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Assignment of the Chloramphenicol Resistance Gene to Mitochondrial Deoxyribonucleic Acid and Analysis of Its Expression in Cultured Human Cells

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The mitochondrial deoxyribonucleic acids (mtDNA's) from human HeLa and HT1080 cells differed in their restriction endonuclease cleavage patterns for *Hae*II, *Hae*III, and *Hha*I. *Hae*II digestion yielded a 9-kilobase fragment in HT1080, which was replaced by 4.5-, 2.4-, and 2.1-kilobase fragments in HeLa. *Hae*III and *Hha*I yielded distinctive 1.35- and 0.68-kilobase HeLa fragments. These restriction endonuclease polymorphisms were used as mtDNA markers in HeLa-HT1080 cybrid and hybrid crosses involving the cytoplasmic chloramphenicol resistance mutation was used. mtDNA's were purified and digested with the restriction endonucleases, the fragments were separated on agarose gels, and the bands were detected by ethidium bromide staining and Southern transfer analysis. Three cybrids and four hybrids (four expressing HeLa and three expressing HT1080 chloramphenicol resistance) contained 2- to 10-fold excesses of the mtDNA of the chloramphenicol-resistant parent. One cybrid, which was permitted to segregate chloramphenicol resistance and was then rechallenged with chloramphenicol, had approximately equal proportions of the two mtDNA's. Only one hybrid was discordant. These results indicated that chloramphenicol resistance is encoded in mtDNA and that expression of chloramphenicol resistance is related to the ratio of chloramphenicol-resistant and -sensitive genomes within cells.

Although a number of genetic loci, including the ribosomal ribonucleic acid genes, the transfer ribonucleic acid genes, and the cytochrome oxidase subunit II gene have been assigned to human mitochondrial deoxyribonucleic acid (mtDNA) by molecular approaches (1, 2, 9, 15, 46), there is still no way to reliably assign genetic variants to this important genomic element. Previous studies on human tissue culture cells resistant to the mitochondrial ribosome inhibitor chloramphenicol (36) have shown that the resistance properties of these variants are encoded on cytoplasmic elements. This was accomplished by fusing cytoplasmic fragments prepared from a chloramphenicol-resistant (CAP^r) cell line to chloramphenicol-sensitive (CAP^s) cells and demonstrating the transfer of chloramphenicol resistance by the combination of selection in chloramphenicol and selection for the recipient cell nuclei (cybrid formation) (6, 40). Although it provided for the first time a method for identifying cytoplasmic genes in cultured cells, this procedure did not identify the cytoplasmic element carrying the mutation.

Recently, it has been discovered that human mtDNA's have a high degree of sequence variation among individuals. This has been detected by studying the variations in the numbers of fragments generated by various restriction endonucleases (4, 5, 27), as well as by direct comparison of sections of mtDNA sequences (9, 10, 15).

In this report, I describe experiments in which mtDNA sequence variants detected by restriction endonucleases were used as a means of distinguishing the mtDNA's of various human tissue culture cell lines. This innovation permitted me to make a correlation between the expression of human chloramphenicol resistance and the predominance in a cell of the mtDNA of the CAP^r parent. Thus, for the first time mtDNA has been demonstrated to be the cytoplasmic element coding for human chloramphenicol resistance, and the expression of chloramphenicol resistance has been shown to be a function of the intracellular ratio of CAP^r and CAP^s mtDNA's.

MATERIALS AND METHODS

Cell lines. For this study, I chose two human cell lines of widely different origins, HeLa (16) and HT1080 (32). HeLa cells are highly aneuploid, lack a Y chro-

mosome, and produce glucose-6-phosphate dehydrogenase variant A (20, 26), whereas HT1080 cells are diploid, have a Y chromosome, and produce glucose-6-phosphate dehydrogenase variant B (32).

The HeLa strains used included the parental line S3 (31), its CAP^r derivative 296-1 (36), the thymidine kinase-deficient mutant BU25 (21), and the CAP^r cybrid HEB7A, which was formed by the fusion of 296–1 cytoplasts with BU25 (40). The HT1080 strains used were HT1080 and a hypoxanthine phosphoribosyltransferase-deficient variant, HT1080-6TG (11), which was generously provided by C. Croce. These lines were recloned to yield HT1080C and HT1080-6TG1, respectively.

Cell culture and selection conditions. All cells were grown attached in Eagle minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% bovine serum (Irvine Scientific, Santa Ana, Calif.). Fusions, initial cybrid and hybrid isolations, and segregation experiments were all performed in medium containing fetal calf serum. This medium was further supplemented with 4 mM glutamine for the fusion of enHEB7A and HT1080C. Expansion of the RIB (CAP^r HT1080 × CAP^s HeLa) hybrids for mtDNA isolation was performed primarily in medium containing 10% fetal calf serum, whereas the other strains were expanded in medium containing fetal calf serum, newborn calf serum, or calf serum. Cells were removed from substrates either with 0.5 mM ethylenediaminetetraacetic acid in calcium- and magnesium-free phosphate-buffered saline or by brief treatment with 1× pancreatin (GIBCO). All cultures were maintained free of antibiotics, except for the antibiotics used in genetic selections. Stock cell lines were screened periodically for mycoplasmas by using Hoechst 33258 fluorescence (8).

For hypoxanthine-aminopterin-thymidine selection, media were supplemented with 0.1 mM hypoxanthine, 0.5 μM aminopterin, 16 μM thymidine, and 3 μM glycine (22, 38). When required chloramphenicol was added at a concentration of 50 μg/ml, and 5-bromodeoxyuridine and 6-thioguanine were added at concentrations of 0.1 mM. The combinations 6-thioguanine plus chloramphenicol and 5-bromodeoxyuridine plus chloramphenicol were added 3 days after cybrid fusion, whereas the combination hypoxanthine-aminopterin-thymidine plus chloramphenicol was added immediately after cybrid fusion and 1 day after hybrid fusion. Stock solutions of oligomycin (10 mg/ml in ethanol) and antimycin A (0.1 mg/ml in ethanol) were prepared. Other stock solutions have been described previously (41). All reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

Isolation of HT102W. A CAP^r mutant of HT1080, HT102W, was isolated by inoculating 1.4×10^6 HT1080C cells onto the surfaces of 0.5% Noble agar (Difco Laboratories, Detroit, Mich.) plates at densities ranging from 5×10^5 to 5×10^4 cells per 90-mm petri dish. The agar medium was composed of Eagle minimal essential medium supplemented with 4 mM glutamine, 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.1 mM hypoxanthine, 0.5 μM aminopterin, 16 μM thymidine, 3 μM glycine, and 50 μg of chloramphenicol per ml. Agar (20 ml) was poured into each dish, and the plates were incubated in high humidity at 37°C in a 5% CO_2 atmosphere. Methotrexate induces petites in yeast (43, 44), and it was hoped that the aminopterin might increase mammalian mtDNA mutations. After 28 days of incubation, a patch of approximately 56 colonies was observed in one dish containing 5×10^4 cells. All other plates remained negative. At 47 days, three colonies were transferred into liquid Eagle minimal essential medium supplemented with 4 mM glutamine, 10% fetal calf serum, 0.1 mM hypoxanthine, 0.5 μM aminopterin, 16 μM thymidine, 3 μM glycine, and 2.5 μg of chloramphenicol per ml. All of these lines grew, and after about 10 doublings they were rechallenged with 50 μg of chloramphenicol per ml in the same medium. Subsequently, they were removed from medium containing hypoxanthine, aminopterin, and thymidine, and one of them was cloned twice in medium containing chloramphenicol (cloning efficiencies, 11 and 13%). The final clone was designated HT102W. It is not known why numerous colonies appeared in one area of one plate and nowhere else. This could have been due to variations in the incubation conditions or agar medium or to the spreading of a clump of preexisting mutant cells.

Enucleation and cell fusion. HT102W and HEB7A were enucleated by using Ficoll gradients (42). The enucleated HT102W (enHT102W) preparation contained 77% cytoplasts, and the enucleated HEB7A (enHEB7A) preparation contained 83% cytoplasts. Cybrid fusions were prepared in suspension by using 500 hemagglutinating units of β-propiolactone-inactivated Sendai virus as described previously (6). Hybrid fusions of attached cells were performed with 50% polyethylene glycol 1500 (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y.) (12).

Cloning efficiencies, calculation of population doublings, and chloramphenicol stability tests. In cloning efficiency experiments, cells were inoculated in duplicate into four- or six-well clustered tissue culture trays over a range of cell densities. All wells of an experiment were stained with 1% methylene blue in 70% isopropanol (37) when the most rapidly growing colonies were prominent. Colonies containing 30 to 40 cells or more were counted, and the numbers were normalized to colonies per 100 cells inoculated (cloning efficiency) and averaged.

The number of cell population doublings and the doubling times were calculated from passage and cloning data by using the initial and final cell numbers and the length of growth between transfers (41). The estimates of the numbers of doublings during expansion for WEH5 and the RIB cultures must be considered minima since large proportions of the cells were lost due to chloramphenicol toxicity and poor cell adhesion.

Cytogenetics. Chromosome counts and Y chromosome analyses were performed as previously described (40). Atebrin slides were examined with a Zeiss Universal microscope by using incident illumination and 435-nm excitation and 460-nm reflector optics.

Glucose-6-phosphate dehydrogenase isozyme analysis. The human glucose-6-phosphate dehydrogenase isozymes AA, AB, and BB were separated on 60-ml 12% agarose gels (13 by 18 cm; Seakem ME; FMC Corp., Rockland, Maine). The gels were poured

onto Gel Bond film (FMC Corp.) and contained 50 μg of nicotinamide adenine dinucleotide phosphate per ml, 0.4 mM ethylenediaminetraacetic acid, 1.5 mM boric acid, and 14 mM tris(hydroxymethyl)aminomethane (pH 9.1). The gel reservoirs contained 10 times the buffer concentration of the gels, and the reservoir buffer and gel staining procedures have been described previously (35). Electrophoresis was carried out with a Shanon electrophoresis system at 4°C for 4 h at a constant voltage (150 V). All three isozymes could be resolved by this procedure.

mtDNA isolation and preparation of DNA probes. mtDNA's were purified by using cesium chloride-ethidium bromide gradients from mitochondria isolated by differential centrifugation (3). When high-purity mtDNA was required for probes or when the initial SW50.1 purification seemed inadequate, the closed circular DNA was collected and rebanded in a Sorvall TV-865 vertical rotor (17). mtDNA was stored at 4°C in 1 mM ethylenediaminetetraacetate–10 mM tris(hydroxymethyl)aminomethane (pH 8.0).

A ^{32}P-labeled mtDNA probe was prepared from 1 μg of HT1080 mtDNA by incubating this DNA in a 30-μl reaction mixture containing 10 U of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 8 μM deoxycytidine triphosphate, 8 μM deoxyguanosine triphosphate, 8 μM thymidine triphosphate, and 80 μCi of [α-^{32}P]deoxyadenosine 5'-triphosphate (PB 164; >350 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (23). This incorporation reaction was continued for 2 to 3 h at 15°C until maximum incorporation was achieved. Then, 10 μg of *E. coli* transfer ribonucleic acid was added, the reaction mixture was extracted with phenol, and the high-molecular-weight DNA was purified on a Sephadex G-50 column by elution with 50 mM NaCl–1 mM ethylenediaminetetraacetate–20 mM tris(hydroxymethyl)aminomethane (pH 8.0).

Restriction endonuclease digestions, Southern transfers, and hybridization. Restriction endonuclease digestions and agarose gel electrophoresis have been described previously (17). All gels were 1.0% agarose except for the *Hae*II gel (Fig. 3), which was 0.7%. *Hae*II was purchased from Bethesda Research Laboratories, Rockville, Md., and all other enzymes were obtained from New England Biolabs, Beverly, Mass. Each gel was photographed for ethidium bromide fluorescence (0.01 μg/ml) by removing it from its mold and placing it on a model C-61 Chromato-Vue transilluminator (Ultra-violet Products, San Gabriel, Calif.). The fluorescent DNA was photographed by using a Polaroid MP-3 camera fitted with a Wratten no. 8 gelatin filter (Eastman Kodak Co., Rochester, N.Y.) and T-55 positive-negative film (Polaroid Corp., Cambridge, Mass.), which was exposed at maximum aperture for 15 min. A molecular weight standard was included in all gels; this consisted of approximately 0.5 μg of lambda DNA digested with *Hin*dIII (25) mixed with about 1 μg of ΦX174 DNA digested with *Hin*cII (33).

mtDNA fragments were transferred to diazobenzyloxymethyl paper (34, 39), and the gel prints were prehybridized overnight in a plastic bag at 42°C in 5 ml of a solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), 50 μM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1× Denhardt solution, 0.2 mg of sonicated salmon sperm DNA per ml, and 0.01% sodium dodecyl sulfate (pH 7.1). After prehybridization, the solution in each bag was exchanged for 3 ml of hybridization solution (50% formamide, 6× SSC, 25 mM HEPES, 1× Denhardt solution, 1.3 mg of sonicated salmon sperm DNA per ml, 0.1% sodium dodecyl sulfate, pH 7.1) containing 1.5×10^6 cpm of denatured ^{32}P-labeled mtDNA probe, and the bags were resealed and incubated at 42°C for 3 days. The papers were then washed (39), dried, and autoradiographed at −90°C with Dupont Cronex 4 film and Dupont Cronex Lightning Plus intensification screens (E. I. Du Pont de Nemours & Co., Inc., Wilmington, Del.).

Determination of the cellular proportions of HeLa and HT1080 mtDNA's. To quantitate the mtDNA in each band on the photographic negatives and autoradiographic films, each channel was scanned at 550 nm for the optical density of the grain with a Cary model 219 spectrophotometer fitted with a gel scanner and a film holder (Varian Associates, Palo Alto, Calif.). For gels of parental mtDNA's (see Fig. 1) and for gels of cybrid and hybrid mtDNA's digested with *Hae*II and *Hae*III (see Fig. 3 and 4), the T-55 negatives of ethidium bromide fluorescence were scanned, whereas for gels of cybrid and hybrid mtDNA's digested with *Hha*I (see Fig. 5) the autoradiographic film was analyzed. Base lines for T-55 film peaks were chosen as the average base lines around the peaks of interest. Base lines for the *Hha*I autoradiographs were chosen as the minima between the peaks at 0.53 and 0.39 kilobases (kb). The relative number of molecules (N_i [i is fragment size in kilobases]) was calculated by dividing the area under the peak (A_i) by the size of the fragment (M_i) in kilobases: $N_i = kA_i/M_i$.

The proportions of parental mtDNA's in cybrids and hybrids were calculated by dividing the N_i value of each strain-specific polymorphic band by the N_i value of the common nonpolymorphic bands. The polymorphic bands for *Hae*II were at 9 kb for HT1080 and at 2.1 and 2.4 kb for HeLa; those for *Hae*III and *Hha*I were at 1.35 and 0.68 kb, respectively, for HeLa. The nonpolymorphic bands used for comparison were at 1.3 and 1.4 kb for *Hae*II, 1.70 kb for *Hae*III, and 0.82 kb for *Hha*I. For *Hae*II digests, $N_{1.35} \equiv (N_{1.3} + N_{1.4})/2$ and $N_{2.25} \equiv (N_{2.1} + N_{2.4})/2$. The proportion of HT1080 mtDNA (P_{HT}) was $N_9/N_{1.35}$, and the proportion of HeLa mtDNA (P_{He}) was $N_{2.25}/N_{1.35}$. For *Hae*III P_{He} was $N_{1.35}/N_{1.70}$, and for *Hha*I P_{He} was $N_{0.68}/N_{0.82}$. $P_{HT} = 1 - P_{He}$.

There are two possible sources of error in such analyses, nonlinear film response (28, 30) and differential degradation of restriction fragments (29). In preliminary trials, I selected mtDNA quantities for gels which generally gave film responses proportional to molecular weights (see figure legends for quantities used). Three control channels were then included in each experimental gel (two for HeLa and one for HT1080). Since the *Hae*II digests had the widest range of measured fragment sizes, these data were analyzed most thoroughly (see Fig. 3). An examination of the HT1080 and HeLa channels showed that there were differential reductions in the peaks for the higher-

molecular-weight fragments. Hybridization of a mtDNA probe to the Southern transfer of the *Hae*II gel revealed that the control channels contained significant quantities of degraded mtDNA (see Fig. 3). To determine the relationships among the peak losses for the various restriction fragments, the relative numbers of molecules ($N_i/N_{1.35}$) for all HeLa and HT1080 restriction fragments from 1.35 to 9 kb were determined and plotted against their molecular weights. A linear plot of $y = -0.0526x + 1.051$ was obtained for nine points, with a sample variance about y ($s^2_{y/x}$) of 0.011 and a correlation coefficient of −0.967. The y-intercept was more than 1 because $N_{1.35}/N_{1.35}$ was defined as 1. This straight line showed that peak loss was directly proportional to molecular weight over this sevenfold range of fragment sizes. Consequently, the 9-kb fragment was four times more likely to be lost than the 2.25-kb fragment. This relationship was true regardless of the slope of the line. Since differential film response would not be expected to be linear, the best explanation for the observed peak loss was the random breakage of the mtDNA's before digestion.

To determine the extent of peak loss for the fragments in each of the *Hae*II channels (see Fig. 3), the number of molecules lost (N_L) was calculated as follows: $N_L = N_{1.35} - (N_9 + N_{2.25})$. According to this measure, the control mtDNA's underwent significant degradation, whereas the freshly isolated closed circular cybrid and hybrid mtDNA's underwent little or no degradation. This conclusion was confirmed by the absence of background hybridization in the cybrid and hybrid channels of the autoradiograph in Fig. 3. To correct for the peak reductions which were observed, I generated a theoretical curve which related various mixtures of HeLa and HT1080 mtDNA's to the proportions of N_L which would be contributed by loss of N_9 and $N_{2.25}$, assuming a fourfold differential in reduction rate. The proportion of HeLa mtDNA (P_{HeO}) and the proportion of HT1080 mtDNA (P_{HTO}) in each cybrid and hybrid were then estimated as follows: $P_{HeO} = N_{2.25}/(N_9 + N_{2.25})$, and $P_{HTO} = N_9/N_9 + N_{2.25})$. The resulting values were used to locate on the curve the proportions of N_L contributed by N_9 and $N_{2.25}$, respectively. These values were then multiplied by the N_L calculated for each sample, and the appropriate values for N_9 and $N_{2.25}$ were added back. The resulting values were then divided by $N_{1.35}$ to give the final P_{He} and P_{HT} values shown below (see Table 4). When this procedure was used, corrected values for P_{He} and P_{HT} for the cybrid and hybrid samples did not differ by more than 4% from observed P_{HeO} and P_{HTO} values. Thus, the *Hae*II determinations of the HeLa and HT1080 mtDNA's in the cybrid and hybrid samples (see Table 4) were based on two independent and reciprocal measurements. In turn, these were compared with a third cumulative measure. The small discrepancies which were found were then corrected by using an independent experimentally derived relationship.

In the *Hae*III and *Hha*I experiments differential degradation was not a significant source of error. This was because the molecular weights of the polymorphic and nonpolymorphic bands were very similar and the undegraded *Hae*II cybrid and hybrid samples were tested. However, only a HeLa polymorphic fragment could be quantitated with these enzymes. This meant that small amount of HT1080 mtDNA had to be measured by variations within a large HeLa peak. Because of this deficiency, the determinations obtained with these enzymes were less accurate than those obtained with *Hae*II.

RESULTS

Sequence polymorphisms of HeLa and HT1080 mtDNA's. The objectives of this study were to assign formally the chloramphenicol resistance mutation to human mtDNA and to examine the molecular basis of the expression of this mutation in this multiple-copy genetic system (3). To accomplish this, it was necessary to be able to distinguish the mtDNA's of CAP^r and CAP^s cells. HeLa and HT1080 cells were chosen because of their different ethnic origins and ease of culture. The mtDNA's of these cell lines were screened for sequence differences by digestion with nine different restriction endonucleases. Of the enzymes tested, *Hae*II, *Hae*III, and *Hha*I gave readily distinguishable patterns (Fig. 1). *Hae*II digestion resulted in the most striking

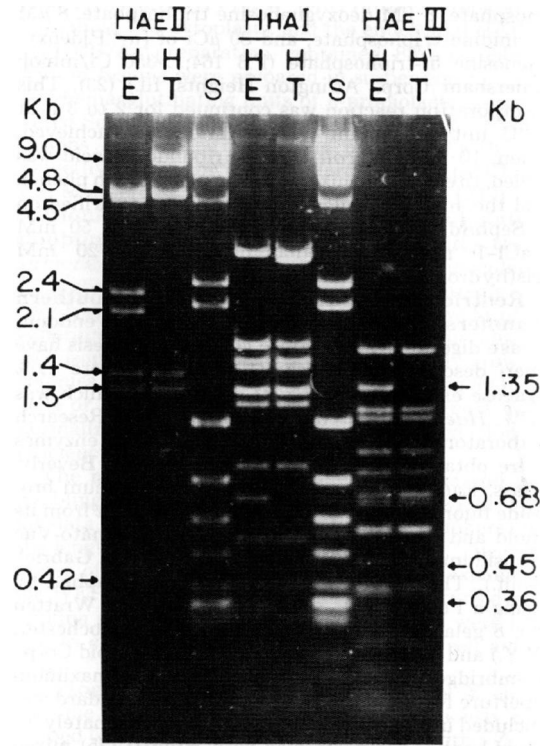

FIG. 1. *Restriction endonuclease cleavage polymorphisms of HeLa and HT1080 mtDNA's. HE, HeLa BU25 mtDNA; HT, HT1080C mtDNA. HaeII channels contained 0.4 μg of mtDNA, and HaeIII and HhaI channels contained 1.0 μg. S, Combined lambda and ΦX174 size standards (see text) (the left channel contained one-half the DNA of the right channel).*

difference. HT1080 had a five-fragment pattern, with 55% of the genome contained in one large 9-kb fragment. In contrast, HeLa had a seven-fragment pattern, with the 9-kb fragment cut into 4.5-, 2.4-, and 2.1-kb fragments. To determine whether any of these bands were double, the number of molecules in each peak was determined densitometrically. All bands were in roughly equal molar ratios, except the region of the HeLa channel from 4.5 to 4.8 kb, which was double. The 4.5- and 4.8-kb HeLa fragments could be resolved in many instances (see Fig. 3). Since the 9-kb fragment and the 2.4- and 2.1-kb fragments were unique to HT1080 and HeLa, respectively, and they did not overlap with any other fragments, it was clear that *Hae*II digestion could distinguish the two mtDNA's unambiguously when they were mixed in hybrid or cybrid cells.

The HeLa and HT1080 mtDNA's also showed clear differences in their *Hha*I restriction sites. HeLa had 0.68- and 0.36-kb *Hha*I fragments which HT1080 lacked (Fig. 1). A densitometric analysis of these digests indicated that the HeLa 1.8- and 1.3-kb bands contained about twice the number of molecules found in the remaining bands down to 0.34 kb (see Fig. 5 for *Hha*I fragment sizes), a result consistent with previous findings (5). In contrast, HT1080C mtDNA had double bands at 2.9 and 1.3 kb but nowhere else. The sum of the 1.8-kb fragment from the HeLa doublet plus the unique 0.68- and 0.36-kb fragments is close to 2.9 kb, the size of the extra HT1080 fragment. Mapping data for *Hha*I revealed that the HeLa 1.8- and 0.68-kb fragments were the result of the acquisition of a new *Hha*I site in a larger fragment (designated B) found in most human mtDNA's. Furthermore, fragment B is adjacent to a fragment K, which corresponds to the 0.36-kb fragment (5). Thus, the most likely explanation for the HT1080 and HeLa mtDNA differences observed is that HT1080 mtDNA lacks two adjacent sites found in HeLa mtDNA, with the result that HT1080 mtDNA has a 2.84-kb fragment composed of fragments B and K.

The fragment patterns for HeLa and HT1080 mtDNA's after digestion with *Hae*III were also distinctive. HeLa mtDNA had a unique 1.35-kb fragment which HT1080 mtDNA lacked, whereas HT1080 mtDNA had a 0.45-kb fragment which HeLa mtDNA lacked (Fig. 1). A densitometric analysis revealed that all of the bands down to the 0.81-kb band were due to single mtDNA fragments (see Fig. 4 for fragment sizes). Although there were several differences in the numbers of fragments in the remaining bands, it was impossible to relate these differences to changes in specific restriction endonuclease sites.

Although there are a number of differences in the *Hha*I and *Hae*III sites for the HeLa and HT1080 mtDNA's, only the HeLa 0.68-kb *Hha*I fragment and the HeLa 1.35-kb *Hae*III fragment could be unambiguously observed and quantitated by the methods used in this study. However, these bands provided adequate information to detect both parental mtDNA's within fused cells when they were compared with adjacent nonpolymorphic bands.

Characterization of CAP^r HT1080 cells. To capitalize on the mtDNA restriction endonuclease site polymorphisms described above, CAP^r HeLa 296-1 cells and CAP^r HT1080C HT102W cells were studied. The cloning efficiency of HT102W was not affected by 50 μg of chloramphenicol per ml, was reduced slightly by 100 μg of chloramphenicol per ml, and was reduced to zero by 150 μg of chloramphenicol per ml (Fig. 2). In contrast, the cloning efficiency of 296-1 was not affected by chloramphenicol concentrations in excess of 150 μg/ml. Both parental lines were inhibited by 25 μg of chloramphenicol per ml (Fig. 2). The growth of all four lines was inhibited by 50 μg of antimycin A per ml and by 0.01 μg of oligomycin per ml, suggesting that mitochondrial respiration was still functional (45).

The chloramphenicol resistance mutation of HT102W was shown to be cytoplasmic by fusing the enucleated cytoplasmic fragment of this line to HT1080-6TG1 and selecting in a medium containing 6-thioguanine and chloramphenicol. Cybrids appeared at a high frequency (Table 1). One of these, WER1A, had 46 chromosomes,

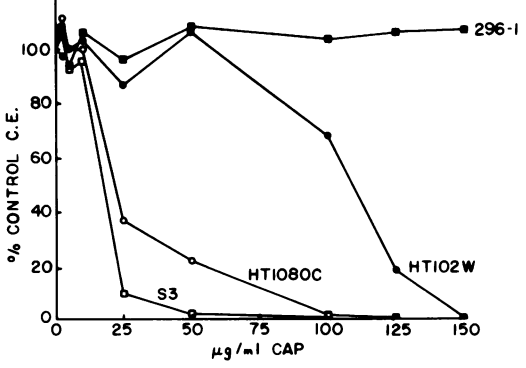

FIG. 2. *Cloning efficiencies (C.E.) of CAP^r mutants and their CAP^s parents in varying concentrations of chloramphenicol. The data are presented as percentages of the cloning efficiency in the absence of chloramphenicol. The control cloning efficiencies and the number of days grown in the drug were as follows: S3, 0.35 and 13 days; 296-1, 0.73 and 14 days; HT1080C, 0.30 and 17 days; and HT102W, 0.41 and 14 days.*

TABLE 1. *Genetics crosses*

Type of cross	Fusion[a]	Selection[b]	Frequency[c]	Designation
Cybrid	enHT102W × HT1080-6TG1	TG + CAP	5.65×10^{-4}	WER
Cybrid	enHEB7A × HT1080C	HAT + CAP	5.12×10^{-4}	HEH
Cybrid	enHT102W × BU25	BrdU + CAP	1.3×10^{-3}	WEH
Hybrid	HEB7A × HT1080-6TG1	HAT + CAP	6.5×10^{-5}	TIR
Hybrid	WER1A × BU25	HAT + CAP	4.2×10^{-5}	RIB

[a] The prefix -en indicates enucleated cells. All fusions were accompanied by inoculating each parental cell preparation separately into the same selective medium; no colonies were observed.

[b] The selective conditions used are described in the text. TG, 6-Thioguanine; CAP, chloramphenicol; HAT, hypoxanthine-aminopterin-thymidine; BrdU, 5-bromodeoxyuridine.

[c] Frequency was calculated as the mean number of colonies relative to the total number of cells and cell fragments inoculated in 8 to 25 flasks. All colony frequencies were proportional to the number of cells inoculated, except for enHT102W × HT1080-6TG1, which showed a marked colony inhibition when there were more than 10^5 cells per 25-cm^2 flask. Colonies were counted between 20 and 30 days after fusion. Observed colonies generally gave rise to continuous cell lines, except in the case of enHT102W × BU25, where colonies stopped growing after 20 days; only 1% of these colonies survived and yielded established lines by 43 days.

TABLE 2. *Nuclear characteristics of parental cells, cybrids, and hybrids*

Cell line	Origin[a]	Drug resistance marker(s)[b]	Chromosome no.[c]	Y chromosome[d]	Glucose-6-phosphate dehydrogenase isozyme[e]
HT1080C	HT1080 parent		48.6 (8.8)	+ (9/9)	B
HT1080-6TG1	HT1080 parent	TG^r	46.4 (2.2)	+ (11/12)	B
HT102W	HT1080 parent	CAP^r	44.4 (3.2)	+ (5/5)	B
WER1A	HT1080-HT1080 cybrid	CAP^r TG^r	46.4 (2.3)	+ (11/11)	B
S3	HeLa parent		64.8 (12.9)	− (0/10)	A
BU25	HeLa parent	$BrdU^r$	58.8 (10.4)	− (0/10)	A
296-1	HeLa parent	CAP^r	66.1 (11.2)	− (0/10)	A
HEB7A	HeLa-HeLa cybrid	CAP^r $BrdU^r$	57.1 (6.2)	− (0/10)	A
HEH2A	HeLa-HT1080 cybrid	CAP^r HAT^r	45.2 (10.4)	+ (10/10)	B
HEH7	HeLa-HT1080 cybrid	CAP^r HAT^r	46.2 (9.7)	+ (7/12)	B
WEH1A	HT1080-HeLa cybrid	CAP^r $BrdU^r$	60.6 (8.6)	− (0/10)	A
WEH5	HT1080-HeLa cybrid	CAP^r $BrdU^r$	56.5 (16.5)	− (0/10)	A
TIR21	HeLa-HT1080 hybrid	CAP^r HAT^r	86.9 (3.8)	+ (9/12)	A, B
TIR22	HeLa-HT1080 hybrid	CAP^r HAT^r	100.0 (7.2)	+ (9/10)	A, B
RIB21	HT1080-HeLa hybrid	CAP^r HAT^r	97.2 (22.5)	+ (8/13)	A, B
RIB22	HT1080-HeLa hybrid	CAP^r HAT^r	94.1 (14.5)	+ (5/10)	A, B
RIB24	HT1080-HeLa hybrid	CAP^r HAT^r	93.3 (17.9)	+ (7/10)	A, B

[a] HeLa-HT1080 and HT1080-HeLa indicate the strain origins of the cells fused, with the CAP^r parent listed first.

[b] The drug concentrations tested are given in the text. TG, 6-Thioguanine; CAP, chloramphenicol; BrdU, 5-bromodeoxyuridine; HAT, hypoxanthine-aminopterin-thymidine.

[c] The numbers of chromosome spreads counted were as follows: HT1080C, 25; HT1080-6TG1, 45; HT102W, 30; WER1A, 45; S3, 53; BU25, 29; 296-1, 50; HEB7A, 53; HEH2A, 25; HEH7, 25; WER1A, 50; WEH5, 30; TIR21, 33; TIR22, 29; RIB21, 50; RIB22, 50; RIB24, 50. The numbers in parentheses are standard deviations. Values less than 0.5 or more than 1.5 times the overall mean were deleted from final calculations as aberrant. These values were as follows: WER1A, 89, 91, 92, 93, and 97; S3, 112 and 117; HEB7A, 188, 114, 32, 178, 108, 127, and 112; and BU25, 118.

[d] The numbers in parentheses are number of karyotypes containing a Y chromosome/total number of karyotypes analyzed.

[e] All hybrids expressed the AA and AB forms; none expressed the BB form. This was probably due to the greater glucose-6-phosphate dehydrogenase activity in BU25 relative to HT1080-6TG1, with the result that all of the B form was complexed with the A form.

which was consistent with a HT1080-6TG1 nuclear origin (Table 2).

An analysis of the stability of chloramphenicol resistance in the HT102W mutant and in WER cybrids gave a surprising result. In contrast to previously described HeLa and mouse L-cell CAP^r mutants and their intrastrain cybrids (7, 41), chloramphenicol resistance in these cells

TABLE 3. *Stability of chloramphenicol resistance in HT102W and cybrids*[a]

Origin	Cell line[b]	No. of doublings[c]		Cloning efficiency[d]		+CAP/−CAP ratio[e]	Doubling time in flasks (h)[f]			
		+CAP	−CAP	+CAP	−CAP		+CAP			−CAP (mean)
							Initial	Mean	Final	
CAP^r mutant	HT102W	>100	0	22.0	27.9	0.79		37 (38.9)		29 (51.9)
			59	0.98	12.9	0.08				
enHT102W × HT1080-6TG1	WER1A	21	0	2.7	2.0	1.35				
			22	14.5	49.4	0.29	62 (10.9)	59 (19.8)	56 (8.9)	46 (37.3)
			37	14.5	12.8	1.13	48 (9.4)	52 (25.0)	42 (12.1)	
	WER2A	21	0	6.9	8.7	0.79				
			23	3.4	37.8	0.09	75 (14.1)	58 (51.5)	50 (17.8)	28 (40.4)
	WER4	16	0	2.6	3.6	0.72				
			22	11.3	27.9	0.41	47 (18.3)	48 (52.9)	51 (17.4)	29 (101)
enHEB7A × HT1080	HEH2A	26	0	2.0	2.8	0.71				
			23	23.3	41.3	0.56	36 (13.5)	34 (39.0)	31 (16.5)	28 (46.6)
	HEH7	12	0	5.6	9.6	0.58[g]				
			24	6.3	23.5	0.27[g]	29 (14.1)	30 (28.6)	31 (16.5)	29 (26.2)
enHT102W × BU25	WEH1A	22	0	17.4	23.7	0.73				
			24	10.3	22.2	0.46	45 (14.5)	40 (30.3)	37 (10.9)	27 (46.3)
	WEH5	18	0	30.0	24.2	1.24				
			23	23.3	39.7	0.59	52 (13.3)	47 (26.3)	46 (8.4)	27 (46.7)
			78	37.4	3.7	0.10	77	64	52	34 (33.6)

[a] Each culture was selected in medium containing chloramphenicol (+CAP) and then removed from selection (−CAP) for the indicated number of doublings. Its residual resistance was then assayed by challenging it with chloramphenicol both by cloning with and without chloramphenicol and by serial passage in flasks with and without chloramphenicol.

[b] Cultures whose designations end in a letter were derived from individual clones. WEH4 was derived from a flask with 69 colonies.

[c] Numbers of population doublings with and without chloramphenicol. HT102W was maintained in selection for 275 days, the last 222 of which the medium contained 50 μg of chloramphenicol per ml. The HT102W estimate with chloramphenicol is a minimum value.

[d] Cloning efficiencies in percentages. Colonies were counted between 10 and 19 days after inoculation.

[e] Ratio of cloning efficiencies.

[f] Weighted mean doubling times (41) during passage. The initial doubling times are the values for the first three to four passages, and the final doubling times are the values for the last one to three passages in chloramphenicol-containing medium. The numbers in parentheses indicate the number of doublings over which the growth rate was averaged. For WEH5 cells grown without chloramphenicol for 78 doublings, the values for growth with chloramphenicol are the means of two independent determinations. The values averaged for doubling times and number of doublings were as follows: initial values, 101.7 h (7.8 doublings) versus 61.2 h (11.4 doublings); mean values, 86.9 h (12.2 doublings) versus 54.7 h (31.7 doublings); and final values, 61.1 h (4.3 doublings) versus 48.6 h (10.3 doublings). WER2A doubling times increased to between 82 and 86 h within four doublings after challenge. After 78 doublings without chloramphenicol, the WEH5 doubling time increased to between 82 and 114 h within one to four doublings after challenge.

[g] Cloning was done in medium containing 4 mM glutamine rather than the standard 2 mM glutamine.

was unstable. Growth of HT102W in the absence of chloramphenicol for 59 doublings resulted in a 10-fold reduction in cloning efficiency in chloramphenicol (Table 3). Likewise, for cybrid WER2A cloning in the absence of chloramphenicol reduced its ability to reclone in chloramphenicol 10-fold and substantially decreased its ability to grow in the presence of this drug (Table 3). WER1A also showed an initial loss of resistance but seemed to regain resistance on subsequent passage, whereas WER4 seemed to be reasonably stable (Table 3). These results indicated that not all of the mitochondria in HT102W and its cybrids were CAP^r and that the expression of chloramphenicol resistance and thus presumably the proportions of resistant and sensitive mitochondria were able to vary in the absence of selective pressure.

HeLa-HT1080 cybrid and hybrid crosses. The appropriate HeLa and HT1080 cell lines were then used for a series of interstrain cybrid and hybrid fusions. In the cybrid crosses, the HeLa CAP^r gene was transferred to HT1080 cells by fusion with enucleated HEB7A cytoplasms and selection in medium containing hypoxanthine, aminopterin, thymidine, and chloramphenicol. Cybrids occurred at a high frequency and readily grew into continuous cybrid lines, which were designated HEH (Table 1). Likewise, HT1080 chloramphenicol resistance was transferred to BU25 cells by fusion with enucleated HT102W cytoplasms and selection in a medium containing 5-bromodeoxyuridine and chloramphenicol (Table 1). This fusion also yielded a high proportion of cybrids (designated WEH), but most of these seem to be unstable for chloramphenicol resistance. Only 1% of the initial colonies grew into continuous lines.

All parental lines and cybrids were then examined for chromosome number, glucose-6-phosphate dehydrogenase isozymes, and the presence of a Y chromosome (Table 2). As expected, all HT1080-derived lines contained approximately 46 chromosomes, produced glucose-

6-phosphate dehydrogenase variant B, whereas all HeLa-derived lines were aneuploid, produced glucose-6-phosphate dehydrogenase variant A, and lacked a Y chromosome.

Two HEH cybrid lines, HEH2A and HEH7, were chosen for additional study. HEH2A was derived from a single clone, whereas HEH7 was derived from a mixture of 118 clones, all of which arose in one flask. Both of these lines had 46 chromosomes, produced glucose-6-phosphate dehydrogenase B, and had a Y chromosome, as expected for cybrids having an HT1080C nucleus (Table 2). Likewise, the WEH cybrids, WEH1A and WEH5, were also examined. WEH1A was derived from a single clone, whereas WEH5 arose from a mixture of five clones which survived in a flask originally containing 283 colonies. Both of these lines contained approximately 58 chromosomes, produced glucose-6-phosphate dehydrogenase A, and lacked a Y chromosome, as expected if their nuclei were derived from BU25 (Table 2).

Next, I examined the stabilities of the chloramphenicol resistance of these four interstrain cybrids (Table 3). Surprisingly, all of the cybrid cultures remained substantially resistant after cloning without chloramphenicol; HEH2A and WEH1A showed 21 and 37% reductions in the proportion of CAP^r cells, respectively, whereas HEH7 and WEH5 showed reductions of about 52%. The HEH cultures also showed little or no reduction in growth rate when they were challenged in flask cultures, whereas the WEH cybrids did show slight transient reductions (Table 3). To determine whether these reductions were indicative of segregation, the WEH5 clone grown without chloramphenicol was passaged for an additional 55 doublings without chloramphenicol and challenged again. This time the cloning efficiency with chloramphenicol was reduced 92%, and the growth of the flask culture was inhibited severely. Thus, like WER2A, WEH5 was unstable for chloramphenicol resistance.

Two comparable interstrain hybrid fusions were also prepared (Table 1). Hybrids bearing HeLa chloramphenicol resistance (TIR) were isolated by fusion of HEB7A to HT1080-6TG1 cells and selection in medium containing hypoxanthine, aminopterin, thymidine, and chloramphenicol. Hybrids expressing HT1080 resistance (RIB) were prepared by fusion of WER1A to BU25 cells and selection in medium containing hypoxanthine, aminopterin, thymidine, and chloramphenicol. Five clones were chosen for further study. A cytogenetic analysis (Table 2) revealed that all of these clones contained 87 to 100 chromosomes (slightly less than the 104 expected from the sum of HT1080-6TG1 and BU25) and that the majority of cells in all of the hybrids still carried a Y chromosome. The hybrid nature of these clones was confirmed unequivocally by using glucose-6-phosphate dehydrogenase isozymes. All five hybrids expressed homomeric and heteromeric forms.

Origin of mtDNA's in parents, cybrids, and hybrids. The mtDNA's of all of the parental, cybrid, and hybrid lines shown in Table 2 were then purified, and their *Hae*II, *Hae*III, and *Hha*I cleavage patterns were examined. The *Hae*II fragment patterns of the hybrid and cybrid mtDNA's are shown in Fig. 3, those of *Hae*III are shown in Fig. 4, and those of *Hha*I are shown in Fig. 5. In all three of these figures an ethidium-bromide-stained gel photograph is shown. The *Hae*II and *Hha*I digest gels were also transferred to diazobenzyloxymethyl paper and hybridized with mtDNA probes. The resulting autoradiographs are shown to the right of the ethidium bromide-stained gels.

A survey of the mtDNA's of the parental lines confirmed that all of the lines derived from HT1080 (HT1080-6TG1, HT102W, and WER1A) were identical in their cleavage patterns to HT1080C, whereas all lines derived from HeLa (BU25, 296-1, and HEB7A) were indistinguishable from S3 (Fig. 1).

The mtDNA's from the HEH cybrids (HEH2A and HEH7) and the WEH1A cybrid were examined next. Each line was grown in medium containing 50 μg of chloramphenicol per ml from the time of fusion until the mtDNA was purified. *Hae*II digestion of both HEH cybrids yielded prominent bands for the HeLa 2.4- and 2.1-kb fragments (Fig. 3). This was confirmed by the presence of strong HeLa bands for the 1.35-kb *Hae*III fragment (Fig. 4) and the 0.68-kb *Hha*I fragment (Fig. 5). However, *Hae*II digestion also produced some 9-kb material, indicating that some HT1080 mtDNA molecules were still present in these cybrids at the time of mtDNA isolation (Fig. 3).

An examination of WEH1A mtDNA gave the complementary result. *Hae*II digestion (Fig. 3) revealed a prominent 9-kb band but very weak 2.4- and 2.1-kb bands. Likewise, both the *Hae*III 1.35-kb HeLa fragment and the *Hha*I 0.68-kb HeLa fragment were barely detectable. Thus, as would be expected if chloramphenicol resistance was encoded in mtDNA, the HEH2A and HEH7 cybrid lines contained a preponderance of mtDNA from the CAP^r HeLa parent, whereas the WEH1A cybrid contained predominantly mtDNA from the CAP^r HT1080 parent.

To obtain a numerical ratio for the HeLa and HT1080 mtDNA's in these cell lines, the grain densities in the appropriate mtDNA channels were scanned on the photographic negatives or the autoradiographs of the gels shown in Fig. 3

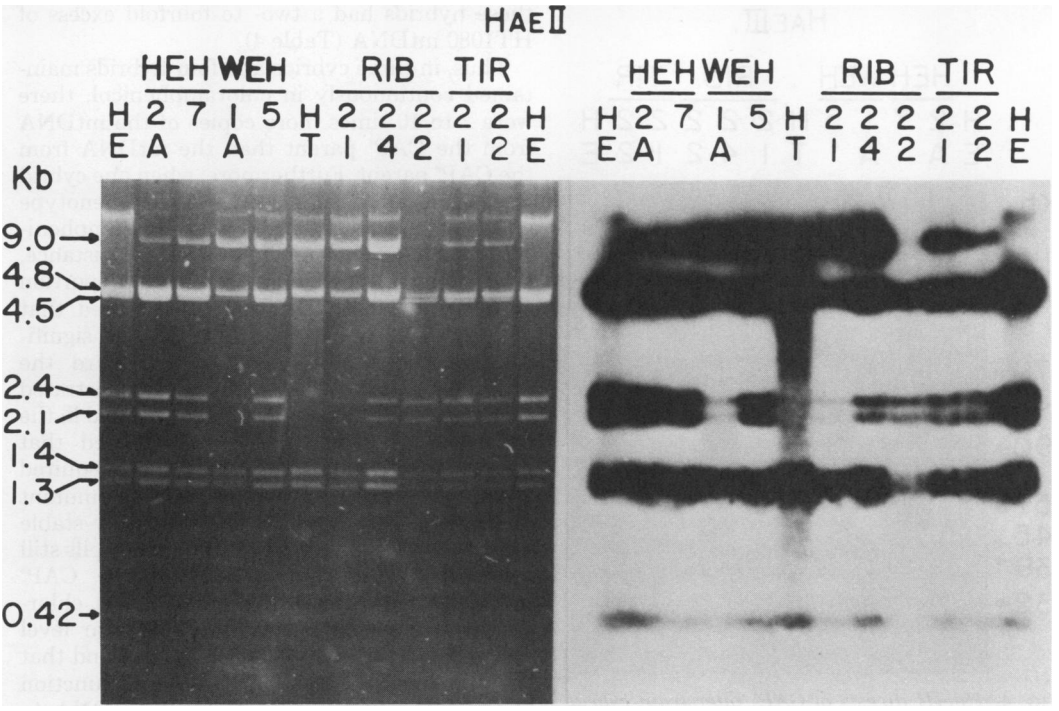

FIG. 3. *HaeII digests of CAP^r interstrain cybrid and hybrid mtDNA's. HE, HeLa S3 mtDNA; HT, HT1080C mtDNA. All channels contained approximately 0.4 μg of mtDNA. The lengths of cell propagation in chloramphenicol from fusion to mtDNA isolation were as follows: HEH2A, 98 days or about 39 doublings; HEH7, 49 days or about 28 doublings; WEH1A, 121 days or about 29 doublings; TIR21, more than 114 days or 34 doublings; TIR22, 125 days or about 50 doublings; RIB21, 177 days or more than 31 doublings; RIB24, 198 days or more than 38 doublings; and RIB22, 173 days or more than 27 doublings. WEH5 was selected for 18 doublings in chloramphenicol, grown for 78 doublings without chloramphenicol, and challenged for 59 days (about 10 doublings) with chloramphenicol.*

through 5. The proportion of each parental mtDNA was then determined by dividing the number of molecules in the polymorphic peaks by the total number of molecules in the sample (see above).

Quantitation of the ratio of HeLa mtDNA to HT1080 mtDNA in the HEH2A and HEH7 cybrid lines confirmed the impression obtained from the photographs and autoradiographs (Table 4). Both cell lines had a 6- to 11-fold excess of mtDNA from the CAP^r HeLa parent (85 and 92%) relative to the CAP^s HT1080 parent (15 and 8%). Likewise, WEH1A had a fivefold excess of CAP^r HT1080 mtDNA (83%) over CAP^s HeLa mtDNA (17%). Thus, in all cybrids selected continuously in chloramphenicol, the mtDNA of the CAP^r parent was present in considerable excess relative to the mtDNA of the CAP^s parent.

To investigate the association between chloramphenicol resistance and the CAP^r mtDNA further, I used the instability of chloramphenicol resistance in the WEH5 cybrid. The WEH5 subclone grown without chloramphenicol, which was permitted to segregate 92% of its original chloramphenicol resistance (Table 3), was rechallenged with chloramphenicol for approximately 10 doublings before mtDNA isolation. This procedure permitted the isolation of mtDNA from cells soon after they acquired the ability to grow in chloramphenicol. An *Hae*II analysis of this mtDNA revealed a prominent 9-kb band, which was consistent with the presence of a substantial proportion of the mtDNA from the CAP^r HT1080 parent (Fig. 3). However, in contrast to the HEH and WEH cybrids maintained continuously in chloramphenicol, this line also had high proportions of the 2.4- and 2.1-kb fragments from the CAP^s HeLa parent. The presence of HeLa mtDNA was further confirmed by a prominent 1.35-kb band present in *Hae*III digests (Fig. 4) and a prominent 0.68-kb band present in *Hha*I digests (Fig. 5). Quantitation of the mtDNA confirmed these observations, indicating that 40% of the mtDNA of this cybrid was from the CAP^r HT1080 parent and 60% was

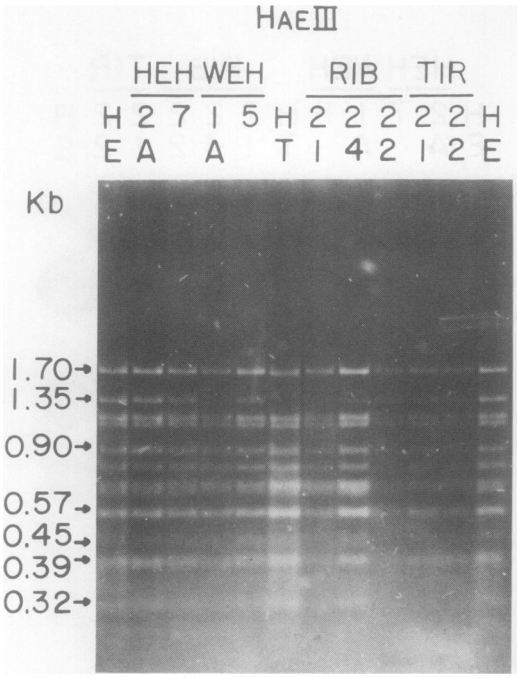

FIG. 4. *HaeIII digests of CAP^r interstrain cybrid and hybrid mtDNA's. HE, HeLa S3 mtDNA; HT, HT1080C mtDNA. All channels contained approximately 0.5 μg of mtDNA. The origins of the mtDNA's were as described in the legend to Fig. 3.*

from the CAP^s HeLa parent (Table 4). Thus, while undergoing a transition from CAP^s to CAP^r, this WEH5 cybrid had a mixture of CAP^r and CAP^s mtDNA's. This result would be expected if chloramphenicol resistance were encoded in the mtDNA and if the expression of the CAP^r phenotype were related to the intracellular proportions of CAP^r and CAP^s mtDNA's.

To confirm these cybrid results and to control for possible effects due to the varying nuclear origins, the mtDNA's of a series of CAP^r hybrid lines were also examined. The TIR hybrid mtDNA's were inherited primarily from the CAP^r HeLa parent. This was evident from the strong HeLa 2.4- and 2.1-kb *Hae*II bands (Fig. 3), the prominent 1.35-kb *Hae*III band (Fig. 4), and the 0.68-kb *Hha*I band (Fig. 5). Both hybrids had a five- to sixfold excess of HeLa mtDNA (Table 4).

Likewise, the mtDNA's of the RIB21 and RIB24 hybrids were inherited primarily from the CAP^r HT1080 parent. Both cell lines had prominent 9-kb HT1080 bands and relatively weak 2.4- and 2.1-kb HeLa bands after *Hae*II digestion (Fig. 3). Likewise, the HeLa 1.35-kb *Hae*III band and the 0.68-kb *Hha*I band were considerably reduced in intensity (Fig. 4 and 5). Quantitation of the mtDNA's revealed that these hybrids had a two- to fourfold excess of HT1080 mtDNA (Table 4).

Thus, in three cybrids and four hybrids maintained continuously in chloramphenicol, there were 2 to 10 times more copies of the mtDNA from the CAP^r parent than the mtDNA from the CAP^s parent. Furthermore, when one cybrid was permitted to segregate the CAP^r phenotype and was then rechallenged with chloramphenicol to yield an intermediate level of resistance, it was found to have an intermediate proportion of CAP^r mtDNA. These results showed that chloramphenicol selection resulted in a significant bias in the mtDNA ratio toward the mtDNA of the CAP^r parent and demonstrated that chloramphenicol was indeed encoded in the mtDNA. However, they also indicated that expression of the CAP^r phenotype only required about 50% of the cellular mtDNA to be mutant and that a fully resistant and relatively stable phenotype could be achieved while the cells still retained significant quantities of the CAP^s mtDNA. These observations imply that chloramphenicol selection acts at the cellular level rather than at the mitochondrial level and that the cellular phenotype appears to be a function of the intracellular ratio of CAP^r mtDNA to CAP^s mtDNA.

One additional hybrid, RIB22, was examined, and it was markedly different from the other cell lines. This hybrid contained little of the mtDNA of the CAP^r parent and a 20-fold excess of the mtDNA of the CAP^s HeLa parent. This was clear both from the prominent HeLa fragments (2.4 and 2.1 kb for *Hae*II, 1.35 kb for *Hae*II, and 0.68 kb for *Hha*I) (Fig. 3 through 5) and from the barely detectable HT1080 9-kb *Hae*II fragment (Fig. 3 and Table 4). An analysis of the growth characteristics of RIB22 in varying concentrations of chloramphenicol revealed that its growth rate and cloning efficiency were enhanced 300 to 500% by 10 to 150 μg of chloramphenicol per ml. This behavior was unlike the behavior of any of the parents or their CAP^r cybrid or hybrid derivatives and suggested that the basis of chloramphenicol resistance in RIB22 may be fundamentally different from that of the other cell lines.

DISCUSSION

The discovery of three restriction endonuclease site polymorphisms between HeLa and HT1080 mtDNA's has provided for the first time genetic markers known to specifically reside on human mtDNA. These polymorphisms provide new markers for identifying cells of HeLa origin (20, 26), and with these polymorphisms it is now possible to trace the fate of mtDNA molecules in hybrids and cybrids and to correlate mtDNA

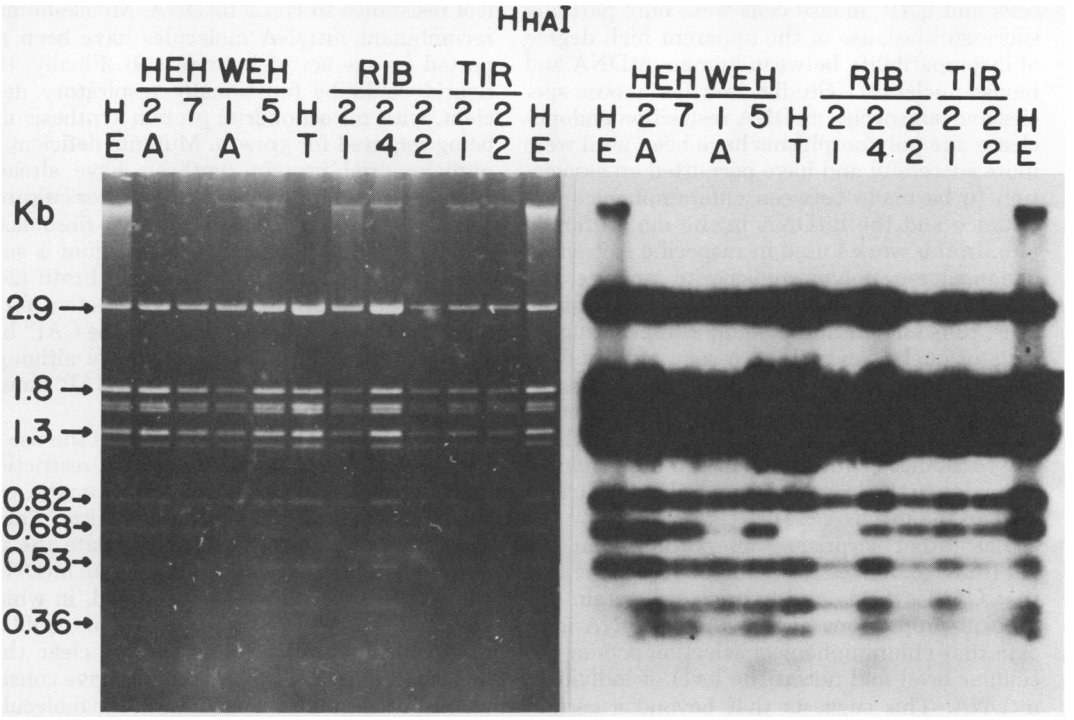

FIG. 5. *HhaI digests of CAP^r interstrain cybrid and hybrid mtDNA's. HE, HeLa S3 mtDNA; HT, HT1080-6TG1 mtDNA. All channels contained approximately 0.5 μg of mtDNA. The origins of the mtDNA's were as described in the legend to Fig. 3.*

TABLE 4. *Relative contents of HeLa and HT1080 mtDNA's in cybrids, hybrids, and parents*

Cell line	Relative content (%) as determined with:						Mean relative content (%)	
	HaeII[a]		*HaeIII*[a]		*HhaI*[b]			
	HeLa	HT1080	HeLa	HT1080	HeLa	HT1080	HeLa	HT1080
HEH2A	91	9	85	15	100[c]	0	92	8
HEH7	85	15	69	31	100[c]	0	85	15
WEH1A	27	73	9	91	14	86	17	83
WEH5	58	42	62	38	59	41	60	40
TIR21	83	17	78	22	86	14	82	18
TIR22	79	21	100[c]	0	78	22	86	14
RIB21	29	71	9	91	18	82	19	81
RIB22	93	7	91	9	100	0	95	5
RIB24	23	77	27	73	45	55	32	68
HT1080	0	100	0	100	0	100	0	100
HeLa	100	0	99	1	96	4	98	2
HeLa	100	0	95	5	100[c]	0	98	2

[a] *HaeII* and *HaeIII* values were calculated from densitometric readings of Polaroid negatives of the ethidium bromide-stained gels in Fig. 3 and 4 (see text).

[b] *HhaI* values were calculated from densitometric readings of the autoradiograph in Fig. 5 (see text).

[c] Several *HaeIII* and *HhaI* estimates for the relative HeLa content were greater than 100%. These included the *HaeIII* value for TIR22, which was 106%, and the *HhaI* values for HEH2A (121%), HEH7 (109%), and HeLa (104%).

inheritance with the inheritance of cytoplasmic genes. By using this innovation, it has been possible to link the inheritance of the human CAP^r locus with the inheritance of mtDNA and to begin to understand the expression of the CAP^r phenotype in terms of a proportionality of mutant and wild-type mtDNA's.

Previous efforts in our laboratory to investigate the basis of chloramphenicol resistance with hybrids and cybrids between CAP^r human

cells and CAP^s mouse cells were only partially successful because of the apparent high degree of incompatibility between human mtDNA and mouse nuclei (17). Studies in which mouse species and subspecies mtDNA restriction endonuclease site polymorphisms have been used were more successful and have permitted an association to be made between chloramphenicol resistance and the mtDNA in the mouse family (18). In this work I used intraspecific restriction endonuclease polymorphisms to analyze the basis of chloramphenicol resistance in human cells, thus eliminating possible complicating effects of nucleus-cytoplasm incompatiblity. The observed association of chloramphenicol resistance with mtDNA is consistent with observations in yeast that mitochondrial chloramphenicol resistance is due to one of two base changes occurring in the large ribosomal ribonucleic acid gene of the mtDNA (14).

The rather surprising observations that the HT102W mutant is unstable for resistance and that CAP^r cybrids and hybrids still retain significant proportions of the CAP^s mtDNA indicate that chloramphenicol selection acts at the cellular level and not at the level of individual mtDNA. This suggests that beyond a certain ratio, there may be little selective advantage for enrichment of CAP^r mtDNA. Therefore, most CAP^r mammalian cells may still retain some CAP^s mtDNA's. Further studies with the CAP^s WEH5 segregant should permit an analysis of the actual molecular changes which occur during the acquisition of resistance and should indicate whether prolonged chloramphenicol selection can lead to a homogeneous population of mutant mtDNA's. Studies on the fate of CAP^r and CAP^s mtDNA's in freely segregating HeLa-HT1080 hybrids should also permit an analysis of the dynamics of segregation of human mtDNA's and their associated genes and may provide further insights into the basis of cellular mitochondrial phenotypes in this complex problem of intracellular population genetics.

The basis of the chloramphenicol resistance of RIB22 remains unexplained. There are several possible explanations for the 20-fold excess of the CAP^s parental mtDNA. First, the presence of the CAP^s mtDNA could be due to the chloramphenicol resistance of HT102W not being coded in the mtDNA. This possibility seems unlikely since the data from WEH1A, WEH5, RIB21, and RIB24 lead to the opposite conclusion. Second, the resident CAP^s HeLa mtDNA could have mutated to chloramphenicol resistance and replaced the HT1080 mtDNA. Third, there could have been a molecular recombinant which transferred HT1080 chloramphenicol resistance to HeLa mtDNA. Mouse-human recombinant mtDNA molecules have been reported in one set of hybrids (19). Finally, the hybrid could be functionally respiratory deficient, with mitochondrial protein synthesis not being required for growth. Mutants deficient in mitochondrial protein synthesis have already been described for human and hamster cultured cells (13, 45), and chicken embryo fibroblasts grow in chloramphenicol if the medium is supplemented with tryptose phosphate broth (24). Indeed, this latter alternative is attractive, for it would also explain why human-mouse CAP^r hybrids could grow in chloramphenicol although very poorly, and yet retain only the CAP^s mouse mtDNA (17).

In conclusion, this report describes the use of a new set of mtDNA genetic markers, restriction endonuclease polymorphisms, for somatic cell genetic studies on human mitochondria. These genetic markers were used to correlate the inheritance of the CAP^r locus and human mtDNA, and a new procedure was developed, in which human mtDNA's from different origins could be quantitated within cells. It is now clear that these new markers and techniques have considerable potential for the analysis of the molecular basis of a number of important questions in human mitochondrial genetics, such as the basis of mitochondrial drug resistance, the dynamics of mutant mtDNA selection, and the mechanism of mitochondrial gene segregation.

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