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Genetics

Cytoplasmic inheritance of erythromycin resistance in human cells

(mitochondria/cytoplast-cell fusion/*in vitro* mitochondrial protein synthesis/carbomycin cross-resistance/somatic cell genetics)

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ABSTRACT **An erythromycin-resistant mutant, ERY2301, was isolated from ethidium bromide-treated HeLa cells in the presence of erythromycin at 300 μg/ml. ERY2301 cells were enucleated and the anucleate cytoplasts were fused with D98/AH-2, a hypoxanthine phosphoribosyltransferase-deficient variant of HeLa cells. The resultant cybrids were isolated in a double selective medium containing erythromycin and 6-thioguanine. Cybrid formation occurred at a frequency of 10^{-3} to 10^{-4}. *In vitro* protein synthesis by intact and Triton X-100 treated mitochondria isolated from ERY2301 was resistant to the macrolide antibiotics erythromycin and carbomycin, but was sensitive to chloramphenicol. These results suggest that the site of erythromycin resistance in ERY2301 may be at the level of mitochondrial protein synthesis and indicate that this trait is cytoplasmically inherited and, therefore, presumably encoded in the mitochondrial genome.**

The biogenesis of mitochondria results from the coordinated action of two distinct genetic systems (1, 2). The majority of proteins located in the mitochondria are encoded by nuclear genes and translated on extramitochondrial cytoplasmic ribosomes, whereas the proteins synthesized on mitochondrial ribosomes are presumably encoded by the mitochondrial DNA. The selection and characterization of cytoplasmically inherited mutations conferring resistance to various antibiotics that inhibit mitochondrial protein synthesis and respiration have been essential in the study of mitochondrial genetics in lower eukaryotes (3, 4).

In recent years mitochondrial mutants in mammalian cells have been described. Resistance to the drug chloramphenicol (CAP), an inhibitor of mitochondrial peptidyltransferase, has been demonstrated to be cytoplasmically inherited in mouse and human cells (5–7) by enucleating the CAP-resistant cells, fusing the anucleate cytoplasts to CAP-sensitive cells, and selecting for CAP-resistant cybrids. Cytoplasmic inheritance of resistance to rutamycin (8), an inhibitor of mitochondrial adenosinetriphosphatase, and antimycin A (9), an inhibitor of electron transport at cytochrome *b*, and mutants deficient in mitochondrial protein synthesis (10) have also been reported.

Mutants of *Saccharomyces cerevisiae* resistant to the protein synthesis inhibitor erythromycin have been described; they were shown to have an erythromycin-resistant mitochondrial protein-synthesizing system (11, 12). Evidence has accumulated that the erythromycin resistance loci in these mutants map in the region of the mitochondrial DNA coding for 21S ribosomal RNA (13, 14). In contrast, cytoplasmically inherited erythromycin resistance in *Paramecium aurelia* has been associated with an altered profile of mitochondrial ribosomal proteins (15).

There has been considerable controversy concerning the effect of erythromycin on mammalian mitochondrial protein synthesis. Towers *et al.* reported that isolated rat liver mitochondria were insensitive to the effects of erythromycin (16). They concluded that this insensitivity to erythromycin reflects a phylogenetic difference between the mitochondrial protein-synthesizing system of lower eukaryotes and that of mammalian cells. Kroon and de Vries (17), however, showed that only intact rat liver mitochondria were insensitive to erythromycin. This resistance to erythromycin was due to the impermeability of the mitochondrial membranes to the antibiotic. The latter interpretation of Kroon and de Vries is supported by the findings that ribosomes isolated from rat liver mitochondria are sensitive to erythromycin (18, 19).

We have found that the growth of human cells in culture is inhibited by erythromycin and we report here the isolation and preliminary characterization of an erythromycin-resistant HeLa cell line in which the resistant phenotype is cytoplasmically inherited and presumably mitochondrially encoded.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HeLa, subline Stone, is a wild-type HeLa cell population. D98/AH-2 is a variant of HeLa deficient in hypoxanthine phosphoribosyltransferase (HPRT; EC 2.4.2.8) (20) and is resistant to 6-thioguanine (6-TG) at 8 μg/ml. $D98^{OR}$ is a ouabain-resistant variant of D98/AH-2 (21) and is resistant to 1 μM ouabain. Cell populations were maintained at 37°C as monolayer cultures in Eagle's minimal essential medium (Flow Laboratories, McLean, VA) supplemented with 5% calf serum (Irvine Scientific, Irvine, CA), 2 mM L-glutamine (Sigma), and 25 mM Hepes buffer, pH 7.4 (Calbiochem), hereafter designated growth medium. All cell populations were routinely tested for the presence of mycoplasma contaminants by cultural methods, uridine/uracil incorporation (22), and the 4,6-diamidino-2-phenylindole assay (23) and were found to be free of any detectable mycoplasma contamination in these experiments.

Selection of Erythromycin-Resistant Cells. HeLa cells were plated at 1×10^6 cells per 75-cm^2 flask and allowed to attach overnight. The cells were then treated with ethidium bromide at 0.5 μg/ml for one cell generation, after which they were allowed to recover in growth medium. At this concentration of ethidium bromide the nonreplicating mitochondrial DNA within cells is thought to be repeatedly nicked and closed (24). This treatment resulted in approximately 50% survival of the cells. The growth medium was then supplemented with erythromycin lactobionate (ERY) at 200 μg/ml for two population doublings. The concentration was then increased to 300 μg/ml. Six to eight weeks later several colonies of cells survived,

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase; 6-TG, 6-thioguanine; ERY, erythromycin lactobionate; CARB, carbomycin; CAP, chloramphenicol; HAT, hypoxanthine/aminopterin/thymidine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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only one of which continued to grow in ERY at 300 μg/ml. This clone was selected for further study and is designated ERY2301.§

Growth Curves. The ability of the cell lines to grow in the presence of inhibitors of mitochondrial protein synthesis was assayed by plating approximately 1×10^5 cells per 25-cm^2 flask containing 3 ml of growth medium, or growth medium supplemented with ERY at 300 μg/ml. In separate experiments the cells were also grown in the presence of carbomycin (CARB) at 10 μg/ml or CAP at 50 μg/ml. Subsequent to day 3, the cells received fresh medium every 2 days. Cells were harvested with 0.1% trypsin and 7 mM EDTA in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline and counted in a hemocytometer.

Cloning Efficiency. Approximately 200 cells per 25-cm^2 flask were plated in growth medium. The next day, the medium was replaced with 3 ml of growth medium or growth medium supplemented with various concentrations of ERY or CAP. Colonies containing 50 or more cells were counted after 7–10 days.

Cell Enucleation. ERY2301 cells were enucleated in gelatin-coated 25-cm^2 flasks (26) at 14,000 × *g*, according to Veomett *et al.* (27). Enucleation ranged from 78 to 92%. The preparations were further enriched for anucleate cytoplasts by treating the monolayers with 7 mM EDTA and differentially detaching the residual nucleated cells by sharply hitting the side of the flask. The anucleate cytoplasts were removed by vigorous pipetting and counted in a hemocytometer.

Cell Fusion. To obtain cybrids, 5×10^5 D98/AH-2 cells were mixed with 5×10^5 or 3×10^6 anucleate HeLa ERY2301 cytoplasts in 0.5 ml of serum-free growth medium plus 0.1 ml of inactivated Sendai virus (1000 hemagglutinating units/ml). Cells were plated in growth medium at approximately 5×10^4 or 1.25×10^4 cells per 25-cm^2 flask. The next day the medium was replaced with growth medium containing ERY at 300 μg/ml. After 5 days had been allowed for any residual HPRT activity contributed by the anucleate ERY2301 cytoplasts to decay, 6-TG was added to the selective medium at 8 μg/ml. Two to three weeks later surviving colonies were counted and selected colonies were picked for further analysis.

Hybrids were obtained by plating mixed cultures of 1×10^6 D98^{OR} cells and 1×10^6 ERY2301 cells in 60-mm petri dishes. After 24-hr incubation at 37°C the growth medium was removed and the cell monolayer was treated with polyethylene glycol, 1000 M_r, for 30 sec. After overnight recovery, the cells were plated in growth medium at approximately 5×10^5 cells per 75-cm^2 flask. The next day the medium was replaced with hypoxanthine/aminopterin/thymidine (HAT) medium (28) supplemented with 0.5 μM ouabain and ERY at 300 μg/ml. Approximately 3 weeks later colonies were picked for further analysis.

Chromosome Analysis. Exponential-phase cells were treated with colchicine and metaphase chromosomes were prepared by the method of Nelson-Rees and Flandermeyer (29).

***In Vitro* Mitochondrial Protein Synthesis.** Cells were homogenized and the mitochondria were isolated by differential centrifugation of the 5000 × *g* membrane fraction according to a combination of procedures described by Attardi and coworkers (30, 31). The final pellet was resuspended in 250 mM sucrose/5 mM $MgCl_2$/1 mM dithiothreitol/10 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) buffer, pH 7.8. Mitochondrial protein synthesis was measured by the incorporation of [3,5-^{3}H]leucine into the cold trichloroacetic acid-insoluble fraction at 37°C and pH 7.8, using a modification of Spolsky and Eisenstadt's procedure (32).

RESULTS

Selection of ERY-Resistant Cells. The resistant clone ERY2301 arose approximately 2 months after the stepwise increase to 300 μg of ERY per ml. The time course of selection, during which the resistant mitochondria presumably repopulated the cells, is similar to that reported for the selection of CAP-resistant cells (5, 32). From the onset of the selection period the mass culture of the cells grew for approximately 1 week. After this initial restricted growth phase, the majority of the cells detached from the surface of the flask and failed to proliferate upon replating in growth medium. The gradual loss of adherent cells continued until only a few colonies survived. These colonies, with the exception of ERY2301, were composed of large multinucleate cells, which failed to proliferate upon subculture. ERY2301 retained the normal HeLa morphology and continued to proliferate serially in the presence of ERY at 300 μg/ml.

Growth Curves. As seen in Fig. 1*A*, wild-type HeLa cells failed to proliferate significantly in the presence of ERY at 300 μg/ml or CARB at 10 μg/ml. The cells underwent approximately two to three population doublings and then ceased to divide, becoming larger and more elongated before they died. On day 9 there were no viable HeLa cells adhering to the surface or floating in either the ERY or the CARB medium, as evidenced by lack of cell attachment and growth after replating in growth medium. ERY2301 grew in ERY at 300 μg/ml and CARB at 10 μg/ml at approximately 50–70% of the growth rate seen in the absence of ERY or CARB (Fig. 1*B*). After being cultured in the absence of ERY for 40 population doublings, ERY2301 grew at the same rate in ERY at 300 μg/ml as parallel ERY2301 cells maintained in the presence of ERY. Both HeLa and ERY2301 displayed a gradual cessation of growth in the presence of CAP at 50 μg/ml and after 9–11 days began to die (Fig. 1). On day 11, there were fewer than 1×10^5 adherent HeLa cells, and they failed to proliferate after they were re-

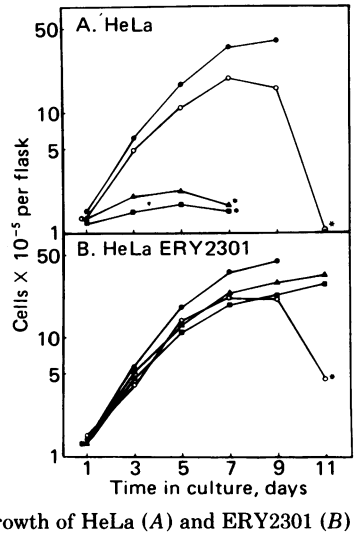

FIG. 1. Growth of HeLa (*A*) and ERY2301 (*B*) in the presence of inhibitors of mitochondrial protein synthesis. ●, Growth medium; ▲, ERY at 300 μg/ml; ■, CARB at 10 μg/ml; ○, CAP at 50 μg/ml. Each point is the mean of triplicate samples. *, All cells failed to proliferate when replated (see text).

§ We have isolated several further ERY-resistant clones by using a different selection system. HeLa cells were exposed to low levels of ethidium bromide to reduce the mitochondrial target size (25). After the removal of ethidium bromide the cells were exposed to ethyl methanesulfonate for one cell generation during the period of stimulated mitochondrial DNA synthesis. Seven clones were isolated 3–4 weeks after stepwise selection to ERY at 300 μg/ml.

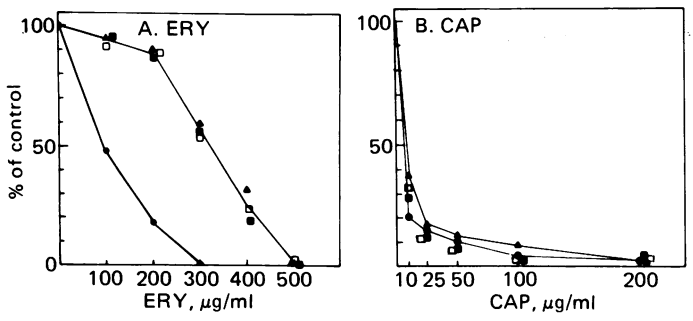

FIG. 2. Cloning efficiency of HeLa (●), ERY2301 (▲), ESCy7 (□), and ESCy10 (■) in the presence of various concentrations of ERY (*A*) and CAP (*B*). The means of the control colony counts were 151 colonies for HeLa, 164 colonies for ERY2301, 99 colonies for ESCy7, and 101 colonies for ESCy10. Each point represents the percent of control calculated from the mean of triplicate samples.

plated in growth medium containing CAP at 50 μg/ml. ERY2301 cells counted on day 11 also failed to proliferate after they were replated in CAP medium.

Cloning Efficiency. The results depicted in Fig. 2 show that ERY2301 cells formed colonies in ERY at 300 μg/ml after seeding of approximately 200 cells per 25-cm^2 flask. Wild-type HeLa cells did not grow in ERY at 300 μg/ml, but did form colonies in ERY at 200 μg/ml. However, this colony formation was significantly less efficient than that of ERY2301 and the colonies died when the ERY concentration was increased to 300 μg/ml. The ability of both HeLa and ERY2301 cells to form colonies was greatly reduced by the presence of CAP (Fig. 2*B*). Continued incubation in the presence of CAP did not give rise to any CAP-resistant HeLa or ERY2301 colonies.

***In Vitro* Mitochondrial Protein Synthesis.** The results in Table 1 show that mitochondrial protein synthesis in wild-type HeLa cells was inhibited approximately 50% by ERY at 400 μg/ml and approximately 80% by CARB at 100 μg/ml or CAP at 200 μg/ml. Protein synthesis by mitochondria isolated from ERY2301, however, exhibited significantly increased resistance to both ERY and CARB, but remained sensitive to CAP. All preparations were insensitive to the cytoplasmic protein synthesis inhibitor cycloheximide, indicating that the bulk of the incorporation of [^{3}H]leucine was due to the mitochondrial protein-synthesizing system and not contaminating rough endoplasmic reticulum. In order to demonstrate that the ERY-resistant mitochondrial protein synthesis was not due to impermeability of the mitochondrial membranes, the mitochondrial preparations were resuspended in 0.01% Triton X-100, a nonionic detergent that causes swelling and disruption of mitochondria (33). As illustrated in Table 1, the level of inhibition by ERY, CARB, and CAP in the presence of 0.01% Triton X-100 was similar to the inhibition seen in the intact mitochondrial preparations of both HeLa and ERY2301.

Transfer of ERY Resistance. A double selective system was employed in the isolation of ERY-resistant cybrids. D98/AH-2 carries the recessive nuclear allele for HPRT deficiency and is, therefore, resistant to 6-TG at 8 μg/ml but will not grow in ERY at 300 μg/ml. HeLa ERY2301 is wild type with respect to HPRT activity but is ERY resistant. In selective medium containing ERY at 300 μg/ml and 6-TG at 8 μg/ml, only fusions between anucleate ERY2301 cytoplasts and D98/AH-2 cells will survive if ERY resistance is cytoplasmically inherited as a dominant trait. The results shown in Table 2 reveal that under these selective conditions no ERY2301 cells survived and only one D98/AH-2 colony arose. This colony may represent a spontaneous ERY-resistant variant. The frequency of cybrid formation was between 10^{-3} to 10^{-4} and was dependent on the ratio of cytoplasts to whole cells. The frequencies are similar to the reported frequencies of CAP-resistant cybrid formation in mouse L cells (5, 34) and human cells (7, 32). The possibility that the surviving cybrids were formed by the fusion of two nucleated cells with the concomitant loss of the wild-type HPRT locus was ruled out by chromosome analysis depicted in Table 3. All six cloned cybrids, as well as the two pooled cybrid populations, displayed a single genome karyotype within the range of the D98/AH-2 recipient. The surviving cybrid colonies from the mock fusion of anucleate ERY2301 cytoplasts and D98/

Table 1. Protein synthesis by isolated mitochondria

Source of mitochondria	Protein synthesis, % of control incorporation*				
	Cycloheximide (300 μg/ml)	ERY 400 μg/ml	ERY 800 μg/ml	CARB (100 μg/ml)	CAP (200 μg/ml)
HeLa	89 ± 3	56 ± 5†	53 ± 5	22 ± 3	15 ± 3
HeLa + 0.01% Triton X-100‡	92 ± 5	54 ± 6	51 ± 6†	21 ± 4	12 ± 2
ERY2301	91 ± 3	71 ± 8†	70 ± 3	50 ± 4	18 ± 3
ERY2301 + 0.01% Triton X-100‡	91 ± 3	70 ± 3	71 ± 9†	44 ± 4	15 ± 3
D98/AH-2	89 ± 2	ND	54 ± 3	27 ± 4	16 ± 2
ESCy7	89 ± 2	ND	68 ± 6	53 ± 6	17 ± 1
ESCy10	91 ± 3	ND	69 ± 1	49 ± 1	19 ± 1

* The incorporation of [^{3}H]leucine into the acid-insoluble fraction was followed for 30 min and expressed as cpm/mg of mitochondrial protein. The mean incorporation in the absence of inhibitors was 12,739 cpm/mg for HeLa; 12,963 cpm/mg for HeLa + 0.01% Triton X-100; 13,088 cpm/mg for ERY2301; 12,180 cpm/mg for ERY2301 + 0.01% Triton X-100; 12,431 cpm/mg for D98/AH-2; 11,376 cpm/mg for ESCy7; and 12,408 cpm/mg for ESCy10. Values are the mean ± standard deviation of the percent of [^{3}H]leucine incorporation in the absence of inhibitors for each mitochondrial preparation and were calculated from at least three separate experiments. ND, not done.

† The null hypothesis that there is no difference between the means for HeLa and ERY2301 can be rejected at the 99% confidence level.

‡ The final mitochondrial pellet was resuspended in 250 mM sucrose/5 mM $MgCl_2$/1 mM dithiothreitol/10 mM Tricine buffer, pH 7.8/0.01% Triton X-100 and kept on ice for 30 min prior to the assay.

Table 2. Cytoplasmic transfer of ERY resistance

Fusion parents*	Whole cells to cytoplasts	Fusing agent	Cells plated	Colonies
D98/AH-2 + enERY2301	1:1	Sendai virus	500,000	181
D98/AH-2 + enERY2301	1:6	Sendai virus	500,000	508
D98/AH-2 + enERY2301	1:1	None	1,000,000	13†
D98/AH-2 + ERY2301	1:1	None	1,000,000	0
D98/AH-2	—	None	2,000,000	1
ERY2301	—	None	2,000,000	0

* enERY2301 refers to anucleate ERY2301 cytoplast.
† Colonies appeared after an additional 2-week incubation (see text).

AH-2 cells arose several weeks after the cybrid colonies generated from virus-mediated fusion had established themselves and presumably occurred through rare spontaneous fusion events.

To further demonstrate that ERY resistance is inherited as a dominant or codominant trait, ERY-resistant hybrids were isolated by using a double selective system. $D98^{OR}$ will not grow in HAT medium, ERY at 300 μg/ml, or both, whereas ERY2301 is sensitive to 0.5 μM ouabain but will grow in HAT medium, ERY at 300 μg/ml or both. In HAT medium containing 0.5 μM ouabain and ERY at 300 μg/ml, only hybrids derived from the fusion between $D98^{OR}$ and ERY2301 will survive if that ERY resistance is dominant or codominant. Our results would suggest that this is indeed the case. Hybrid formation occurred at a frequency of approximately 10^{-6}. The three hybrids selected showed a chromosome constitution consistent with the fusion of the two parental cells (Table 3).

Characterization of ERY-Resistant Cybrids. Two cybrids were chosen for further study. Both ESCy7 and ESCy10 displayed growth characteristics similar to ERY2301 when grown under cloning conditions (Fig. 2) and as mass populations (Fig. 3). In Table 1, it is seen that *in vitro* protein synthesis by mitochondria isolated from ESCy7 and ESCy10 exhibited the same degree of resistance to ERY and CARB as ERY2301 mitochondria, whereas mitochondria isolated from D98/AH-2 resembled wild-type HeLa cells in their response to these antibiotics.

Table 3. Chromosome analysis of parent, cybrid, and hybrid cell populations

Cell line	Chromosome number* Mode	Range
Parental		
ERY2301	66	64–67
D98/AH-2	61–62	56–65
$D98^{OR}$	61	57–66
Cybrid		
ESCy7	60	58–62
ESCy10	61	57–64
ESCy11	61	57–63
ESCy14	58	55–61
ESCy15	61	57–64
ESCy16	59	55–62
Mass cybrid (1:1)†	62	55–64
Mass cybrid (1:6)†	61	57–63
Hybrid		
CDH5	100	96–104
CDH7	114	112–122
CDH8	108	104–112

* Values were based on at least 30 metaphase spreads.
† Pooled cybrid populations from fusions with ratios of 1:1 and 1:6 D98/AH-2 cells to ERY2301 cytoplasts, respectively.

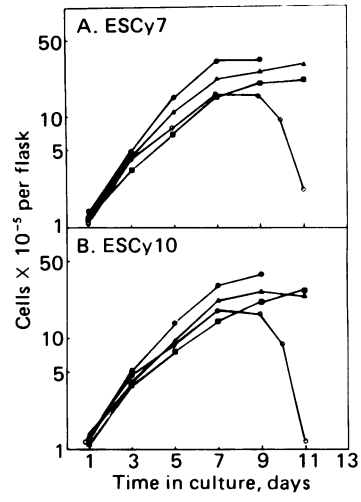

FIG. 3. Growth of cybrids ESCy7 (*A*) and ESCy10 (*B*) in the presence of ERY, CARB, or CAP. ●, Growth medium; ▲, ERY at 300 μg/ml; ■, CARB at 10 μg/ml; ○, CAP at 50 μg/ml. Each point is the mean of triplicate samples.

DISCUSSION

An ERY-resistant mutant HeLa, ERY2301, was isolated approximately 2 months after ethidium bromide treatment of HeLa cells and selection in ERY at 300 μg/ml. Although there is no evidence that the ethidium bromide treatment was mutagenic in this system, similar treatments have been employed in the selection of CAP-resistant mutants in mammalian cells (5, 32). Furthermore, ethidium bromide has been shown to be an effective inducer of petite mutants in yeast (35).

The growth characteristics of ERY2301 demonstrated cross-resistance to ERY at 300 μg/ml and CARB at 10 μg/ml, but sensitivity to CAP at 50 μg/ml. The cross-resistance to CARB is not surprising because ERY and CARB are thought to have the same binding site on the larger ribosomal subunit (36). Cross-resistance to various macrolide antibiotics has also been reported for yeast mitochondrial mutants (12). The growth inhibition of HeLa cells by ERY at 300 μg/ml and CARB at 10 μg/ml agrees with results previously reported for HeLa cells (37) and mouse L cells (38). HeLa cells in the presence of inhibitory concentrations of ERY and CARB underwent at most three population doublings and died within 1 week. In contrast, both HeLa and ERY2301 grew at a decreasing rate in the presence of CAP at 50 μg/ml for 9–11 days before they began to die. It has been reported that high concentrations of CAP (i.e., greater than 200 μg/ml) have a direct inhibitory effect on respiration (39), whereas ERY and CARB do not directly affect respiration even at much higher concentrations (37). Therefore, we were able to use concentrations of ERY and CARB that were more effective inhibitors of cell proliferation than CAP in our cultural conditions without producing unwanted side effects.

The fusion experiments clearly demonstrate that ERY resistance is cytoplasmically inherited. ERY resistance was inherited as a dominant or codominant trait in hybrids, and cybrids were successfully produced from fusions between ERY2301 cytoplasts and ERY-sensitive D98/AH-2 whole cells, followed by selection in the presence of 6-TG and ERY. It seems reasonable, therefore, to suggest that the ERY-resistant phe-

notype may be encoded in the mitochondrial DNA. The possible contribution of cytoplasmic DNA species other than mitochondrial DNA has been previously discussed (5). The molecular nature of ERY resistance in these HeLa cells is not yet known.

The results of the *in vitro* protein synthesis assays suggest that both ERY and CARB affect mitochondrial protein synthesis and that resistance to these inhibitors is due to an alteration in the mitochondrial protein-synthesizing apparatus of ERY2301 and not due to permeability changes in the mitochondrial membranes. We base the latter inference on the fact that, in the presence of ERY or CARB, protein synthesis was inhibited to the same degree in both intact and 0.01% Triton X-100 treated mitochondria isolated from HeLa cells, whereas ERY2301 mitochondria demonstrated significant resistance to ERY and CARB in both of these conditions. Thus, unlike the intact rat liver mitochondria, in which a permeability barrier to ERY has been noted (17), intact HeLa mitochondria appear to be permeable to ERY.

Mitochondrial protein synthesis assays were carried out at pH 7.8 because HeLa mitochondrial protein synthesis was insensitive to ERY at pH 7.4 (unpublished data). ERY, with a pK_a of 8.6, is also more inhibitory at alkaline pH in bacterial *in vitro* protein synthesis systems (40), and it has been postulated that only the nonprotonated molecule of ERY is inhibitory.

As is evident from Fig. 1 and Table 1, higher concentrations of the drugs ERY, CARB, and CAP were needed to inhibit cell-free mitochondrial protein synthesis than were needed to inhibit cell growth. The differences reported here between the concentration of drug needed to inhibit growth of clones or mass populations and that needed to inhibit cell-free mitochondrial protein synthesis also have been reported for CAP (31) and CARB (38, 41).

Irrespective of the concentration of ERY used, only partial inhibition of HeLa mitochondrial protein synthesis was obtained. This partial inhibition of protein synthesis in ERY-sensitive cells has also been reported in bacterial and yeast systems (11, 12, 40, 42). The reasons for this have not been elucidated in any of these systems.

This report describes the isolation of an ERY resistance marker in mammalian cells that is cytoplasmically inherited and presumably mitochondrially encoded. The addition of this drug resistance marker to the repertoire of cytoplasmically inherited markers already available should significantly enhance the investigations of nucleocytoplasmic interactions and mitochondrial recombination in mammalian cell systems.

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1. Schatz, G. & Mason, T. (1974) *Annu. Rev. Biochem.* **43,** 51–87.
2. Borst, P. & Grivell, L. A. (1978) *Cell* **15,** 705–723.
3. Linnane, A. W., Haslam, J. M., Lukins, H. B. & Nagley, P. (1972) *Annu. Rev. Microbiol.* **26,** 163–198.
4. Linnane, A. W. & Nagley, P. (1978) *Plasmid* **1,** 324–345.
5. Bunn, C. L., Wallace, D. C. & Eisenstadt, J. M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1681–1685.
6. Wallace, D. C., Bunn, C. L. & Eisenstadt, J. M. (1975) *J. Cell Biol.* **67,** 174–188.
7. Mitchell, C. H. & Attardi, G. (1978) *Somatic Cell Genet.* **4,** 737–744.
8. Lichtor, T. & Getz, G. S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 324–328.
9. Harris, M. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5604–5608.
10. Wiseman, A. & Attardi, G. (1979) *Somatic Cell Genet.* **5,** 241–262.
11. Linnane, A. W., Lamb, A. J., Christodoulou, C. & Lukins, H. B. (1968) *Proc. Natl. Acad. Sci. USA* **59,** 1288–1293.
12. Grivell, L. A., Netter, P., Borst, P. & Slonimski, P. P. (1973) *Biochim. Biophys. Acta* **312,** 358–367.
13. Nagley, P., Molloy, P. L., Lukins, H. B. & Linnane, A. W. (1974) *Biochem. Biophys. Res. Commun.* **57,** 232–239.
14. Faye, G., Kujawa, C. & Fukuhara, H. (1974) *J. Mol. Biol.* **88,** 185–203.
15. Beale, G. H., Knowles, J. K. C. & Tait, A. (1972) *Nature (London)* **235,** 396–397.
16. Towers, N. R., Dixon, H., Kellerman, G. M. & Linnane, A. W. (1972) *Arch. Biochem. Biophys.* **151,** 361–369.
17. Kroon, A. M. & de Vries, H. (1971) in *Autonomy and Biogenesis of Mitochondria and Chloroplasts*, eds. Boardman, N. K., Linnane, A. W. & Smillie, R. M. (North Holland, Amsterdam), pp. 318–327.
18. Ibrahim, N. G. & Beattie, D. S. (1973) *FEBS Lett.* **36,** 102–104.
19. Greco, M., Pepe, G. & Saccone, C. (1973) in *The Biogenesis of Mitochondria*, eds. Kroon, A. M. & Saccone, C. (Academic, NY), pp. 367–376.
20. Szybalski, W., Szybalska, E. H. & Ragni, G. (1962) *Natl. Cancer Inst. Monogr.* **7,** 75–89.
21. Weissman, B. E. & Stanbridge, E. J. (1977) *J. Cell Biol.* **75,** 382a.
22. Schneider, E. L., Stanbridge, E. J. & Epstein, C. J. (1974) *Exp. Cell Res.* **84,** 311–318.
23. Russell, W. C., Newman, C. & Williamson, D. H. (1975) *Nature (London)* **253,** 461–462.
24. Smith, C. A., Jordan, J. M. & Vinograd, J. (1971) *J. Mol. Biol.* **59,** 255–272.
25. Wiseman, A. & Attardi, G. (1978) *Mol. Gen. Genet.* **167,** 51–63.
26. Richler, C. & Yaffe, D. (1970) *Dev. Biol.* **23,** 1–22.
27. Veomett, G., Shag, J., Hough, P. V. C. & Prescott, D. M. (1976) *Methods Cell Biol.* **13,** 1–6.
28. Littlefield, J. W. (1964) *Science* **145,** 709–710.
29. Nelson-Rees, W. A. & Flandermeyer, R. R. (1977) *Science* **195,** 1343–1344.
30. Attardi, B., Cravioto, B. & Attardi, G. (1969) *J. Mol. Biol.* **44,** 47–70.
31. Lederman, M. & Attardi, G. (1970) *Biochem. Biophys. Res. Commun.* **40,** 1492–1500.
32. Spolsky, C. M. & Eisenstadt, J. M. (1972) *FEBS Lett.* **25,** 319–324.
33. Greville, G. D. & Chappell, J. B. (1959) *Biochim. Biophys. Acta* **33,** 267–269.
34. Bunn, C. L. & Eisenstadt, J. M. (1977) *Somatic Cell Genet.* **3,** 335–341.
35. Slonimski, P. P., Perrodin, G. & Croft, J. H. (1968) *Biochem. Biophys. Res, Commun.* **30,** 232–239.
36. Pestka, S. (1971) *Annu. Rev. Microbiol.* **25,** 487–562.
37. Dixon, H., Kellerman, G. M. & Linnane, A. W. (1972) *Arch. Biochem. Biophys.* **152,** 869–875.
38. Bunn, C. L. & Eisenstadt, J. M. (1977) *Somatic Cell Genet.* **3,** 611–617.
39. Freeman, K. B. (1970) *Can. J. Biochem.* **48,** 469–478.
40. Mao, J. C.-H. & Wiegand, R. G. (1968) *Biochim. Biophys. Acta* **157,** 404–413.
41. Mitchell, C. H., England, J. M. & Attardi, G. (1975) *Somatic Cell Genet.* **3,** 215–234.
42. Grivell, L. A., Reijnders, L. & de Vries, H. (1971) *FEBS Lett.* **16,** 159–163.