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An Established Avian Fibroblast Cell Line Without Mitochondrial DNA

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Abstract—*An established avian fibroblast cell line (LSCC-H32) has been found to be inherently resistant to the growth-inhibitory effect of ethidium bromide, when supplied with exogenous uridine. After long-term exposure to ethidium bromide (90 days), the cell population has been transferred to drug-free medium for 60 days, and then seeded at low cell density. Three clones have been isolated and propagated in drug-free medium for 5, 6, and more than 12 months, respectively. It was found that none of these cell lines had detectable cytochrome* c *oxidase activity and that they were virtually devoid of cytochromes* aa_3 *and* b. *Mitochondrial DNA was quantitated by DNA–DNA reassociation kinetics with a probe of chicken liver mitochondrial DNA. A mean number of 300 copies of mitochondrial DNA per cell was found in LSCC-H32 cells. Analysis of DNA extracted from cell populations exposed to ethidium bromide for 90 days and then transferred to drug-free medium for long periods of time revealed no mitochondrial DNA molecules by reassociation kinetics or by Southern blot hybridization of HindIII- or AvaI-digested total cellular DNA.*

INTRODUCTION

The extent to which the mitochondrial genome (mtDNA) is involved in the establishment, maintenance, and promotion of vertebrate cell phenotypic traits is largely unknown. Several groups (1–3) have constructed intraspecific cybrids using drug resistance specified by mtDNA as selective markers and sometimes demonstrated that the cytoplasm of the donor cells can prevent or modulate phenotypic traits expressed by the recipient cells. Although some conclusions can be drawn on the extent of contribution of mtDNA, these studies are impaired by the fact that variable amounts of mtDNA from both parents are retained and expressed in cybrids (4) and that cytoplasmic factors other than mtDNA may be involved in this process.

To circumvent these disadvantages and also to provide a novel means to study the contribution of mtDNA to cell phenotype (5, 6), we have worked for some time on the development of vertebrate cell systems devoid of mtDNA. In the course of these studies, we have observed that primary chicken embryo fibroblasts (CEF) populations were inherently resistant to the growth-inhibitory effect of ethidium bromide (EtdBr), when supplied with exogenous pyrimidines (7). The drug is a well-known inhibitor of mtDNA replication (8), and in the presence of this agent, the mtDNA content of CEF was progressively diluted during succeeding cell generations by a factor of $\frac{1}{2}n$ (9), where n is the number of cell doublings. Continuous cell proliferation in the presence of EtdBr resulted in cell popula-

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tions apparently devoid of mtDNA (9). The cells were respiration deficient and retained this phenotype until senescence whether or not they were transferred to EtdBr-free medium (9, 10). The fact that primary CEF populations treated (9, 10) or not (9, 11) with EtdBr have a finite life-span in vitro was found to be of some inconvenience, however, in carrying out long-term studies. Recently, Kaaden et al. (12) have been successful in establishing a chicken embryo fibroblast cell line (LSCC-32). Here we report the isolation from LSCC-H32 cells, after long-term exposure to EtdBr, of an established cell line devoid of characteristic mtDNA molecules.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Growth Curves. Primary CEF populations were prepared from 8- to 9-day-old embryos obtained from Spafas (Norwick, Connecticut) and cultured as previously described (7, 9). Cell line LSCC-H32 was established from primary chicken embryo cells spontaneously transformed at 32°C (12). The fertilized eggs used for the preparation of the primary cell culture were obtained from a commercial Leghorn breeding flock. LSCC-H32 cells were cultivated in Ham's F12 medium supplemented with 15% fetal calf serum. Penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (0.5 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 and 5% CO_2. Cells were passaged twice a week, and the medium was changed three times a week. The number of cell generations in each passage was calculated as reported (13). The growth curves obtained were used to determine the mean doubling time of the cell populations.

Cytochrome c *Oxidase, Cytochrome Spectra, and Protein Determinations.* These assays were performed as previously described (9, 13).

Isolation of mtDNA from Chicken Liver. mtDNA was extracted from the mitochondrial fraction prepared from the liver of White Leghorn chickens (Spafas) and isolated by centrifugation through a two-step CsCl–EtdBr gradient essentially as described (9, 14).

Extraction of Total Cellular DNA. Trypsinized cells (2.0×10^8 to 5×10^8 cells) were used to extract total cellular DNA as described (9). For the measurement of DNA–DNA reassociation kinetics and for Southern blot hybridization, the DNA preparations were processed as previously described (9).

Preparation of mtDNA Probes. mtDNA isolated from White Leghorn chicken liver was labeled with [^{32}P]dCTP by nick translation (9).

Measurement of DNA–DNA Reassociation Kinetics, and Southern Blot Hybridization. The methods used to carry out these studies have been previously described (9). To calculate the number of mtDNA copies per cell by reassociation kinetics, the molecular weight of LSCC-H32 cell nuclear DNA was taken as 2.6×10^{12} (4.3 pg of DNA per diploid cell).

RESULTS

To verify if immortalized chicken embryo fibroblasts were also inherently resistant to the growth-inhibitory effect of EtdBr when supplied with exogenous pyrimidines, LSCC-H32 cells were cultivated for three weeks in Hams's F12 medium with or without 4.0 μg uridine/ml, and then EtdBr at 0.4 μg/ml was added. In the absence of uridine, cell proliferation was found to cease after about four generations, and growth arrest was followed by cell death. On the other hand, in uridine-containing medium, the doubling time of the drug-exposed cells was found to increase after three cell doublings and stabilize to about 42 h as compared to 30 h for control cells. During the treatment, cytochrome *c* oxidase activity was measured as a