CYTOPLASMIC TRANSFER OF CHLORAMPHENICOL RESISTANCE IN HUMAN TISSUE CULTURE CELLS

DOUGLAS C. WALLACE, C. L. BUNN, and J. M. EISENSTADT
From the Department of Human Genetics, Yale University, New Haven, Connecticut 06510

ABSTRACT
The cytoplasmic inheritance of human chloramphenicol (CAP) resistance has been demonstrated by removing the nuclei of cells of the CAP-resistant HeLa strain 296-1 (enucleation) and fusing them to a CAP-sensitive HeLa strain lacking nuclear thymidine kinase. Plating the fusion products in bromodeoxyuridine and CAP resulted in the growth of about 150 colonies/10^6 parent cells plated. Permanent cell lines (cybrids) grown from such fusions have been designated HEB. A recloned HEB cybrid (HEB7A) has also been enucleated and fused to hypoxanthine phosphoribosyl transferase (HPRT)-deficient HeLa cells (S3AG1) and HPRT-deficient lymphocytes (WAL-2A). Cybrids were selected in thioguanine and CAP. In the fusion of enucleated (en) HEB7A to S3AG1, 1,200 colonies/10^6 parents were observed. Fusion of enHEB7A to WAL-2A was done in mass culture and cybrids were obtained on three separate occasions. In every case the parental controls were negative.

All isolates tested from the above fusions have the CAP-resistant characteristics, in vivo and in vitro, of the enucleated parent and the nuclear characteristics of the CAP-sensitive parent, such as chromosome number, morphology, and specific isozyme and chromosome markers. Therefore, it can be concluded that CAP resistance is coded in the cytoplasm and not in the nucleus of 296-1 cells. Furthermore, this resistance can be transferred to cells of widely different origin and differentiated state. These studies represent the first genetic evidence of cytoplasmic inheritance in human cells.

Human mitochondria from HeLa cells contain closed circular DNA molecules approximately 4.8 μm in length or about 9.4 × 10^6 daltons (d) molecular weight (34). These genomes carry one gene for each rRNA (1.7−2.1 × 10^6 d of DNA) and 12 4S RNAs (0.6 × 10^6 d) (1, 2, 45). About 14 proteins of 3.0 × 10^6 d could be coded in the remaining 6.9 × 10^6 d of mitochondrial DNA (mtDNA).

A genetic investigation of the specific coding functions of mtDNA requires the isolation of mitochondrial mutants. Previously, this laboratory described a stable mutant HeLa cell line, 296-1, whose growth and mitochondrial protein synthesis in vitro is resistant to levels of chloramphenicol (CAP) which eliminate mitochondrial protein synthesis of the sensitive parent (18, 39). Mitochondrial protein synthesis of this mutant in vitro is unaffected by CAP even in the presence of 0.01% Triton X-100, suggesting that the resistance does not result from mitochondrial membrane permeability. The question remains whether the mutation arose in the mtDNA and, therefore, would be cytoplasmically inherited.

A mitochondrially coded erythromycin-re-
sistant mutation in Paramecium has been correlated with an altered pattern of mitochondrial ribosomal proteins (5, 42). Mitochondrial coding of mutations to antibiotic resistance in Paramecium has been demonstrated by injecting isolated mitochondria from resistant cells into sensitive ones. Generation of resistant cells suggests that the gene is carried with the mitochondria (4, 5, 22). CAP resistance has been shown to be mitochondrially coded in Paramecium (4) and in yeast (9), and cytoplasmically inherited in Tetrahymena (35).

In our previous paper we described a method to demonstrate that CAP resistance in mouse cells was cytoplasmically coded (7). The nuclei of CAP-resistant cells were removed and the resulting cytoplasmic fragments were fused to CAP-sensitive cells. Development of a CAP-resistant line indicated that the determinant of CAP resistance was carried in the cytoplasm. In this paper we have used such a system to demonstrate the first genetic evidence of cytoplasmic inheritance in human cells. A preliminary report of these results has been presented elsewhere (43).

MATERIALS AND METHODS

Cell Lines

The human HeLa cell clone, S3 (14, 32), was used in this study. The isolation and growth characteristics of a CAP-resistant S3 line, designated 296-1, have been described (39).

The HeLa cell line BU25 was also derived from S3 (19). This line is resistant to high levels of bromodeoxyuridine (BrdU) and possesses only 2–5% of the wild-type thymidine kinase (TK).

Three other drug-resistant lines have been isolated for this study. These all lack the salvage enzyme hypoxanthine phosphoribosyl transferase (HPRT).

S3AG1 was isolated after treatment of $4 \times 10^6$ S3 cells with $1 \times 10^{-4}$ M ICR-372 (an acridine mustard mutagen, the gift of Dr. Creech, see reference 10) for 72 h. The cells were washed three times with saline and grown an additional 72 h in nonselective medium. They were then challenged with $3 \mu g/ml$ AG and washed three times in saline and allowed to grow in nonselective medium for 16 days. They were then challenged with $3 \mu g/ml$ AG, and the survivors were transferred into medium containing $6 \mu g/ml$ AG. The resulting culture was then cloned twice in $6 \mu g/ml$ AG, first in petri dishes and then in 0.12% agar.

Line 300-4b was derived from 296-1 and is CAP resistant. Approximately $5 \times 10^6$ cells were treated with $5 \times 10^{-4}$ M ICR-372 for 24 h. Survivors were washed three times in saline and allowed to grow in nonselective medium for 16 days. They were then challenged with $3 \mu g/ml$ AG, and the survivors were transferred into medium containing $6 \mu g/ml$ AG. The resulting culture was then cloned twice in $6 \mu g/ml$ AG, first in petri dishes and then in 0.12% agar.

One of the resulting clones was grown in $2 \times 10^{-4}$ M ICR-372 for 24 h and washed three times. The cells were challenged immediately with $12 \mu g/ml$ AG and the survivors were allowed to grow for 17 days. $5 \times 10^6$ of these cells were then challenged with $60 \mu g/ml$ AG. These cells were unaffected by this level and so were cloned in petri dishes. A single clone was designated 300-4b. The line grows indefinitely in $60 \mu g/ml$ AG and in $1 \times 10^{-4}$ M TG. No detectable HPRT activity was observed in 300-4b (Table 1).

Line 300-4b is HAT sensitive, although a low reversion rate of about 0.002% is observed after treatment with inactive Sendai virus and incubation for over 20 days. A similar observation on HAT reversion rate has been made for the line D98/AH (41). This line was obtained from Detroit-98, a presumptive HeLa line (13), by stepwise selection of resistance to AG and azahypoxanthine. In HAT, 0.1% of the D98/AH cells were observed to revert to resistance.

WAL-2A is an HPRT-negative derivative of the human diploid lymphocyte line WI-L2 (24). The latter strain was grown in suspension for 24 h in $2.5 \times 10^{-6}$ M ICR-372. After growth in the absence of selective agent

| Strain* | HPRT Activity of Parents and Their AG- and TG-Resistant Mutants |
|---------|---------------------------------------------------------------|
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* In each pair of strains the parent is listed above the AG- and TG- resistant mutant. WI-L2 was recloned before selection of WAL-2A, yielding line WI-L2A.

Specific activity units are nanomoles hypoxanthine converted to IMP per hour per milligram protein at 24°C. Zero indicates no detectable activity—less than 0.02 nmol/h/mg protein.

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for 24 h, the entire culture was challenged with 3 \( \mu \text{g/ml} \) AG. Cells which grew in AG were then cloned in 0.12% agar once in 3 \( \mu \text{g/ml} \) AG, once in 5 \( \mu \text{g/ml} \) AG, and once in 1 \( \times 10^{-4} \text{ M TG} \). The resulting strain, designated WAL-2A, grows in 1 \( \times 10^{-4} \text{ M TG} \), is HAT sensitive, and shows no HPRT activity (Table I). WI-L2 contains a Y chromosome and the glucose-6-phosphate dehydrogenase (G6PD) variant B, while HeLa lacks a Y chromosome and is G6PD variant A (13, 30, 31, Table VII).

**Enucleation**

The method of enucleation used in this study is designed to obtain large numbers of enucleated fibroblasts (11). All enucleations were performed in MEM-E + 10% fetal calf serum + 50 \( \mu \text{g/ml} \) CAP. In the first fusion between enucleated (en) 296-1 and BU25 cells, the method was modified by preincubation of the attached cells in medium containing 20 \( \mu \text{g/ml} \) cytochalasin B for 3 h at 37°C, followed by centrifugation in 20 \( \mu \text{g/ml} \) cytochalasin B in an SW27 rotor at 10,000 rpm for 45 min. This treatment was followed by a 3-h recovery period in the absence of cytochalasin B. In all subsequent enucleations, the method was modified by preincubation for 2 h at 10 \( \mu \text{g/ml} \) cytochalasin B, followed by a 45-min centrifugation at 15,000 rpm in an SW 27 rotor in the presence of 2 \( \mu \text{g/ml} \) cytochalasin B. Recovery was allowed for 1 h in the absence of cytochalasin B. The resulting enucleated fragments, cytoplasts (36), were then removed from the tubes with Viokase (Grand Island Biological Co., Grand Island, N.Y.) and washed once in saline before fusion. The percentage of cytoplasts relative to nuclei and whole cells was determined by counting cells stained with lactopropionic orcein (12).

**Cell Fusion**

Between 3 and 5 \( \times 10^6 \) cells of each parent were fused at a 1:1 ratio with 1,000 hemagglutination units (HAU) of inactive Sendai virus, as previously described (7). All parental controls of enucleation experiments were also virus treated at approximately the same virus-to-cell ratio to check for virus-induced mutation. Fusions in which WAL-2A cells were used employed a modified lymphocyte trypsin pretreatment (40). WAL-2A cells were washed once in saline and then incubated for 6 min at 37°C in 0.001% trypsin in saline. The trypsin was inactivated by adding 1.5 vol of medium containing serum, and then the cells were washed once again in saline. All other cell lines were grown in monolayers. Before fusion, the line was harvested with Viokase and washed once in saline.

**Media**

The medium used for fibroblastic cell fusions was MEM-E supplemented with 10% fetal calf serum, 100 U/ml of penicillin, and 100 \( \mu \text{g/ml} \) streptomycin. The one exception was in the fusion of 300-4b and BU25, selected in HAT + CAP, where 5% fetal calf serum plus 5% calf serum was used. In fusions involving the lymphocyte line WAL-2A, RPMI 1640, containing 592 mg/l-glutamine and supplemented with 15% heat-inactivated fetal calf serum plus penicillin and streptomycin, was employed.

**Cloning**

Two methods of cloning cells were used in this study. The semisolid agar method involved suspending the cells in 0.12% Noble agar (Difco, Detroit, Mich.) prepared in growth medium containing serum. Clones were also picked from petri dishes and flask surfaces with stainless steel cloning rings (33).

**Karyotyping**

Late exponential phase cells were arrested with 0.1 \( \mu \text{g/ml} \) colchicine and swollen in hypotonic buffer for 10–15 min. The cells were then fixed with methanol-acetic acid (3:1) and spread on slides. Chromosomes were counted after staining with lactoacetic orcein (44). To identify the Y chromosome, cells were stained by a modification of the Q band method (30). Slides were stained for 15 min in 0.5% Atebrin (Bio/Medical Specialties, Santa Monica, Calif.), and then rinsed for 3 min in running distilled water. The slides were then drained and mounted in Tris-maleate buffer (pH 5.6), and the cover slip was sealed with nail polish. The slides were examined with a Zeiss transmission fluorescence microscope fitted with a dark-field condenser and illuminated with an HBO 200 mercury vapor lamp. The excitation filter BG12, with or without BG38, was used with barrier filters 50 or 47.

**Enzyme Assays**

HPRT activity was measured in whole cell extracts (25) after the transfer of the phosphorylated ribose group from 5-phosphoribosyl-1-pyrophosphate to radioactive hypoxanthine (15). TK activity was assayed by examining the phosphorylation of labeled thymidine from ATP. Standard assay conditions were used except that 3-phosphoglycerate was omitted from the reaction (21, 27). Preparation of TK cell extracts was modified by reducing the thymidine level to 60 \( \mu \text{g/ml} \) in all solutions (27). Cell debris from homogenized cells in the TK extract was removed by a 40-min centrifugation at 35,000 g. Inosinic acid and thymidylic acid were separated from their labeled precursors by differentially binding the phosphorylated compounds to DEAE paper (6).

G6PD isozyme variants A and B were separated by cellulose acetate strip electrophoresis (38). Enzyme extracts were prepared from cells washed twice in 0.85% saline, homogenized in distilled water, and clarified by centrifugation for 60 min at 35,000 g. The Folin method was used for all protein determinations (28).
Mitochondrial Protein Synthesis In Vitro

Cells were grown in the absence of CAP in glass roller bottles and harvested with Viokase. The mitochondria were purified by differential centrifugation and the isolated mitochondria were tested for their ability to incorporate [3H]leucine into hot TCA-insoluble material in vitro. The experimental details for mitochondrial purification and protein synthesis in vitro have been previously described (39). The specific activity of the L-[4,5-3H]leucine (New England Nuclear, Boston, Mass.) was 40.7 Ci/mmol and the scintillation counter efficiency was about 30%.

Growth and Sensitivity Curves

To determine the sensitivity of cells to BrdU and TG, cells were grown in 30 μg/ml BrdU or 1 x 10^-4 M TG until growth ceased. HeLa cells are sensitive to both drugs and continuous growth stops within 4 days. WAL-2A and its derivatives are sensitive to BrdU, but will grow for 5-7 days in 50 ml of stationary medium before growth ceases. Growth rates in various selective media and sensitivity to various levels of CAP were determined as previously described (39).

RESULTS

Experimental Design

A schematic representation of the method used for demonstrating cytoplasmic inheritance is shown in Fig. 1. HeLa cells which are resistant to CAP and sensitive to BrdU (TK present) are enucleated. The resulting cytoplasts are harvested and fused to human CAP-sensitive cells which are resistant to BrdU. The fusion mixture is then inoculated into medium containing BrdU and CAP.

CAP kills the CAP-sensitive parents and BrdU kills any CAP-resistant parents that have escaped enucleation. The BrdU also kills any nuclear-nuclear fusion product involving a nucleated CAP-resistant cell, since the TK gene will be expressed and confer sensitivity to BrdU on the hybrid cell.

The only cells that can grow, therefore, are the products of a fusion between a CAP-resistant cytoplasm and a BrdU-resistant cell. Such cells which grow into continuous cell lines have been previously designated cytoplasmic hybrids or cybrids (7). Similar experiments can be done by use of HPRT as the nuclear marker and TG as the selective agent.

Demonstration of Cytoplasmic Inheritance of CAP Resistance in 296-1

To demonstrate cytoplasmic inheritance of CAP resistance, 296-1 cells were enucleated (en296-1) and fused to BU25 which lacks TK and is CAP-sensitive. Selection was carried out in 25 μg/ml BrdU and 50 μg/ml CAP. As shown in Table II, colonies arose in about 18 days in flasks containing the fusion mixture, while no colonies developed when the parents were plated alone. The number of colonies appearing in each flask follows a dilution sequence and the frequency of colony formation is about 150 cybrids/10^4 total parents plated. Cybrids from these fusions are designated HEB.

![Diagram](https://example.com/diagram.png)

**Fig. 1** Procedure used for construction of human cybrids. Superscript R indicates that the cell line is resistant; an S indicates that it is sensitive.
TABLE II
Fusion of en296-1 and BU25*

| Cells plated   | No. cells per flask | No. flasks | Mean no. colonies per flask | Clones per 10^6 cells |
|---------------|---------------------|------------|-----------------------------|-----------------------|
| en296-1       | 1 x 10^6            | 2          | TNTC§                       | —                     |
| BU25          | 2.5 x 10^4          | 4          | 63                          | 126                   |
| BU25          | 1 x 10^4            | 4          | 35                          | 140                   |
| en296-1       | 2.6 x 10^4          | —          | 0                           | 0                     |
| BU25          | 8 x 10^4            | —          | 0                           | 0                     |

* Cybrids were formed by the fusion of the parental cells, enucleated (en) 296-1 and BU25. The medium contained the inhibitors 25 μg/ml BrdU and 50 μg/ml CAP to select for the cybrids. Cells were plated at different cell densities and colonies were counted after 18 days.

§ TNTC means too numerous to count.

These numbers represent the total number of cells plated in all flasks.

Fig. 2 shows the effects of the selective system on growth of the parental lines and a recloned cybrid, HEB7A, from this experiment. The growth of strain 296-1 is unaffected by 50 μg/ml of CAP in the medium. When it is grown in 30 μg/ml BrdU plus 50 μg/ml CAP, however, the culture undergoes about one cell division when growth ceases. BU25, in the presence of 30 μg/ml BrdU and 50 μg/ml CAP, grows exponentially until the delayed effect of CAP is expressed, at which time the culture dies. In contrast, the growth of the cybrid, HEB7A, is unaffected by the combination of 30 μg/ml BrdU and 50 μg/ml CAP. Thus, the cybrid exhibits the nuclear characteristics of BU25 and the CAP resistance of 296-1.

A summary of all experiments on the cytoplasmic inheritance of 296-1 CAP resistance is shown in Table III. The first line describes the above experiment. The second is a repeat of that experiment using 30 μg/ml BrdU and 50 μg/ml CAP. In both instances, colonies arose at a frequency of 150/10⁶ parental cells plated. Fusion of 296-1 without previous enucleation to BU25 followed by selection in 30 μg/ml BrdU and 50 μg/ml CAP resulted in a very low frequency of colony formation, about 2.2/10⁶ parents plated. This result rules out the possibility that cybrid formation is the result of a nuclear-nuclear fusion followed by loss of the 296-1 chromosomes coding for TK. The few colonies that do appear could be explained by the loss of 296-1 chromosomes resulting from premature chromosome condensation after fusion of cells in different stages of the cell cycle. It has been observed that when HeLa cells in G₁ or S phase 296-1 fused with a late G₂ or M phase BU25 could result in loss of the 296-1 chromosomes coding for TK and might explain the appearance of the colonies observed.

The fourth line in Table III shows that when the replicating (16). G₁ or S phase 296-1 fused with a late G₂ or M phase BU25 could result in loss of the 296-1 chromosomes coding for TK and might explain the appearance of the colonies observed.

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HPRT-negative derivative of 296-1, 300-4b, is fused to BU25 and selection takes place in HAT plus 50 μg/ml CAP, colonies appear at a rate slightly greater than one-third that of cybrid formation. Cultures resulting from this fusion have been designated HAB.

Plating the virus-treated parental cell lines alone results in no colonies which grow in BrdU and CAP. Similarly, 300-4b or BU25 do not form colonies in the presence of HAT plus CAP in the time indicated.

Table IV gives the nuclear characteristics of the parents and representative hybrids and cybrids from the previous fusions. It is clear that the chromosome numbers of the HAB hybrids are about 120, which is close to the sum of the chromosome numbers of the two parents, BU25 and 300-4b. On the other hand, the chromosome numbers of the HEB cybrids are equivalent to that of BU25, less than that of 296-1, and not nearly that found in the hybrids. Thus, the HEB clones are not the product of a nuclear-nuclear fusion.

Morphology was distinctive here. The 296-1 cell is rounded and highly refractile while the BU25 cell is large, flattened, and gray. All of the cybrids had the flat morphology of BU25. Thus, the HEB cybrids have the nuclear characteristics of BU25 and not 296-1.

The effects of increasing concentrations of CAP on the growth of the parents 296-1 and BU25 and the cybrid HEB7A are shown in Fig. 3. The 296-1 line is resistant to levels of CAP that completely inhibit BU25. HEB7A shows resistance to CAP similar to 296-1. Comparable results have been obtained for two other cybrids, HEB3A and HEB4A. Thus, cybrid formation generates a cell that has the same CAP resistance as the CAP-resistant donor.

The ability of isolated mitochondria of 296-1, BU25, and HEB7A to incorporate labeled leucine into mitochondrial protein was examined in vitro. The effects of CAP and cycloheximide (CHX) were then tested (Table V). CAP-resistant mitochondrial protein synthesis was found for both the CAP-resistant parent, 296-1, and the cybrid, HEB7A. Mitochondrial protein synthesis of BU25 was found to be CAP sensitive. Protein synthesis in all three types of mitochondria was found to be resistant to CHX, proving that the differences cannot be explained by contaminating cytosol ribosomes. These experiments in vitro prove that the resistance transferred with the cytoplast still resides in the mitochondria. This rules out the possibility that CAP resistance is the result of some secondary change occurring in the recipient cell during cybrid formation.

These data, in conjunction with the absence of colonies formed when the CAP-sensitive parent is plated alone, the high frequency of generation of CAP-resistant colonies during cybrid formation, would suggest that these cybrids are primarily nuclear fusions.

### Table III

| Cells       | Enucleation* | Total cells fused| Selective medium| Colonies/10^6 cells| Time of count| Culture designation |
|-------------|--------------|------------------|-----------------|--------------------|--------------|---------------------|
| 296-1 × BU25| 52           | 6 × 10^5         | BrdU + CAP      | 150                | 18           | HEB                 |
| 296-1 × BU25| 34           | 1 × 10^7         | BrdU + CAP      | 150                | 25           | HEB                 |
| 296-1 × BU25| 0            | 1 × 10^7         | BrdU + CAP      | 2.2                | 26           | —                   |
| 300-4b × BU25| 0            | 1 × 10^7         | HAT + CAP       | 59                 | 13           | HAB                 |
| en296-1     | 52           | 1 × 10^8         | BrdU + CAP      | 0                  | 18           | —                   |
| en296-1     | 34           | 3 × 10^8         | BrdU + CAP      | 0                  | 25           | —                   |
| BU25        | 0            | 3 × 10^8         | BrdU + CAP      | 0                  | 18           | —                   |
| BU25        | 0            | 5 × 10^8         | HAT + CAP       | 0                  | 25           | —                   |
| 300-4b      | 0            | 5 × 10^8         | HAT + CAP       | 0                  | 13           | —                   |
| BU25        | 0            | 5 × 10^8         | HAT + CAP       | 0                  | 13           | —                   |

* The percentage of the total parental cells plus fragments which were cytoplasts.
† In all cases, where two cell types were employed, the ratio of parents was 1:1. Thus, the number of cells of each parent was 50% of the number reported. Virtually all of the fused cells were then inoculated into the selective medium.
§ The concentrations of the selective drugs were 25 or 30 μg/ml BrdU and 50 μg/ml CAP.
¶ The mean number of colonies observed in a number of flasks.
# Colonies were counted as soon as they were clearly visible to minimize the inclusion of satellite colonies in the counts.
**TABLE IV**

*Karyotypes and Morphology of Parents, Cybrids, and Hybrids from the en296-1 × BU25 System*

| Cell line     | Source* | X     | SD  | N excluded | Morphology |
|---------------|---------|-------|-----|------------|------------|
| Parents       |         |       |     |            |            |
| 296-1 HeLa   | S3 CAP  | 64.2  | 2.3 | 26         | 3.7        | rounded    |
| BU25 HeLa    | S3 BrdU| 59.3  | 1.7 | 29         | 9.4        | flat       |
| 300-4b 296-1 | TGR     | 65.2  | 3.4 | 30         | 0          | —          |
| Cybrids       |         |       |     |            |            |
| HEB3A en296-1| BU25    | 56.9  | 4.0 | 24         | 23         | flat       |
| HEB4A en296-1| BU25    | 57.8  | 2.5 | 24         | 23         | flat       |
| HEB7A en296-1| BU25    | 58.2  | 4.3 | 27         | 0          | flat       |
| Hybrids       |         |       |     |            |            |
| HAB2 300-4b  | BU25    | 118.6 | 8.2 | 30         | 0          | —          |
| HAB3 300-4b  | BU25    | 119.6 | 8.5 | 30         | 0          | —          |
| HAB4 300-4b  | BU25    | 118.5 | 10.0| 30         | 0          | —          |

* The origin of the cell lines and the markers they contain, or the fusions from which the cybrids and hybrids were derived. Superscript R indicates resistance to the drug named.

† The mean chromosome number (X) for each parent or fusion product, the standard deviation (SD) from the mean, and the number of chromosome smears (N) sampled.

§ When the chromosome count was extremely high, close to double the mean, or extremely low, the count was excluded from the sample as aberrant. The percentage of the total sample excluded on this basis is listed here.

‖ See text.

and the extreme difficulty experienced in obtaining CAP-resistant colonies originally (39), eliminate the possibility that cybrid formation occurs as a result of mutation of the CAP-sensitive parent to CAP resistance.

**Other Systems for Demonstrating Cytoplasmic Inheritance in Human Cells**

By using 296-1 and the recloned cybrid HEB7A as donors, CAP resistance has been transferred to

![Graph](image-url)  

**Fig. 3** Effect of increasing concentration of CAP on the growth of the two parents, 296-1 and BU25, and the cybrid HEB7A. CAP sensitivity is measured as a percentage of the growth after 8 days of test cultures in CAP relative to control cultures in the absence of CAP. Filled triangles (▲) represent the CAP-resistant parent 296-1. Open circles (○) represent the CAP-sensitive parent BU25, and the filled circles (●) represent the cybrid. In the case of BU25, the approximately 10% of cells remaining at CAP concentrations of 50 μg/ml and above do not indicate a small percentage of resistant cells. These cells are shriveled and incapable of developing into colonies in the presence of CAP. However, they may remain attached to the substrate for some time.
two other human cell lines. The two recipient lines, S3AG1 and WAL-2A, are HPRT negative, TG resistant, and CAP sensitive. TG was used in these experiments because AG was found to produce poor selection in HeLa cells. TG, on the other hand, is extremely toxic to HeLa cells, eliminating virtually all 296-1 cells exposed to $10^{-4}$ M in 7 days.

The transfers of CAP resistance from enucleated HeLa cells to S3AG1 are summarized in Table V.

### Table V

**Effect of Antibiotics on Mitochondrial Protein Synthesis In Vitro of Parents 296-1 and BU25 and the Cybrid HEB7A**

| Source of mitochondria* | Treatment | 296-1 | BU25 | HEB7A |
|-------------------------|-----------|-------|------|-------|
| None                    | 100†      | 100   | 100  |
| + 100 µg/ml CAP         | 79        | 24    | 85   |
| + 200 µg/ml CHX         | 103       | 98    | 97   |

* Mitochondria were purified from the various cell lines (see Materials and Methods) and the incorporation of [3H]leucine into protein was followed for 45 min at 30°C. The incorporation of [3H]leucine in the absence of antibiotics was 17,400 cpm/mg mitochondrial protein for 296-1, 9,120 cpm/mg protein for BU25, and 19,800 cpm/mg protein for HEB7A.
† All values are expressed as a percentage of [3H]leucine incorporation in the absence of inhibitors.

Table VI. In the first experiment, cells of the cybrid, HEB7A, were enucleated and fused to S3AG1 cells. Each parent was also virus treated and all preparations were diluted and inoculated into flasks of MEM-E containing 50 µg/ml of CAP. After 5 days, the medium was further supplemented with $10^{-4}$ M TG.

A cytoplast from a cell containing HPRT should confer on a cybrid not only its CAP resistance but also temporary TG sensitivity, HPRT being co-transferred. Thus, the addition of high levels of TG are delayed to permit the decay of the enzyme and the re-expression of the TG-resistant genotype in the cybrid.

After only 13 days of incubation, colonies were clearly visible in every fusion flask while none were seen in either parental control. The colony number was directly related to the number of fused cells plated. The overall cybrid frequency was 1,200 cybrids/10^6 parental cells. This is eight times the frequency seen in the fusion en296-1 × BU25. Cultures developing from this fusion were named BES.

The nuclear characteristics of the parents (HEB7A and S3AG1) and of the BES cybrids (BES3, BES5, and BES7) are shown in Table VII. The cybrids BES3, BES5, and BES7 each have a chromosome number close to that of S3AG1 and greater than that of HEB7A. Also, HEB7A has inherited the flat morphology of BU25, while
### TABLE VII

**Characteristics of Parents and Cybrids From the Fusions enHEB7A × S3AGI and enHEB7A × WAL-2A**

| Cell line | Source* | Number of chromosomes* | Sensitivity to drugs | Enzyme specific activity | Presence of Y chromosome\(
|-----------|---------|------------------------|----------------------|--------------------------|-------------------------|
|           |         | \(X\) SD N excluded Morphology | TG\| BrdU\| HPRT\| TK** variant|\| \|
| Parents   |         |                        |                       |                          |                         |
| HEB7A     | HeLa S3 CAP\# BrdU\# | 58.2 4.3 27 0 epithelial, flat | S R 200 0.52 A |                         |                         |
| S3AGI     | HeLa S3 TG\#          | 67.5 2.7 29 3.3 epithelial, flat | R S 0 4.4 ND |                         |                         |
| WAL-2A    | WI-L2A TG\#           | 45.6 1.2 41 6.8 lymphocyte | R S 0 2.6 B |                         |                         |
| Cybrids   |         |                        |                       |                          |                         |
| BES3      | enHEB7A × S3AGI       | 64.8 2.1 30 0 epithelial, rounded | R S 0 5.1 ND |                         |                         |
| BES5      | enHEB7A × S3AGI       | 65.3 3.3 30 0 epithelial, rounded | R S 0 9.6 ND |                         |                         |
| BES7      | enHEB7A × S3AGI       | 65.3 1.8 30 0 epithelial, rounded | R S 0 9.3 ND |                         |                         |
| HEW2      | enHEB7A × WAL-2A      | 45.4 0.89 41 0 lymphocyte | R S 0 4.1 B |                         |                         |
| HEW3      | enHEB7A × WAL-2A      | 45.6 0.9 29 3.3 lymphocyte | R S 0 3.1 B |                         |                         |
| HEW4      | enHEB7A × WAL-2A      | 45.5 1.3 28 6.7 lymphocyte | R S 0 2.7 B |                         |                         |
| HEW5      | enHEB7A × WAL-2A      | 46.0 0.67 30 0 lymphocyte | R S 0 4.1 B |                         |                         |

\* See Table IV.

1 An epithelial cell is defined as a HeLa cell which grows attached to the substrate. A lymphocyte is a cell which grows in suspension.

§ R means that the cell is resistant and grows indefinitely in the drug at concentrations of at least 10\(-9\) M TG. S indicates that the cell line is sensitive and that the cells are destroyed within 6 days at 10\(-5\) M.

\(\dagger\) R indicates that the line is resistant to 30\(\mu\)g/ml BrdU and will grow indefinitely at this level. S indicates that the line is sensitive and is killed by 30\(\mu\)g/ml BrdU.

Growth of HeLa cells stops in about 4 days, while growth of WAL-2A and HEWs stops in about 6 days.

\(\ddagger\) The units represent nanomoles hypoxanthine converted to IMP/hour/milligram protein at 24\(\circ\)C. See Table I.

** The units represent nanomoles of thymidine phosphorylated/hour/milligram protein at 37\(\circ\)C.

\(\ddagger\dagger\) The A variant is the faster-migrating band while the B is the slower. ND indicates that the assay was not done because the two parents were identical.

\(\ddagger\ddagger\) A negative (\(\_\_\_\)) indicates that the line lacks a Y chromosome. A positive (+) means that a Y chromosome is present. ND indicates that the assay was not done because the parents were identical.
S3AG1 and the three BES cybrids are rounded. HEB7A is BrdU resistant and lacks most of its TK activity. Since cybrids were selected with TG, BrdU resistance represents a nonselected marker. All of the BES cybrids are BrdU sensitive and have TK activity at least 10 times that of HEB7A, to be expected if their nuclei were derived from S3AG1.

The BES cybrids and S3AG1 are resistant to TG but HEB7A is not. Examination of HPRT activity shows that HEB7A exhibits 200 U of activity; S3AG1 has none, like the BES cybrids. The high rate of cybrid formation suggests that the colonies observed are not the results of mutation of HEB7A to TG resistance. All of these results strongly suggest that the BES cybrid nuclei are derived from S3AG1.

The extremely high rate of cybrid formation in this experiment and the absence of any colonies in the S3AG1 virus-treated control make it very unlikely that such results could be explained by mutation. This is further supported by the appearance of cybrid colonies in less than 13 days.

To demonstrate that these cybrids were the result of the fusion of CAP-sensitive, TG-resistant cells with CAP-resistant cytoplasts from TG-sensitive cells, advantage was taken of the fact that such a transfer should impart a transient TG sensitivity to the cybrids. The extreme toxicity of 10^{-4} M TG to sensitive HeLa cells suggested that cybrid formation might be selected against by addition of TG before the re-expression of the HPRT-negative genotype. A reduction in the number of cybrids would demonstrate that the selection for the CAP-resistant determinant also required the addition of active HPRT. Since the sole source of HPRT in this experiment is the cytoplasm of the donor cell, this would show that the CAP resistance was carried in the donor cell’s cytoplasm. HeLa 296-1 was chosen as the donor in this HPRT cotransfer experiment.

HeLa 296-1 cells were enucleated and fused to S3AG1; both parents were also virus treated. All three preparations were inoculated directly into flasks containing medium with both 50 µg/ml CAP and 10^{-4} M TG (Table VI). Of the 8.8 x 10^{8} cells inoculated, only three clones (designated HES) appeared after 23 days, each in a separate flask. No colonies appeared in the parental controls.

To determine if the HES clones could be the product of a fusion between two nucleated cells followed by chromosome loss, nucleated 296-1 cells were fused to S3AG1 cells and the mixture was inoculated directly into 10^{-4} M TG and 50 µg/ml CAP. All flasks were negative at 26 days and remained so despite prolonged incubation. This result, plus the negative parental controls, suggests that HES clones are cybrids.

The enormous decrease in the number of cybrids caused by immediate rather than delayed addition of TG is proof that the CAP resistance is carried in the same structure which imparts the transient TG sensitivity. Thus, CAP resistance must be carried in the HeLa cytoplasts.

The fact that three HES cybrids did appear is evidence that fusion occurred. The appearance of these colonies could be explained if, in these cases, very small cytoplasts fused with the S3AG1 cells. The low level of HPRT added by the small cytoplast would reduce the TG toxicity. The simultaneously low input of the CAP-resistant factor would require a longer period for the selection of a fully resistant cell. This could account for the increase from 13 to 26 days in the time of cybrid appearance. The isolation of the HES clones indicates that both HEB7A and 296-1 can donate CAP resistance to S3AG1 in a TG and CAP selection system. The use of cybrid HEB7A as a donor proves that CAP resistance can be transferred sequentially to one cell line after another.

Four different procedures have been used to select for cybrid formation between enHEB7A and WAL-2A, the human lymphocyte line. In all cases, the cells were grown without agitation in RPMI 1640 (see Materials and Methods). HEB7A cells have been found to attach tenaciously in this medium, while the WAL-2A lymphocytes do not. Thus, a method to enrich for lymphocytes is simply to swirl the Erlenmeyer flask or bottle containing the cells and then pour the supernate into a new container ("pour-off" selection).

Successful selection of CAP-resistant lymphocyte lines has been achieved by selection in 50 µg/ml CAP added immediately, plus either 10^{-4} M or 10^{-4} M TG added immediately after fusion as well as after a 5-day delay. However, it required 67 days for the culture inoculated directly into 10^{-4} M TG to grow. Inoculation of cells into medium containing 10^{-4} M TG or into medium to which 10^{-4} M TG or 10^{-4} M TG was added after a 5-day delay resulted in CAP-resistant lymphocytes in 25–26 days (Table VIII). Virus-treated enHEB7A or WAL-2A under identical conditions did not grow in any of the four experiments. The concerted effect of pour-off plus TG toxicity
Table VIII

Transfer of CAP Resistance to Lymphocyte Line WAL-2A

| Cells                        | Enucleation* | Total cells fused† | Selective system‡ | Growth of culture§ | Time of transfer or cessation of experiment¶ | Culture designation |
|-----------------------------|--------------|--------------------|-------------------|--------------------|---------------------------------------------|---------------------|
| enHEB7A × WAL-2A            | 74           | 8 x 10⁶            | CAP + high TG + pour-off | G                | 67                                          | HEW2                |
| enHEB7A                     | 74           | 4 x 10⁶            | CAP + high TG + pour-off | NG               | 49                                          | —                   |
| WAL-2A                      | 0            | 4 x 10⁶            | CAP + high TG + pour-off | NG               | 87                                          | —                   |
| enHEB7A × WAL-2A            | 74           | 8 x 10⁶            | CAP + low TG + pour-off | G                | 25                                          | HEW3                |
| enHEB7A                     | 74           | 4 x 10⁶            | CAP + low TG + pour-off | NG               | 49                                          | —                   |
| WAL-2A                      | 0            | 4 x 10⁶            | CAP + low TG + pour-off | NG               | 49                                          | —                   |
| enHEB7A × WAL-2A            | 57           | 10⁷               | CAP + low TG delayed + pour-off | G    | 26                                          | HEW4                |
| enHEB7A                     | 57           | 5 x 10⁶            | CAP + low TG delayed + pour-off | NG   | 30                                          | —                   |
| WAL-2A                      | 0            | 5 x 10⁶            | CAP + low TG delayed + pour-off | NG   | 44                                          | —                   |
| enHEB7A × WAL-2A            | 57           | 10⁷               | CAP + high TG delayed + pour-off | G    | 26                                          | HEW5                |
| enHEB7A                     | 57           | 5 x 10⁶            | CAP + high TG delayed + pour-off | NG   | 30                                          | —                   |
| WAL-2A                      | 0            | 5 x 10⁶            | CAP + high TG delayed + pour-off | NG   | 44                                          | —                   |

* See Table III.
† See Table III. All cells indicated were inoculated into the selective medium contained in a single Erlenmeyer flask.
‡ In all cases CAP was used at 50 µg/ml. "High TG" represents 10⁻⁴ M, while "low TG" represents 10⁻⁵ M. "Delayed" means that TG was not added until 5 days after fusion. "Pour-off" refers to selection for the lymphocytic phenotype of WAL-2A. When TG was added at the time of fusion, day 0, the cultures were "poured off" at days 2, 3, 4, and 5. When TG was added 5 days after fusion, "pour-off" was employed on days 2, 3, 4, 5, 9, 14, 15, 16, and 17. As the cells grew, medium was added to keep them in log phase. At periodic intervals the cells were pelleted and resuspended in fresh medium to avoid possible loss of the activity of the inhibitors and depletion of the medium.
§ G indicates that the culture grew, while the NG indicates that it did not grow. Since the lymphocyte fusions were done in mass culture, it was impossible to quantify the yield of cybrids.
¶ The time in days that the fusion cultures were first transferred or that the dead parental cultures were finally discarded.
rapidly eliminated all on HEB7A. WAL-2A alone grew in CAP for about 1 wk at an ever-decreasing rate until all growth ceased. By 3 wk the cells were unable to exclude the vital dye, trypan blue. Prolonged incubation of the WAL-2A cultures in 50 \( \mu \text{g/ml} \) CAP for 87 days with \( 10^{-4} \) M TG, 49 days with \( 10^{-5} \) M TG, and 44 days with the delayed addition of TG resulted in no growth or viability.

In the experiments involving the delayed addition of TG, the fusion mixture was divided after 5 days. Half of the culture received \( 10^{-5} \) M TG and the other half \( 10^{-4} \) M TG. The combined results show that CAP-resistant lymphocytes have been generated in three separate fusions and under four different selective procedures.

The CAP-resistant lines resulting from these fusions have been designated HEW 2, 3, 4, and 5. The cultures have now been grown for several months in 50 \( \mu \text{g/ml} \) CAP with and without TG. The HEW cybrids, although resistant to CAP, did not initially exhibit the same level of resistance as the HEB7A parent, a result distinctly different from our observations of other human and mouse cybrids. Prolonged culture, however, produced a fully resistant line which retains its CAP resistance for over 14 generations of growth in the absence of CAP.

The nuclear characteristics of HEB7A and WAL-2A and the HEW cybrids are shown in Table VII. The chromosome number of HEB7A is 58 which is substantially higher than the diploid 46 seen in WAL-2A. The chromosome number for all the HEW cybrids is 46. HEB7A is an epithelial cell while WAL-2A and HEW 2, 3, 4, and 5 grow in suspension as lymphocytes. WAL-2A and the HEW cybrids each show at least five times the TK activity of HEB7A and all are BrdU sensitive. No HPRT activity is seen in WAL-2A or the HEW lines, all of which are TG resistant.

Finally, invoking two of the criteria for HeLa cell identification suggested by other research groups (13, 31), HEB7A (being of HeLa origin) should contain G6PD, variant A, and lack a Y chromosome. WAL-2A should possess G6PD, variant B, and have a Y chromosome. Both G6PD and the Y chromosome are nonselected markers in this system. Analysis of the parents and cybrids for G6PD isozymes and for the Y chromosome using Q banding shows that HEB7A is indeed G6PD, variant A, and lacks a Y chromosome while WAL-2A and the cybrids HEW 2, 3, 4, and 5 are all G6PD, variant B, and have a Y chromosome. From these data, it is concluded that HEW 2, 3, 4, and 5 contain only the nuclear information of WAL-2A. However, these lines are now CAP resistant and can grow indefinitely in levels of CAP to which the WAL-2A parent remains sensitive, even after virus treatment.

It is clear that the cybrids isolated in all of the above fusions have the nuclear characteristics of the nucleated parent. All cybrids possess these properties irrespective of the selective system used or the cell types involved.

**DISCUSSION**

In these studies we have described a system for demonstrating cytoplasmic inheritance in human cells. This has been accomplished by transferring CAP resistance from a resistant to a sensitive cell via the cytoplasm and in the absence of the nucleus.

The appearance of the cybrids cannot be explained by the mutation of the CAP-resistant parent to BrdU or TG resistance. Virus-treated CAP-resistant cells inoculated into the selective medium by themselves were consistently negative. Also, efforts to obtain cells resistant to BrdU (19), and TG (see above) require mutagenesis and several months of selection, whereas cybrid colonies arise in a few weeks. These results are confirmed by the evidence that the cybrid nucleus is derived from the CAP-sensitive parent. Cybrids were observed to inherit the sensitive parent’s (a) chromosome number, (b) gross morphology, (c) unselected genetic markers such as TK, (d) unique Y chromosome, (e) G6PD isozyme, and (f) selected markers, such as HPRT deficiency. These lines of evidence make a strong case against mutation of the CAP-resistant line to resistance to BrdU or TG. They are entirely consistent with cybrid formation.

Mammalian cells have been shown to contain a specific mitochondrial thymidine kinase which is retained even when the nuclear thymidine kinase is lost (3, 8, 20). Growing a BrdU-resistant and CAP-sensitive cell in BrdU and CAP would result in the incorporation of the BrdU into the mtDNA. It is possible that this BrdU might act as a specific mutagen rendering the cells resistant to CAP. However, the lack of colony formation when virus-treated CAP-sensitive parental cells are plated in BrdU and CAP and the successful formation of cybrids by selection in TG instead of...
BrdU indicate that cybrid formation cannot be explained by specific BrdU mutagenesis of the mtDNA. The high frequency of cybrid formation and the rapidity with which colonies appear in all experiments are inconsistent with mutation of the CAP-sensitive cells to CAP resistance. To isolate CAP-resistant cells, ethidium bromide (EtBr) mutagenesis and several months of selection were required (39).

Cybrid formation has been shown to require the fusion of a CAP-resistant cytoplast with a CAP-sensitive cell. In the fusion of an HPRT-positive and CAP-resistant enucleated cell to an HPRT-negative and CAP-sensitive nucleated cell, it was shown that CAP resistance was cotransferred in the same structure as the HPRT. This HPRT decayed over time. The only structure which could impart unstable HPRT is a cytoplast from the CAP-resistant cell. Thus, transfer of CAP resistance requires the fusion of a donor cytoplast.

The development of CAP resistance is not simply the result of a change in the ratio of the volume of the cytoplasm relative to the nucleus of the CAP-sensitive cell. Fusion of cytoplasts and cells of different volumes was not found to affect cybrid formation. In the fusion en296-1 x BU25, cells of smaller volume were enucleated and fused to larger cells. Conversely, in the fusion enHEB7A x S3AG1, cells of larger volume were enucleated and fused to smaller cells. The nuclei in both fusions are very similar and CAP resistance was readily transferred in both experiments. If cell volume were the only factor in the development of CAP resistance, there should have been major differences in the success of these two experiments. Similarly, the fusion of the very large HEB7A cytoplasts to the very small WAL-2A cells should have readily generated cybrids. On the contrary, this was the more difficult fusion to perform. These data argue against cybrid formation being the result of a simple change in the volume of the cytoplasm relative to the nucleus.

The nature of the CAP resistance of the cybrids was examined in vivo by checking their tolerance to increasing levels of CAP. It was observed that cybrids were resistant to the same high levels of CAP as the CAP-resistant parent. Purified mitochondria from one cybrid were found to synthesize protein in the presence of CAP at the same level as did the mitochondria purified from the CAP-resistant parent. The same CAP concentration strongly inhibited mitochondrial protein synthesis in the sensitive parent. Thus, cybrid formation results in a cell as resistant to CAP as its resistant parent, a resistance which occurs at the level of mitochondrial protein synthesis. The transfer of this mitochondrial resistance requires the fusion of the donor cytoplast.

The appearance of cybrids cannot be explained by nuclear-nuclear fusions followed by segregation of the chromosomes coding for TK or HPRT. A fusion using the same selection system but involving nucleated CAP-resistant cells gave only 1.5% of the number of colonies observed when enucleated cells were used. Furthermore, selection for nuclear-nuclear fusions utilizing HAT and CAP produced about one-third the colonies observed in cybrid formation. Such hybrids contained about twice the number of chromosomes as the cybrids.

The existence of forms of DNA other than mtDNA which are found in the cytoplasm make it impossible to prove by these enucleation experiments that CAP resistance is coded in the mtDNA. Examples of these types of DNA have been mentioned in a previous paper (7). Of the cell lines used in this study, DNA has been found in the cytoplasm of both HeLa and WI-L2 cells. HeLa has a cytoplasmic DNA of about 16S and a small polydisperse circular DNA, both of which originate in the nucleus (23, 37). WI-L2 contains cmDNA synthesized in the nucleus which can be picked up from the cytoplasm by vesicular stomatitis virions (17, 29). The nuclear origin of cytoplasmic DNAs has been confirmed in a number of cultured cells (23).

The fact that these DNAs require a nucleus for their synthesis decreases the probability that they could transfer CAP resistance via cytoplasts. However, it is possible that the cytoplasmic DNA could be transferred via the cytoplast, migrate back to the sensitive nucleus, and recombine with the CAP-sensitive gene. Such a complex model seems unlikely in view of the high frequency of transfers observed. Another possibility is that there are plasmid-like genes that are both extranuclear and extramitochondrial. Such genes could transfer stable CAP resistance. Finally, the gene might be coded in the mtDNA. The last two possibilities cannot be distinguished by the current methods. However, experiments designed to transfer CAP resistance using isolated mitochondria and purified mtDNA are under way in order to clarify this point.
Resistance to CAP is expressed at the level of HeLa mitochondrial protein synthesis in the line 296-1 (39). The mutant was isolated after treatment with the specific mtDNA mutagen, ethidium bromide. CAP resistance has been shown to be cytoplasmically coded in Tetrahymena (35) and mitochondrial DNA coded in yeast (9) and in Paramecium (4). It therefore seems reasonable that CAP resistance in the human line 296-1 is coded in the mtDNA.

This study represents the first genetic evidence of cytoplasmic inheritance in human cells. It describes a genetic system for demonstrating cytoplasmic inheritance in human cells and has shown that CAP resistance can be transferred sequentially from cell to cell and transferred to cells from widely different origins and differentiated states.

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