CHLORAMPHENICOL-RESISTANT MUTANTS OF HUMAN HeLa CELLS*

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1. Introduction

Chloramphenicol is one of a number of antibiotics which specifically inhibit mitochondrial protein synthesis in eukaryotic cells, while not affecting the cytoplasmic system [1,2]. Some mutations conferring resistance to chloramphenicol could directly affect the mitochondrial protein synthesizing system. Studies of respiratory-deficient mutants of yeast and of Neurospora have shown that mitochondrial proteins are products of both cytoplasmic extrachromosomal determinants and of chromosomal genes [3]. The Neurospora poky mutation, which affects mitochondrial ribosomes, exhibits nonMendelian inheritance [4]. The existence of erythromycin-, mikamycin-, and chloramphenicol-resistant yeast mutants which likewise show cytoplasmic inheritance [5–8] suggest that, in mammalian cells also, resistance to chloramphenicol may be mitochondrially coded.

Extrachromosomally inherited mitochondrial mutations in mammalian cells are essential to determine gene locations of mitochondrial proteins and to study nuclear–mitochondrial interactions in mitochondrial biogenesis. This paper describes the isolation and general properties of mutants of human HeLa cells resistant to chloramphenicol.

2. Materials and Methods

2.1. *Strains and growth medium*

All mutants were isolated from the S3 clone of HeLa cells. Each resistant culture was cloned in plastic petri dishes (attachment method), then recloned in semi-solid 0.12% agar (suspension method). All cell lines were maintained as *monolayer cultures* in MEM-E supplemented with 10% foetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [9], in a 37° incubator with a 5% CO_2 atmosphere.

2.2. *Mutagenesis and isolation of mutants*

A large (75 cm^2) flask was seeded with 1×10^6 S3 cells. After allowing the cells to attach, ethidium bromide was added for one cell generation, then washed out with 3 changes of Earle's balanced salt solution, harvested by treating with 0.25% viokase and plated, at various cell densities, in growth medium containing 25 or 50 μg/ml of chloramphenicol. After resistant cells had grown to stationary phase, they were transferred 2–3 times and then cloned.

2.3. *Growth curves*

A number of small (25 cm^2) flasks were seeded with $4–5 \times 10^4$ cells each and incubated for 10–14 days at 37°. Cells from two flasks were harvested and counted in a Coulter counter at approximately daily intervals.

2.4. *Sensitivity curves*

20 Small flasks were seeded with $4–5 \times 10^4$ cells each. The following day, different concentrations (0–200 μg/ml) of chloramphenicol (CAP) were added to duplicate flasks. 5–6 Days later (when control flasks had increased to $1–2 \times 10^6$ cells, i.e., late exponential phase) cells were harvested and counted.

2.5. *Isolation of mitochondria*

Mitochondria were prepared by cell homogenization and differential centrifugation as described by Attardi

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and Attardi [10], except that the mitochondrial pellet was washed again with STM buffer (0.01 M Tris, pH 7.6; 0.01 M $MgCl_2$; 10% (w/v) sucrose) and finally resuspended in STM + 0.001 M 2-mercaptoethanol.

2.6. *Protein synthesis in isolated mitochondria*

[^{3}H]leucine incorporation into an acid-insoluble product was measured at 30° in an incubation mixture containing in 0.25 ml: 50 mM Tricine, pH 7.6; 10 mM $MgCl_2$; 154 mM KCl; 5 mM Na_2HPO_4, pH 7.6; 2 mM EDTA; 2 mM ATP; 10 mM PEP; 50 μM of each amino acid (minus leucine); 25 μg/ml pyruvate kinase; 20 μCi/ml [^{3}H]leucine 51 c/m mole, and 0.6–1.0 mg/ml mitochondrial protein. Other additions are as described in the tables. At intervals up to 60 min, 0.05 ml aliquots were put on Whatman 3MM filter paper discs and prepared for counting in toluene based scintillation fluid by the method of Mans and Novelli [11]. Incorporation data were calculated as pmoles of [^{3}H]leucine incorporated per mg of mitochondrial protein per 30 min. Protein was determined by the Biuret method [12].

3. Results and discussion

3.1. *Isolation of mutants*

To increase the mutation frequency in mitochondrial DNA, cells were treated with ethidium bromide, an intercalating dye specific for mitochondrial DNA [13, 14]. The concentrations of ethidium bromide used and the levels of resistance to chloramphenicol of a series of mutants are described in table 1. Two ethidium bromide concentrations were used, 0.05 μM (a level at which 90% of the cells survive), and 0.5 μM (50% survival). A mutated culture resistant to 25 μg/ml of CAP was isolated from cells treated with the lower concentration of ethidium bromide (mutant culture 294), while two cultures were obtained from cells treated with the higher concentration, one resistant to 25 μg/ml (culture 295) and another to 50 μg/ml of CAP (culture 296). A number of clones were isolated from each resistant culture and tested for growth characteristics and sensitivity to CAP; a representative clone from each group was selected for futher study, strains 294–1, 295–1, and 296–1. 1½ to 3 months after placing ethidium bromide treated cells under selective conditions, resistant cells began to appear. During this selective process, cells continued to grow for about ten days, after which most of them died. The remaining attached cells decreased to about a tenth their normal size for a variable period of time. Eventually they began once again to enlarge, regain a normal morphology, and divide. The slowness of the selection may result from the long time required for sensitive mitochondria to disappear from a cell and for repopulation of the cell with resistant mitochondria, assuming that only one or two mitochondria were originally mutated in a cell containing hundreds of mitochondria. This delayed expression of a mutant phenotype also suggests that the resistances obtained are not the result of an alteration in cell permeability.

3.2. *Growth and sensitivity characteristics*

The generation times of the resistant mutants in the presence or absence of CAP are given in table 1. Exponential growth rates of mutant cells in the presence of CAP are approximately the same as those of sensitive cells in the absence of CAP. In the presence of CAP, however, the maximal monolayer density reached by resistant cells was 4 to 5-fold less than in its absence. The difference in monolayer density was observed when mutants were tested soon after cloning. More recent observation, however, shows that

Table 1
Growth characteristics of mutant strains.

Mutant clone	Ethidium bromide concentration * (μM)	Chloramphenicol concentration ** (μg/ml)	Generation time (hr)	
			−CAP	+CAP
294–1	0.05	25	30	32
294–2	0.05	25	26	30
294–3	0.05	25	41	41
294–4	0.05	25	30	34
294–5	0.05	25	31	34
295–1	0.5	25	28	28
296–1	0.5	50	28	28
296–2	0.5	50	30	28
296–3	0.5	50	28	28

* Ethidium bromide concentration used to mutate S3 cells.
** Concentration of chloramphenicol at which cells were isolated and cloned.

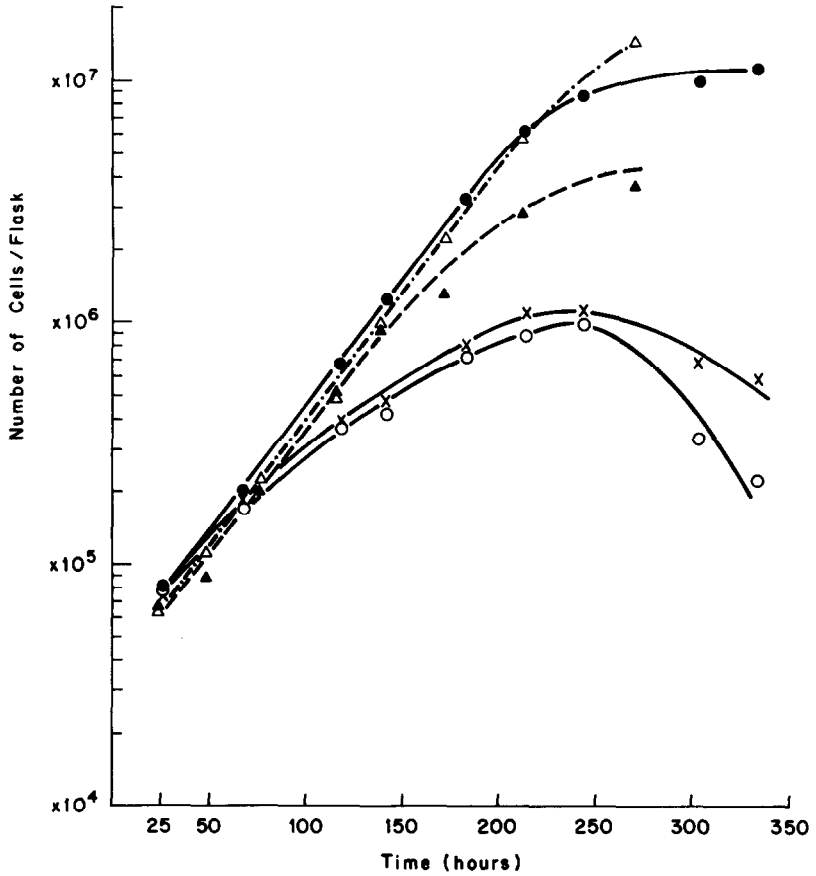

Fig. 1. Growth curves of S3 and 296–1. All cultures were grown in MEM-E + 10% calf serum. Solid lines refer to S3 growth curves; (●——●——●) S3 control; (X——X——X) S3 + 25 μg/ml CAP; (○——○——○) S3 + 50 μg/ml CAP. Broken lines refer to 296–1 growth curves; (Δ·–·–·Δ) 296–1 control; (▲– – –▲) 296–1 + 25 μg/ml CAP.

they are growing to the same density with as without CAP. Fig. 1 shows the growth curves for wild type S3 and for mutant strain 296–1 cells in the presence and absence of CAP. Wild type cells never grow exponentially in the presence of CAP but exhibit a progressively decreasing growth rate from the time they are plated until, after 9–10 days, growth stops altogether and cells begin to die. This is in contrast to reported effects of CAP on growth in suspension cultures, where the growth rate is normal for 5 days before growth ceases [15]. When tested for sensitivity *in vivo* to CAP, mutants fell into two classes (fig. 2). Cells in one group showed complete resistance to CAP concentrations less than or equal to the concentrations at which they were isolated (clones of cultures 295 and 296); in the other class they are partially resistant up to these levels (clones of culture 294). This partial resistance is also reflected in the slightly slower growth rates of clones of 294 in the presence of CAP than in its absence. All mutants cloned from the same resistant culture showed the same sensitivity characteristics.

3.3. *Protein synthesis by isolated mitochondria*

The ability of mutant strains to grow in inhibitory concentrations of CAP (up to 100 μg/ml) could be the result of a mutation affecting the mitochondrial protein synthesizing system, or one excluding the antibiotic from the cell. Therefore, amino acid incorporation into protein by isolated mitochondria was studied in sensitive S3 cells and in three derived CAP resistant mutants, strains 294–1, 295–1, and 296–1. To obtain sufficient quantities, the cells were grown in roller

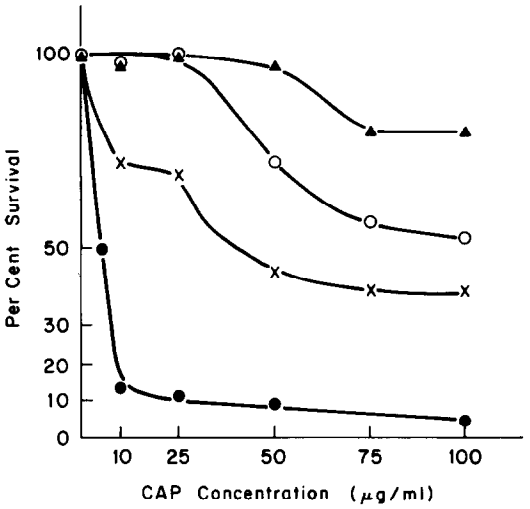

Fig. 2. Cell sensitivity to chloramphenicol. Cells were grown for 5 days, to late exponential phase, in the presence of varying concentrations of CAP. (●—●—●) S3; (X—X—X) 294–1; (○—○—○) 295–1; (▲—▲—▲) 296–1.

bottles to stationary phase. Mutant cultures were grown both with and without CAP to determine any differences in incorporating activity or in sensitivity to CAP. In all the mitochondrial preparations, incorporation was linear for 30–45 min and continued for at least 60 min. Table 2 presents a summary of the activities obtained for all the preparations and of the effects of CAP on incorporation. Incorporation by mitochondria from 295–1 and 296–1 cells grown without CAP is similar to that by wild type mitochondria; however, 294–1 mitochondria have a much lower specific activity. This may reflect a real difference in these cells or it may be just an artifact of the preparation. Mitochondria from cells of all 3 mutants grown in the presence of CAP show a significant increase in specific activity, varying from 1.9 to 3.5-fold in the different mutants. This may be an indication of a mechanism operating in the mutants to increase the number or the activity of ribosomes in the mitochondria in the presence of CAP. The resistance of mitochondrial protein synthesis to cycloheximide suggests that most of the incorporation is by the mitochondrial protein synthesizing system and not the result of cytoribosomal contamination.

CAP inhibits incorporation in the 3 mutant mitochondrial preparations much less than in wild type sensitive mitochondria. The extent of inhibition of all 3 mutants is approximately the same. With 100 μg/ml of CAP, wild type mitochondria have less than 15% of the activity of the control. In contrast, the mutants show 36–48% of control activity. 100 μg/ml was the minimal level which gave maximal inhibition of incorporation. Fig. 3 shows the CAP sensitivity curve for mitochondrial protein synthesis from sensitive S3 cells and from the resistant mutant strain 296–1. These findings indicate that the resistance *in vivo* of the mutants are not caused by mutations which decrease cell membrane permeability to CAP, but are the result of altered mitochondria with increased resistance.

To exclude the possibility that the resistance of the mitochondria was caused by decreased permeability of the mitochondrial membranes to CAP, mitochondria were treated with 0.01% Triton X-100, a nonionic detergent which disrupts mitochondrial membrane structure. That Triton disrupts the membranes is evident from the 1.6 to 5.4-fold increase in specific activity in its presence, resulting from the removal of permeability barriers to exogenous substrates. In the

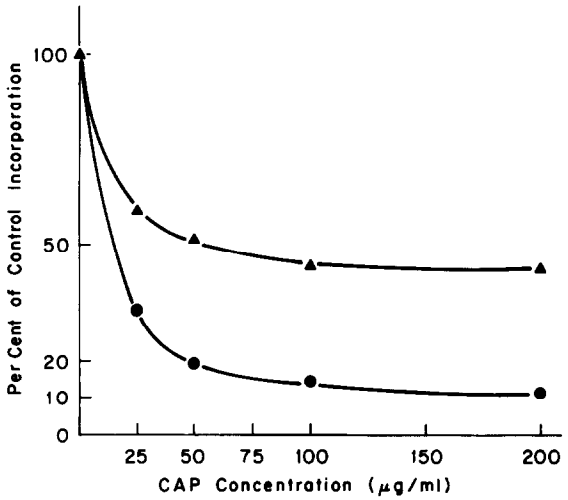

Fig. 3. *In vitro* sensitivity of mitochondrial protein synthesis to chloramphenicol. Protein synthesis *in vitro* by isolated mitochondria was measured as described in Methods. Reactions with varying amounts of CAP were incubated for 30 min at 30°, samples removed and incorporation measured as previously described [11]. The 100% value for S3 was 1.35 pmoles [^{3}H]leucine/mg protein/30 min; for 296–1, it was 3.12. (●—●—●) S3; (▲—▲—▲) 296–1 (grown + CAP).

Table 2
Incorporation of [^{3}H] leucine by isolated mitochondria.

Treatment	S3		294–1				295–1				296–1			
	–CAP[2]		–CAP		+CAP		–CAP		+CAP		–CAP		+CAP	
	S.A.[1]	%[3]	S.A.	%	S.A.	%	S.A.	%	S.A.	%	S.A.	%	S.A.	%
None	1.61	100	0.520	100	0.964	100	0.985	100	3.41	100	1.36	100	2.88	100
+50 μg/ml CAP	0.262	16.3	0.298	57.3	0.496	51.5	–	–	1.18	34.6	0.575	42.3	1.33	46.2
+100 μg/ml CAP	0.204	12.7	0.219	42.1	0.356	36.9	0.477	48.4	1.22	35.8	0.490	36.0	1.17	40.6
+200 μg/ml cycloheximide	1.44	89.4	0.357	68.7	0.794	82.4	0.928	94.2	3.23	94.7	1.05	77.2	2.58	89.6
+.01% Triton X-100	2.64	100	1.52	100	3.27	100	5.33	100	9.00	100	–	–	3.10[4]	100
+.01% Triton + 50 μg/ml CAP	–	–	0.623	41.0	1.31	40.1	1.92	36.0	2.72	30.2	–	–	–	
+.01% Triton 100 μg/ml CAP	0.186	7.0	–	–	–	–	–	–	–	–	–	–	1.46[4]	47.1

[1] Specific activity is measured as pmoles of [^{3}H] leucine incorporated/mg of mitochondrial protein/30 min.
[2] – or +CAP refers to the growth conditions of the cells prior to isolation of mitochondria. Each mutant culture was grown in medium containing the same CAP concentration as that to which it was resistant (see table 1).
[3] The percent value shown is percent of control incorporation.
[4] Triton treatment of 296–1 mitochondria was carried out on a different preparation of mitochondria, in which control specific activity was 2.01 pmoles/mg mitochondrial protein/30 min. 100 μg/ml of CAP decreased this incorporation to 52% of control.

presence of Triton, there is some decrease in resistance of mutant mitochondria to CAP proportional to the increase in sensitivity of S3 mitochondria. This increase probably reflects the ease of entry of CAP as well as other compounds in detergent-treated mitochondria. It suggests that the mutations isolated are in the mitochondrial protein synthesizing system itself, not in the mitochondrial membranes.

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