cell line derived initially from a liver lesion induced in a white Leghorn chicken by the MC29 strain of avian leukemia virus (17), were kindly provided by A. J. Langlois, Department of Surgery, Duke University Medical Center, Durham, NC. Virus released from these cells consisted of both subgroup A and B components. DU24, a subclone of the
DU249 cell line, was isolated from semisolid agar medium with a Pasteur pipette. All continuous cell lines were grown in Ham's F12 medium supplemented with 15% (LSCC-H32) or 10% fetal bovine serum. Penicillin (100 IU/ml), streptomycin, (100 μg/ml), Fungizone (0.5 μg/ml), and uridine (4 μg/ml) were routinely added to the culture medium. Cultures were maintained at 37°C in a humidified incubator with an atmosphere of 95% air: 5% CO₂. Cells were passaged twice a week, and the medium was changed every other day.

Assay of growth. For analysis of saturation density, cells were harvested by trypsinization and plated at a density of 0.5 to 1.0 × 10⁶ cells/60-mm plastic culture dish. Cell number was determined in duplicate dishes at 1- to 2-d intervals with a Coulter counter. Media were first changed every other day and then daily once cultures reached confluence. The colony-forming efficiency of cells on plastic substrate was determined by seeding 500 cells harvested by trypsinization per 60-mm culture dish. The dishes were refed every 3 to 4 d up to 21 d after seeding. Colonies were fixed with methanol, stained with crystal-violet, and scored. Only colonies with a minimum of about 60 cells, representing at least 6 cell divisions, were counted.

Growth curves. The number of generations in each passage of cells exposed or not to EthBr for long periods of time was calculated as reported previously (23), except that the cell medium was changed every other day and the number of cells present in the medium at each refeeding was not counted. Cell attachment efficiency, defined as the proportion of seeded cells that were attached to the plastic surface after 5 h, was determined at some of the passages only and found to range for EthBr-treated cells from 45 to 75% and from 55 to 65% for control cells. An arbitrary cell attachment efficiency value of 60% was used to calculate the relative number of generations made at each passage.

Electron microscopy. Subconfluent monolayers were washed twice with cold 0.1 M Na phosphate buffer, pH 7.4. The cells were scraped from the bottom of the culture dishes using a rubber policeman, concentrated by centrifugation, and fixed on ice for 1 h in 2% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.4. The cells were washed with phosphate buffer, postfixed in 1% OsO₄, (1 h, 4°C), and resuspended in 2% low-melting agarose. The solidified pellets were cut in small pieces, dehydrated in alcohol, and embedded in Araldite. Thin sections (thickness about 50 to 60 nm) were stained with uranyl acetate followed by lead citrate and viewed in a Philips EM-300 electron microscope.

Morphometric analysis. Subconfluent monolayers were prepared for electron microscopy as described above. For each cell line, a single uranyl acetate-lead citrate contrasted thin section was studied. Photographs (negative magnification, ×4000) were taken and negatives were printed at a final magnification of ×23 000. On each of the 85 prints obtained, cellular and mitochondrial profiles were measured using a modular system for quantitative digital analysis (MOP-3, Carl Zeiss Inc.). The data obtained were used to determine the following: the numerical mitochondrial profile per cell; the mean area of cellular and mitochondrial profiles, expressed in μm² × 10⁻²; and the relative mitochondrial volume, i.e. the percentage of cytoplasmic area occupied by mitochondria, expressed in percentage (12).

Cytochrome c oxidase, dihydroorotate dehydrogenase, cytochrome spectra, and protein determinations. The assays and procedure used were as previously described (5,10,23). Briefly, cytochrome oxidase activity was measured by following the oxidation of reduced cytochrome c. Dihydroorotate dehydrogenase was assayed by measuring the conversion of labeled dihydroorotic acid to orotate. The concentration of protein was determined according to the Lowry et al (19) method, using bovine serum albumin as reference standard.

Preparation of mitochondrial DNA. Chicken mtDNA was extracted from the mitochondrial fraction prepared from white Leghorn chicken (Spafas Inc., Norwich, CT) livers, and separated from degraded nuclear DNA by centrifugation through a two-step CsCl-EthBr gradient essentially as described previously (5,32). Isolation of mtDNA and estimation of its concentration in 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA was performed as described (5).

A mitochondrial fraction was prepared from the liver of Japanese quails (5 to 8 wk old) as described before for chickens (5). The mitochondrial fraction was resuspended in 5 mM Tris-hydrochloride (pH 7.2), 1.5 mM MgCl₂, 50 mM NaCl, 0.25 M sucrose, and treated with DNase I (100 μg/ml) and RNase A (100 μg/ml) for 15 min at 37°C. After pelleting, the fraction was washed twice, lysed with 1% sodium dodecyl sulfate, 0.01 M EDTA, 0.01 M Tris (pH 7.4), and incubated with pronase (75 μg/ml; self-digested for 2 h at 37°C) at 37°C for 30 min. The lysate was adjusted to 1 M CsCl, kept on ice for 30 min, and then centrifugated at 12 (Krpm) for 10 min. The supernatant fraction was centrifuged overnight at 40 Krpm (4°C). The pellet was solubilized in 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA, and the soluble material extracted twice with a mixture of phenol; chloroform (1:1) and once with chloroform. After ethanol precipitation, the material was washed twice with 70% ethanol and solubilized in 10 mM Tris-hydrochloride (pH 7.4) and 1 mM EDTA. The mtDNA concentration was estimated by electrophoresis on 0.7% agarose gels with various concentrations of pMtC35 DNA (see below) as reference standards.

![Graph](image)  
**Fig. 1.** The physical map of chicken mitochondrial DNA, linearized at one of two XhoI sites (0), pMtC10, a 4.3 kb fragment cloned into the BamHI site of pBR322; pMtC35, a 7.2 kb fragment cloned into the SalI site of pUC18. A, AalI; B, BamHI; H, HindIII; X, XhoI.