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Cytoplasmic Inheritance of Chloramphenicol Resistance in Mouse Tissue Culture Cells

(enucleation/5-bromodeoxyuridine resistance/cell fusion/mitochondria/somatic cell genetics)

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ABSTRACT **A chloramphenicol-resistant mutant, isolated from mouse A9 cells, was enucleated and fused with a nucleated chloramphenicol-sensitive mouse cell line. Resultant fusion products, cytoplasmic hybrids (or "cybrids"), were selected as resistant to chloramphenicol, and had the nuclear markers and chromosome complement of the chloramphenicol-sensitive parent. These cybrids appeared at the high frequency of 2–8 per 10^4 cells plated. Neither parent produced any colonies when plated under identical selective conditions. Fusion between enucleated chloramphenicol-sensitive cell fragments and the chloramphenicol-sensitive cell produced no resistant colonies, suggesting that chloramphenicol resistance is not due to an increase in the ratio of cytoplasm to nucleus. Furthermore, fusions between resistant and sensitive nucleated cells produced resistant hybrids at a frequency 100 times less than that of resistant cybrids. Thus, these stable chloramphenicol-resistant cybrids result from the fusion of a chloramphenicol-resistant cytoplasm with a chloramphenicol-sensitive cell. It is proposed, therefore, that chloramphenicol resistance is a cytoplasmically inherited characteristic in this mouse cell line.**

It has been established that mitochondria contain, in addition to their own DNA, the biochemical apparatus for translation and transcription of this genetic information. Studies on the genetics of mitochondria are most advanced in yeast, where several antibiotic resistance and respiratory deficiency mutations are known to be coded in mitochondrial DNA (mtDNA) and cytoplasmic genetic recombination has been demonstrated (1, 2). Mammalian cell mtDNA is smaller in size than yeast mtDNA. Hybridization studies have shown that mammalian mtDNA codes for ribosomal RNA and 12 distinct 4S RNAs (3).

A genetic approach to mtDNA function in mammalian cells requires the isolation of mutants with altered mitochondrial properties, and the demonstration of the cytoplasmic inheritance of such properties. Chloramphenicol (CAP) inhibits mitochondrial protein synthesis in human HeLa cells (4), and this laboratory has recently described a HeLa mutant whose mitochondrial protein synthesis is resistant to CAP (5, 6). Further, CAP resistance in yeast is coded by mtDNA (7). However, no method has been described as yet to demonstrate cytoplasmic inheritance in mammalian cells.

A CAP-resistant mutant of the mouse line A9 has been isolated in this laboratory in a manner similar to that for the HeLa CAP-resistant mutant. This paper describes the experimental evidence that CAP resistance in mouse cells is cytoplasmically inherited.

Abbreviations: TK, thymidine kinase; BrdU, 5-bromodeoxyuridine; HPRT, hypoxanthine phosphoribosyltransferase; CAP, chloramphenicol; HAT, hypoxanthine–aminopterin–thymidine; mtDNA, mitochondrial DNA.

MATERIALS AND METHODS

Strains and Culture Conditions. Strains A9 and LMTK$^-$ are subclones of mouse L-cells, a line of aneuploid fibroblasts. Strain A9 is deficient in hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) activity and resistant to 8-azaguanine; LMTK$^-$ is deficient in thymidine kinase (TK; EC 2.7.1.75) activity and resistant to 5-bromodeoxyuridine (BrdU) (8, 9). Both lines are sensitive to 50 μg/ml of CAP and do not grow in hypoxanthine–aminopterin–thymidine (HAT) medium (10).

Strain 501-1 is a CAP-resistant mutant subclone isolated from A9 by Spolsky in this laboratory. Cultures of A9 inoculated at 2×10^6 cells per flask (75 cm^2) were treated with 2.5 μM ethidium bromide for 18 hr. The cells were washed, fed with fresh medium, and incubated for 8 hr, when 50 μg of CAP per ml of medium was added to each flask. Mutant colonies appeared in $2^1/_2$ months, and were then cloned. CAP resistance is a stable characteristic of the mutant 501-1.

Growth media, cloning conditions, and the methods of determining growth curves and sensitivity curves have all been described by Spolsky and Eisenstadt (5). A CAP-sensitive strain in the presence of CAP undergoes approximately four cell divisions before growth ceases. In such cases the mean cell division time is reported.

Enucleation. Strains 501-1 and A9 were enucleated by a modification of the technique described by Croce and Koprowski (11). Cells were treated with 20 μg of cytochalasin B per ml of medium for 3 hr at 37°, and then centrifuged in the same medium at 10,000 rpm in an SW27 rotor for 45 min. The percentage of enucleated cells in the resultant cell population was estimated by staining an aliquot of the cell suspension with lactoacetic-orcein (12) and counting at least 200 cells.

We have chosen to use the nomenclature suggested by Shay *et al.* (13) in referring to the resultant enucleated cell fragments as *cytoplasts* and the nuclear fragments as *karyoplasts.*

Cell Fusion. Five million cells of each parent were mixed in suspension with 1000 HAU of β-propiolactone-inactivated Sendai virus (pH 7.8) and incubated at 4° for 10 min. Nonselective medium was added and the cells were incubated for 30 min at 37°, and then distributed at various concentrations into flasks containing selective media.

The product of the fusion of a cytoplast and a cell will be referred to as a *cytoplasmic hybrid* (*cybrid*) as opposed to *hybrid,* which refers to the fusion of two nucleated cells.

TABLE 1. *Transfer of chloramphenicol resistance by cytoplasts*

Cells	No. of cells plated	Average no. of colonies per flask*	Colonies per 10^6 cells
en501-1 × LMTK⁻	2×10^6	Confluent (2)	
	1×10^6	Confluent (2)	
	5×10^5	Confluent (3)	
	2.5×10^5	70 (3)	280
	1×10^5	18 (3)	180
	8×10^4	14 (1)	175
	5×10^4	5 (2)	100
en501-1	2×10^6	0	0
LMTK⁻	2×10^6	0	0

Cells were fused and plated in selective medium containing BrdU (30 μg/ml of medium) and CAP (50 μg/ml of medium) as described in *Methods*. The prefix "en" denotes a culture previously treated with cytochalasin B to induce enucleation (see *Methods*). This treatment produced 93% enucleation of 501-1. Cells (5×10^6) of LMTK⁻ and 5×10^6 cells of the enucleated preparation of 501-1 were fused. Colonies growing on flasks in the selective medium were counted when clearly visible (see *text*).

* Numbers in parentheses represent the number of flasks counted at that particular cell number.

Chromosome Analysis. Cells were prepared for chromosome examination by treatment in late exponential phase with colchicine. They were then swollen by incubation for 15 min in hypotonic buffer, fixed with methanol–glacial acetic acid (3:1), and stained with lacto-acetic orcein.

RESULTS

Transfer of Chloramphenicol Resistance by Cytoplasts. In order to demonstrate cytoplasmic inheritance of any characteristic, one must be able to distinguish between nuclear gene inheritance and the postulated cytoplasmic gene inheritance. One way of achieving this distinction in mammalian cells is to separate physically the nucleus from the cytoplasm, and to demonstrate the transfer and stable inheritance of a given characteristic by the cytoplasm in the absence of the nucleus. This has recently become possible following the demonstration of cytochalasin B-induced enucleation in mammalian tissue culture cells (11, 13, 14). Such an experimental system would entail the enucleation of the postulated cytoplasmic mutant and its Sendai virus-induced fusion with a nucleated "wild-type" cell, followed by growth under conditions that select for the product of fusion between mutant cytoplast (enucleated cell) and wild-type cell. These growth conditions should also select against both parents, nuclear–nuclear hybrids, and any mutant cells not enucleated by cytochalasin B treatment.

Accordingly, the CAP-resistant mutant, 501-1, was enucleated as described in *Methods* and fused with the CAP-sensitive LMTK⁻. The fusion mixture was then maintained in the presence of 30 μg of BrdU per ml of medium to select against nucleated 501-1 cells and any hybrids resulting from the fusion of nucleated 501-1 and LMTK⁻. Fifty micrograms of CAP per ml of medium was also added to select against LMTK⁻. Hence, the only cells capable of growing in this selective medium would be BrdU-resistant and CAP-resistant, and would presumably result from LMTK⁻ cells having received CAP resistance from the 501-1 cytoplast.

Two fusion experiments between enucleated 501-1 (en-501-1) and LMTK⁻ were performed, and the results of one are shown in Table 1. BrdU-resistant, CAP-resistant colonies appeared in flasks in both experiments at different but high frequencies relative to normal mutation frequencies. Visible colonies appeared in flasks 9 days after fusion, and were counted after 12 days. The number of such colonies was approximately proportional to the number of cells inoculated into each flask (Table 1).

No colonies appeared in any flasks containing one or the other parent under identical conditions of Sendai virus treatment and BrdU and CAP selection medium. The en501-1 cells in the presence of BrdU become large, flat, and gray within 3 days and continue growing at a decreasing rate. If

TABLE 2. *Comparison of transfer of chloramphenicol resistance in cell–cell and cell–cytoplast fusions*

Fusion/parents	% Enucleation (501-1 or A9)	Ratio of parents	Selective medium	No. of cells plated × 10^6 (0 time)	No. of colonies per 10^6 cells (12–14 days)
en501-1 × LMTK⁻	93	1:1	BrdU + CAP	9.7	184
en501-1 × LMTK⁻	57	1.25:1	BrdU + CAP	3.5	832
enA9 × LMTK⁻	84	1:1	BrdU + CAP	8.6	0
501-1 × LMTK⁻	0	1:1	BrdU + CAP	8.7	0*
501-1 × LMTK⁻	0	0.07:1	BrdU + CAP	4.8	0
501-1 × LMTK⁻	0	1:1	HAT + CAP	9.0	176
en501-1	93	—	BrdU + CAP	2.0	0
en501-1	57	—	BrdU + CAP	3.6	0
501-1	0	—	BrdU + CAP	4.4	0
501-1	0	—	HAT + CAP	5.0	0
enA9	84	—	BrdU + CAP	4.4	0
LMTK⁻	0	—	BrdU + CAP	8.7	0
LMTK⁻	0	—	HAT + CAP	5.0	0

The methods of enucleation, cell fusion, and selective techniques were as described for Table 1 and in *Methods*.

* Two colonies appeared after prolonged incubation (see *text*).

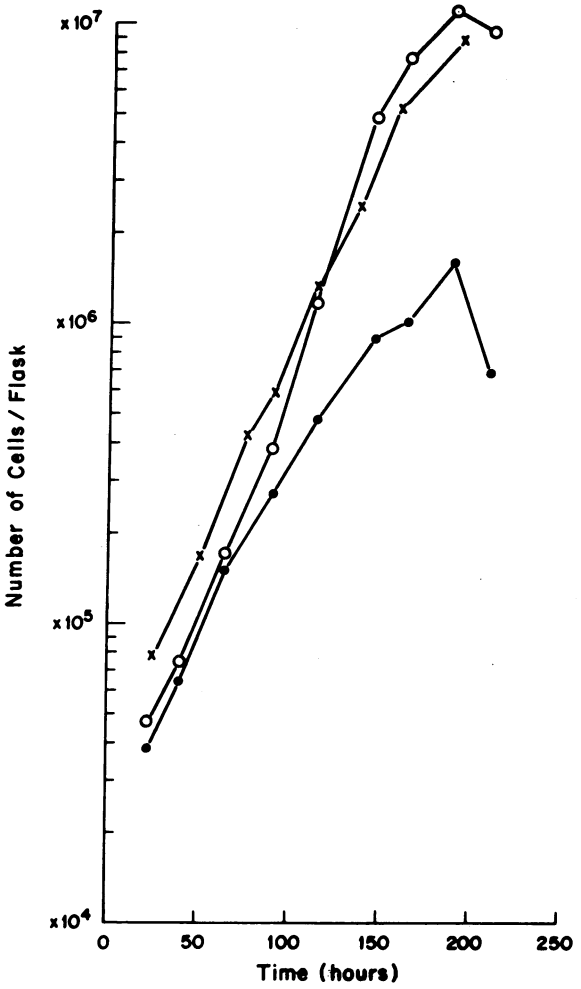

FIG. 1. Chloramphenicol resistance of a LEA clone. Cells (5×10^4) of LEA clone 2a, derived from an en501-1 × LMTK⁻ fusion and LMTK⁻, were inoculated into flasks, and cell counts were taken daily as described in *Methods*. (●) LMTK⁻ in the presence of 50 μg of CAP per ml of medium; (○) LMTK⁻ in the absence of CAP; (×) LEA-2a in the presence of 50 μg of CAP per ml of medium.

such cells reach confluence, they stop growing and die slowly. Otherwise, they become vacuolated and die within 2 weeks. In the experiment described in Table 1, there were occasionally some nonviable large, flat, vacuolated 501-1 cells remaining attached to the flask. Strain LMTK⁻ in the presence of CAP undergoes approximately four successively slower cell divisions, becoming granular and vacuolated in appearance, and then dies. These LMTK⁻ cultures have no viable cells after 10 days in the presence of CAP. The absence of any colonies when parents alone are plated demonstrates that these BrdU-resistant, CAP-resistant colonies have not appeared as a result of mutations of LMTK⁻ cells to CAP resistance, nor of 501-1 cells to BrdU resistance.

The results of further control experiments are shown in Table 2. The fusion of the enucleated CAP-sensitive strain, A9, and LMTK⁻ failed to produce any CAP-resistant clones (Table 2, line 3). Hence CAP resistance depends upon the presence of the enucleated CAP-resistant mutant, and is not due merely to an increase in the amount of cytoplasm relative to the nucleus in the "cybrid".

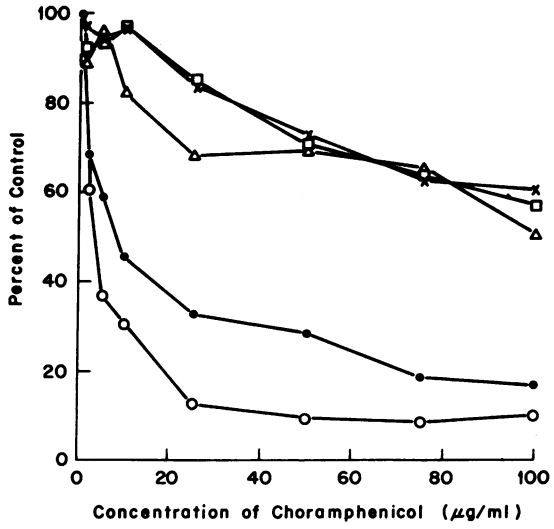

FIG. 2. Sensitivity of LEA clones, 501-1, A9, and LMTK⁻, to chloramphenicol. 5×10^4 cells of each culture were inoculated into flasks. Twenty-four hours later CAP was added in the concentrations indicated to duplicate flasks. Six days later, the cells were harvested and counted as described in *Methods*. Clones LEA-10a and LEA-2a were derived from the fusion of an enucleated culture of 501-1 and LMTK⁻ as described in *Results*. The data are reported as percentages of the number of cells in flasks in the absence of CAP. (×) LEA-10a; (□) LEA-2a; (△) 501-1; (●) LMTK⁻; (○) A9.

A crucial question was whether CAP resistance might have been transferred to LMTK⁻ by fusion with some of the remaining nucleated 501-1 cells. To test this possibility, fusions were performed under identical BrdU and CAP selection conditions with nucleated 501-1 and LMTK⁻ cells. These cells were combined in the ratios of 1:1 and 0.07:1, the latter ratio designed to mimic the 7% nucleated 501-1 cells present in the enucleated 501-1 cell preparation used in Table 1. Only two colonies in 10^6 cells resulted from the 1:1 parent fusion, and none from the 0.07:1 fusion (Table 2, lines 4 and 5). These two colonies appeared after prolonged incubation in selective medium, one after 18 days and the other after 26 days. Both these colonies contained cells resembling LMTK⁻ in morphology. They may have arisen by mutation of LMTK⁻, or from the fusion of a G_1 or S phase 501-1 nucleated cell with a mitotic LMTK⁻ cell, resulting in premature chromosome condensation. Premature chromosome condensation has been described in a variety of animal species (15, 16), and might lead to loss of 501-1 chromosomes as observed for HeLa, Chinese hamster, and chick cells (16, 17). However, either event would be expected to occur at low frequency and could not account for the results presented in Table 1, where BrdU-resistant, CAP-resistant colonies appeared at least 100-fold more frequently. Colonies of large, gray, flat cells also appeared in these nucleated fusion experiments after 14 days, presumably containing nuclear complements of both LMTK⁻ and 501-1, but all of these died by 26 days.

CAP-resistant binucleate cell hybrids were specifically selected in HAT medium after fusion of 501-1 and LMTK⁻ for comparison with the CAP-resistant cybrid clones. The selection of HAT-resistant hybrids from the fusion of H-PRT⁻ and TK⁻ cells has been described by Littlefield (10). These hybrids appeared at a frequency of approximately 2 per 10^4 cells plated (Table 2, line 6), a rate similar to that of

TABLE 3. *Properties of parents, hybrids, and cybrids*

Strains	Cell division time (hr)* −CAP	Cell division time (hr)* +CAP	Chloramphenicol sensitivity† (μg/ml)	Mean‡ chromosome no.	5-Bromo-§ deoxyuridine	8-Azaguanine¶
501-1	19.6	21.4	100	47.2 (3.8, 21)‡	S	R
LMTK⁻	19.2	40.8	8	44.6 (3.8, 33)	R	S
LEA 1a	N.T.	19.4	100	46.6 (5.1, 11)	R	S
LEA 2a	N.T.	19.4	>100	48.1 (3.3, 24)	R	S
LEA 10a	N.T.	19.4	>100	47.6 (2.2, 22)	R	S
ALM 1	N.T.	N.T.	>50	91.2 (14.1, 15)	S	S
ALM 2	N.T.	N.T.	>50	97.3 (10.9, 10)	S	S
ALM 10	N.T.	N.T.	>50	97.8 (15.0, 14)	S	S

LEA cybrid clones were obtained from the fusion of enucleated 501-1 cells with LMTK⁻ cells followed by selection in BrdU and CAP (see *Results*); ALM clones were similarly derived from 501-1 × LMTK⁻ fusions and selection in HAT + CAP medium.

* Cell division time was determined from growth curves of strains in the presence and absence of 50 μg/ml of CAP (see *Methods*). LMTK⁻ does not maintain growth in 50 μg/ml of CAP.

† CAP sensitivity is defined as that concentration of drug which limits cell growth in 6 days to 50% of the control level in the absence of CAP (see *Methods*).

‡ Chromosome staining and counting were performed as described in *Methods*. Numbers in *parentheses* after each mean chromosome number are the standard deviations and number of cells counted, respectively. The following anomalous counts were omitted from calculations of means and standard deviations: 84 from LEA 1a; 78, 97, and 98 from LEA 10a; and 37 from ALM 10. The high chromosome numbers may be due to aberrant chromosome duplication without cell division. The low number would presumably be a nonviable cell.

§ Sensitivity (*S*) to 5-bromodeoxyuridine is defined as the inability of cells to grow continuously in the presence of 30 μg of drug per ml of medium for 14 days. Resistant (*R*) cells are unaffected by this concentration of BrdU.

¶ Sensitivity (*S*) to 8-azaguanine is defined as the inability of cells to grow continuously in the presence of 12 μg of drug per ml of medium for 14 days. Resistant (*R*) cells are unaffected by this concentration of 8-azaguanine.

N.T. indicates not tested.

cybrid colonies (Table 1, and line 1, Table 2). Further characterization of these hybrids will be described in Table 3. No colonies appeared in parental cultures under any of the selective conditions described in Table 2.

Cybrids from the fusion of enucleated 501-1 (derived from strain A9) and LMTK⁻ (derived from strain L) are referred to as LEA strains (L × enucleated A9). Hybrids from nucleated 501-1 × LMTK⁻ fusions are referred to as ALM strains (A9 × LM, both nucleated). Sixteen LEA strains, one from each flask, were then cloned in soft agar in the presence of 50 μg of CAP per ml of medium.

Analysis of the Products of Cell–Cell and Cell–Cytoplast Fusions. If LEA cells are the product of the fusion between LMTK⁻ and a CAP-resistant cytoplasm of 501-1, they should have the following characteristics: (*1*) CAP resistance similar to 501-1; (*2*) a chromosome complement similar to LMTK⁻; (*3*) the BrdU resistance and 8-azaguanine sensitivity of LMTK⁻; and (*4*) a morphology similar to LMTK⁻. The following experiments indicate that cloned LEA cells do indeed possess all these characteristics.

Fig. 1 illustrates that the growth rate of a representative LEA clone, LEA-2a, in the presence of 50 μg/ml of CAP is almost identical with that of LMTK⁻ in the absence of CAP. By contrast, LMTK⁻ in the presence of CAP undergoes four successively slower cell divisions and then dies. Fourteen other LEA clones, and mixed cultures from three flasks each containing more than 50 BrdU and CAP-resistant colonies (Table 1), have growth characteristics identical to LEA-2a in the presence of CAP.

The resistance of LEA clones 2a and 10a to a range of CAP concentrations (0–100 μg/ml of medium), as indicated by extent of growth after 6 days, was compared to that of 501-1, A9, and LMTK⁻. The results are shown in Fig. 2. A third LEA clone gave identical results to LEA-2a and LEA-10a. The LEA clones are clearly as resistant as 501-1 over a wide range of CAP concentrations, and in fact show a slightly increased degree of resistance in the range 10–50 μg of CAP per ml. This may be due to the presence of BrdU (30 μg/ml) in the medium in which LEA clones are routinely maintained. Other experiments in this laboratory suggest that BrdU may slightly increase antibiotic resistance in TK⁻ strains.

Exposure of LMTK⁻ or A9 cells to each level of CAP for periods longer than 6 days would cause total cell death. Hence, the curve in Fig. 2 does not indicate that 17% of LMTK⁻ cells or 10% of A9 cells are CAP-resistant. Longer exposure of LEA clones and 501-1 to 75 or 100 μg of CAP per ml may also cause inhibition greater than that shown in Fig. 2. However, LEA clones and 501-1 can be grown indefinitely in 50 μg of CAP per ml, and show no decrease in viability or alteration in division time.

The CAP sensitivities of LEA clones, ALM strains, and the parent strains 501-1 and LMTK⁻ are compared in Table 3, in terms of cell division time and the CAP concentration that will inhibit to 50% of the control level in 6 days. Clearly, LEA clones are as CAP-resistant as 501-1.

Table 3 also shows that the LEA clones possess a mean chromosome number similar to that of LMTK⁻ and 501-1. This is, however, about half the number of chromosomes of the CAP-resistant binucleate ALM strains that were selected from 501-1 × LMTK⁻ fusions in HAT and CAP. Therefore, LEA clones have not arisen from the fusion of two nucleated cells without chromosome loss. Nor are LEA clones the result of a simple mutation to TK⁻ in 501-1, cells as they are sensitive to 8-azaguanine while 501-1 cells are resistant (Table 3). Finally all LEA clones had morphology similar to LMTK⁻, which was quite different from either 501-1 cells or ALM cells (not shown).

DISCUSSION

The isolation and characterization of CAP-resistant cells resulting from the fusion between enucleated CAP-resistant mouse cells and nucleated, CAP-sensitive mouse cells has been described. These fusion products, or cybrids, appear at high frequency and are stable with respect to CAP resistance. They resemble the nucleated parent cell in terms of chromosome complement and nuclear markers and the enucleated parent cell in terms of CAP resistance. No CAP- and BrdU-resistant cells were obtained from either parent under identical selection conditions. Hence, such cybrid cells do not arise from mutations in either parent. Furthermore, control fusions between enucleated and nucleated CAP-sensitive cells produced no CAP-resistant cells. Fusions between nucleated, CAP-resistant and CAP-sensitive cells under the same selective conditions produced CAP-resistant hybrids at a frequency 100 times lower than the rate of appearance of CAP-resistant cybrids. Consequently, the appearance of these CAP-resistant cybrids at such frequencies could not be explained by the fusion of two nucleated cells, nor by the increase in the amount of cytoplasm per nucleus.

Similar results showing the transfer of CAP resistance have also been obtained in this laboratory with the CAP-resistant human HeLa mutant described by Spolsky and Eisenstadt (5).

Thus, the genetic information for CAP resistance resides in the cytoplasm of mouse cells. This information may be encoded in mtDNA, or possibly in one of the other types of cytoplasmic DNAs reported in mammalian cells. These are spcDNA (18), microsome-associated DNA (19, 20), informational or I-DNA (21), and membrane-associated cmDNA (22). All are suggested to be of nuclear origin. If these classes of cytoplasmic DNA are dependent on the nucleus for function and replication, it is unlikely that they are capable of transferring permanent CAP resistance in the absence of the nucleus.

CAP resistance has been shown to be expressed at the level of mitochondrial protein synthesis in HeLa cells (6). Preliminary experiments indicate that this is also true for the CAP-resistant mouse mutant 501-1 described in this paper. Mitochondrial DNA has been shown to code for CAP resistance in yeast (8). It appears likely that the mutation to CAP resistance in mouse cells may also have occurred in mtDNA.

The mechanism of the transfer process at the cellular or molecular level is unclear, as is the fate of the CAP-sensitive cytoplasm. Fusions between human and mouse cells have shown that human mtDNA is lost along with human chromosomes (23, 24), but may be retained when reverse segregation occurs, that is, when mouse chromosomes are eliminated (25). No selective pressure for either species of mitochondria was used in these fusions (23–25). It is now possible to select for the retention of human mitochondria in interspecific fusions where human chromosomes are lost.

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