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Different nucleotide changes in the large rRNA gene of the mitochondrial DNA confer chloramphenicol resistance on two human cell lines

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ABSTRACT

The nucleotide sequence of the mitochondrial DNA (mtDNA) in the region coding for the 3' end of the large rRNA has been determined for two human cell lines bearing independent cytoplasmic chloramphenicol-resistant (CAP-r) mutations. Comparison of the sequences of these two phenotypically different CAP-r mutants with their CAP-sensitive (CAP-s) parental cell lines has revealed a single base change for each in a region which is highly conserved among species. One CAP-r mutation is associated with an A to G transition on the coding strand while the second contains a G to T transversion 52 nucleotides away. Comparable sequence changes in this region had previously been found for mouse and yeast cell mitochondrial CAP-r mutants. Thus, changes in the large rRNA gene eliminate the inhibition of the ribosome by CAP and different nucleotide changes may result in variations in the drug-r phenotype.

INTRODUCTION

The human mitochondrial DNA (mtDNA) is 16,569 base pairs (bp) in length and codes for two ribosomal RNAs (rRNAs), 22 transfer RNAs (tRNAs), and 13 polypeptides, 5 of which have been assigned a genetic function (1). Virtually all of the genes including the 12S and 16S rRNA genes are separated by one or more tRNAs. The mtDNA is read into a continuous transcript which is processed at the tRNAs into the structural and messenger RNAs (2,3). The 12S and 16S rRNA transcripts are incorporated into the small and large subunits of the mitochondrial ribosome and these ribosomes are used to translate the mtDNA messenger RNAs into proteins.

Mitochondrial ribosomes differ from those of the cytosol in that they are sensitive to the bacterial ribosome inhibitors chloramphenicol (CAP), erythromycin (ERY) and carbomycin (CAR). In yeast, Saccharomyces cerevisiae, cytoplasmically inherited mutants resistant to CAP were reported over twelve years ago (4). These mutations were assigned to the mtDNA and mapped to the large (21S) rRNA gene. Recently, the partial nucleotide sequence of the 21S rRNA from two such mutants,

C^{R}_{321} and C^{R}_{323}, were determined. This analysis revealed two single nucleotide changes separated by 56 bp and altering the 3' end of the mitochondrial 21S rRNA (5).

CAP-resistant (r) mutants have also been isolated for mammalian cells. The first mutant reported was a HeLa S3 derivative, 296-1 (6). The CAP-r mutation of 296-1 was shown to be cytoplasmic by enucleation of the CAP-r cells and fusion of the cytoplasmic fragment to CAP-sensitive (s) cells. Selection in CAP and for the recipient cell nucleus yielded viable CAP-r cell lines (cybrids) (7,8). The CAP-r locus of 296-1 as well as the CAP-r locus of a mutant of the cell line HT1080 (HT102W) were assigned to the mtDNA by demonstrating linkage of the CAP-r phenotype to mtDNA restriction endonuclease site polymorphisms (9). 296-1 and HT102W were also observed to differ in the degree of their CAP resistance (9,10).

Comparison of the nucleotide sequence of mouse and human mtDNAs in the region of the large rRNA surrounding the two yeast CAP-r mutations has revealed a high degree of interspecies sequence homology. Two blocks of sequence homology have been found, one of 10 and one of 13 nucleotides, which are separated by 42 nucleotides. Analysis of the sequence in this same region of the mtDNA of a CAP-r mouse line, 501-1, has also revealed a single nucleotide change close to one of the yeast mutations (11).

In the present paper we describe a comparable analysis of the mtDNA from the two human CAP-r mutants, 296-1 and HT102W. CAP resistance has also been found to correlate with single nucleotide changes in these lines, though the changes were found to be different in the two mutants.

MATERIALS AND METHODS

Cell Lines, Media and Growth Tests

S3 (12), 296-1 (6), HT1080 (13) and HT102W (9,10) were maintained in MEM (Grand Island Biological Company, Grand Island, N.Y., GIBCO) supplemented with 10% bovine serum (Irvine Scientific, Santa Ana, CA). Media for 296-1 and HT102W were further supplemented with 50 μg/ml CAP. Suspension cultures of 296-1 were grown in MEMS (GIBCO) supplemented with 10% bovine serum, 0.2% glucose and 15mM HEPES (pH = 7.4).

Cloning efficiencies and growth curve procedures have been described previously (9). CAP was obtained from Sigma (St. Louis, MO) erythromycin-lactobionate from Abbott Laboratories (North Chicago, IL) and carbomycin was a generous gift from Mr. Nathan Belcher, Pfeitzer

Pharm. Corp., Groton, CO.

MtDNA Purification and Cloning

Mitochondria were isolated by differential centrifugation (14) and the closed circular mtDNA purified by CsCl ethidium bromide (EtBr) gradients (15). An E. coli vector, ST15/8.1, was constructed from pBR325 (16) and plasmid B12 (gift of Mr. M. Fromm and Dr. P. Berg, Department of Biochemistry, Stanford Medical Center, Stanford, CA). B12 carries the PvuII fragment of Herpes Simplex virus containing the thymidine kinase gene which was inserted into the pBR322 BamH1 site using BamH1 linkers. B12 was digested with BamH1 and the Herpes Simplex virus fragment transferred into the BamH1 site of pBR325. One plasmid was chosen in which the EcoRI site of the insert lay between the unique KpnI site of the insert and the EcoRI site of pBR322. Complete digestion of ST15/8.1 with EcoRI and KpnI inactivated the tetracycline (Tc) and CAP resistance loci but not the ampicillin (Ap) resistance locus and provided sites for the insertion of the 1.5 kilobase (Kb) human mtDNA KpnI-EcoRI fragment containing the 3' end of the 16S rRNA gene (see Figure 1).

One microgram of human mtDNA digested with KpnI and EcoRI was mixed with 0.2 μg of comparably digested ST15/8.1. The DNAs were ligated with T4 ligase (New England Biolabs, Beverly, MA) (17) and the ligation mixtures were used to transform E. coli (18). Colonies were screened for Ap-r, CAP-s and Tc-s. The plasmids were then extracted (19) and screened for the appropriate insert. Only two human mtDNA fragments are bounded by KpnI and EcoRI sites, one of 3.4 Kb containing the unique BamHI site and the other of 1.5 Kb containing the rRNA genes (see Figure 1). The plasmid containing the 1.5 Kb insert obtained from S3 mtDNA was designated ST27/10.1, those from 296-1 were ST25/9.1 and ST25/13.1, that from HT1080C was ST27/13.8 and that from HT102W was ST28/8.40. All of these plasmid inserts were confirmed by digestion with XbaI, AvaII and HpaII which yield distinctive fragment patterns for the 16S rRNA (1). Experiments were performed under P1 + EK1 containment consistent with the guidelines for recombinant DNA research set forth by the National Institutes of Health (20).

DNA Sequencing

The DNA sequences were determined by the chemical method of Maxam and Gilbert (21). 500 ml cultures of E. coli bearing the plasmid were grown in LB medium (22) and extracted (23).

The sequences for plasmids ST27/10.1, ST25/13.1, ST27/13.8 and

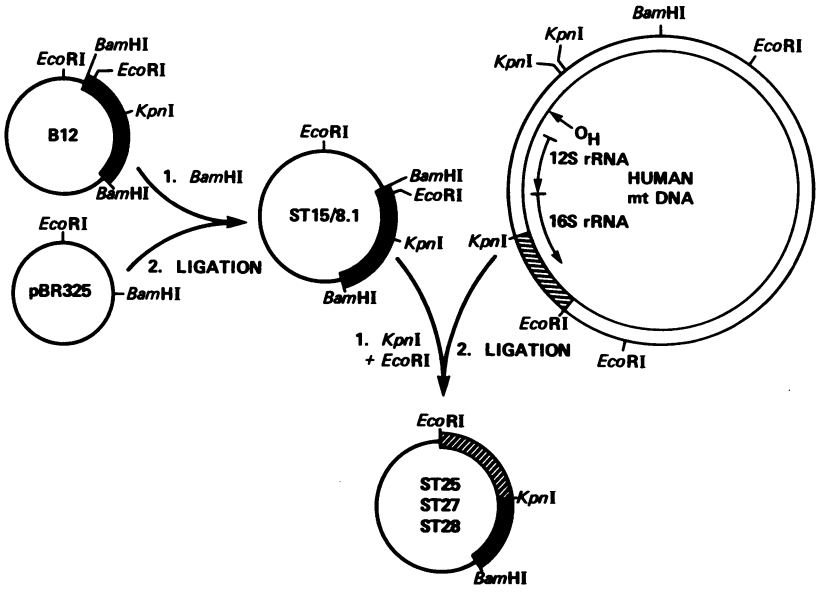

Figure 1: Cloning of the Human MtDNA Fragment Corresponding to the 3' End of the 16S rRNA. The E. coli plasmid sequences, pBR322 in B12 (7.9 Kb) and pBR325 (5.5 Kb), are shown as single lines; the tk gene of HSV is shown by heavy line and the human mtDNA (16.5 Kb) by double line with a hatched area corresponding to the cloned region. Restriction endonuclease cleavage sites used in the clonings are indicated.

ST28/8.40 were determined by sequencing from the unique XbaI site within the 1.5 Kb insert. The plasmid was digested with XbaI and 5' end labeled by digestion with bacterial alkaline phosphatase (21) and treatment with T4 polynucleotide kinase together with [γ-^{32}P] ATP, S.A. > 4,000 Ci/mmole (ICN, Irvine, CA). The linear plasmid was digested with KpnI and EcoRI, the two resulting labeled fragments separated by gel and the L strand sequenced toward the EcoRI site and the H strand sequenced toward the KpnI site (see Figure 2).

These sequences were confirmed for ST25/13.1 and ST27/10.1 by removal of the insert from the plasmid by KpnI and EcoRI digestion, digestion with HpaII, 5' end labeling, and separating and sequencing the HpaII-KpnI fragment. Comparably, the plasmids ST27/13.8 and ST28/8.40, were digested with AvaII, 5' end labeled, digested with EcoRI and the 1.35 Kb EcoRI-AvaII fragment purified and sequenced.

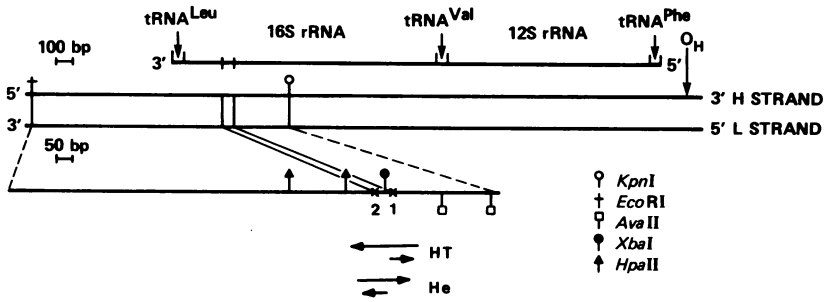

Figure 2: Schematic Map of the Ribosomal RNA Genes and Sequencing Strategy of the DNA Region Corresponding to the 3' End of the 16S rRNA. The tRNA genes ($tRNA^{Phe}$, $tRNA^{Val}$, $tRNA^{Leu}$) and the origin of replication of the heavy strand (O_H) are shown relative to the rRNA genes. The fragment cloned as described in Figure 2 is enlarged in the lower portion of the figure and shows the restriction sites used for sequencing. The direction and extent of sequence determined is indicated (see Materials and Methods). He indicates sequences for the S3 or 296-1 mtDNAs while HT indicates those for HT1080C or HT102W.
Nucleotide differences are indicated by X. (1) corresponds to nucleotide 2939 and is the C to A transversion found in HT102W, (2) corresponds to nucleotide 2991 and is the T to C transition found in 296-1.

RESULTS

Drug Resistant Phenotypes of 296-1 and HT102W

HeLa CAP-r mutant 296-1 grows with essentially the same generation time in 25 and 50 μg/ml CAP and CAP concentrations of at least 150 μg/ml have no effect on the 296-1 cloning efficiency. S3, by contrast, is fully inhibited by 25 μg/ml (6,9) (Table I).

The HT1080C CAP-r mutant, HT102W, grows with a 35 to 37 hour doubling time with and without 50 μg/ml CAP and its growth rate is slightly stimulated in 25 μg/ml CAP. HT102W clones normally at 50 μg/ml CAP but, unlike 296-1, is unable to clone in 150 μg/ml of the drug. Like S3, HT1080C is fully inhibited by 25 μg/ml CAP (Table 1).

Analysis of the cloning efficiencies of S3, 296-1, HT1080C and HT102W in up to 500 μg/ml ERY and 20 μg/ml CAR revealed no significant difference between the CAP-r mutant and its CAP-s parent. S3 and 296-1 were inhibited by 200 μg/ml ERY and 10.0 μg/ml CAR while HT1080C and HT102W were inhibited by 100 μg/ml ERY and 5.0 μg/ml CAR. These results contrast with the cross resistances observed for other CAP-r mutants (24).

Nucleotide Changes in 296-1 and HT102W 16S rRNA Genes

The nucleotide sequences around the central XbaI site were determined

TABLE 1: GROWTH CHARACTERISTICS OF PARENTAL AND CAP-R MUTANT CELL LINES

	DOUBLING TIME (hours)			CLONING EFFICIENCY (%)	
	CAP^0	CAP^{25}	CAP^{50}	CAP^{50}	CAP^{150}
S3	18	--	--	5	0
296-1	17	18	17	108	107
HT1080C	17	--	--	22	0
HT102W	35	31	37	111	0

CAP^0 to CAP^{150} represent concentrations of CAP in μg/ml in which cells were grown. Cloning Efficiency data adapted from (9).

for the cloned 1.5 Kb KpnI-EcoRI mtDNA fragments of S3, 296-1, HT1080C and HT102W. This region encodes the 3' end of the 16S rRNA and encompasses the sites of the single nucleotide changes associated with CAP resistance in yeast mutants C^R_{321} and C^R_{323}.

The sequences of S3 mtDNA from the insert of plasmid ST27/10.1 and 296-1 mtDNA from the insert of ST25/13.1 were examined between nucleotides 2884 and 3064. The S3 mtDNA sequence was found to be identical with the previous L strand sequence obtained for placental mtDNA (1). S3 and 296-1 were found to differ by only a single nucleotide. This was a T to C transition at residue 2991. This sequence difference was confirmed by sequencing the H strand for 85 nucleotides from nucleotide 2954 to 3029. An A to G transition was found at position 2991. The sequence of the H strand for a second clone of 296-1 mtDNA (ST25/9.1) confirmed this result ruling out the possibility that the base change occurred during mtDNA cloning.

The nucleotide sequences in the region of the XbaI site of the 1.5 Kb KpnI-EcoRI fragment of HT1080C mtDNA (plasmid ST27/13.8) and HT102W mtDNA (plasmid ST28/8.40) were determined from nucleotide 2857 to 3047 on the L strand. As found for the HeLa samples, the HT1080C sequence was identical to the placental sequence (1). Likewise, HT102W was found to differ from HT1080C by a single C to A transversion at nucleotide 2939. This change was confirmed by sequencing from nucleotide 2874 to 2944 on the H strand where a G to T transversion was found at this same site.

Thus, in this region of the 16S rRNA the S3 and HT1080C mtDNA sequences have been found to be identical to placental mtDNA while the

296-1 and HT102W sequences were found to differ from their parental sequences by a single nucleotide change. These two changes are themselves separated by 52 nucleotides though both are associated with CAP resistance.

DISCUSSION

Phenotypic and Structural Significance of Two Human CAP-r Mutations

We believe the two different substitutions observed in the two human mutants to be the molecular basis for the CAP-r phenotypes and not to be phenotypically neutral. The identification of other CAP-r mutants in this highly conserved region among different species strongly supports this conclusion and will be detailed below. CAP is known to block the binding of aminoacyl-tRNA to the ribosome and thus inhibit the peptidyl transferase reaction (25). Because of the possible secondary structure of the rRNA in this region (26 and 11) which brings the two sites of mutation within 10 nucleotides of each other, these changes could easily affect the same enzymatic site.

The parallel between the differences in 296-1 and HT102W CAP associated nucleotide changes and the differences in their CAP sensitivity raises the intriguing possiblity that these phenomena are causally related. The phenotypic differences are not simply the product of nuclear background for they are retained in reciprocal cybrid transfers where the nuclei are reversed (9,10). Though they might be explained by differences in the proportion of CAP-r and CAP-s mtDNAs within the cell, this possiblity seems unlikely since the mixing of CAP-s and CAP-r mtDNAs in cybrids and hybrids does not negate the association between the level of CAP resistance and the origin of the cellular mtDNA. The most reasonable conclusion is that the two different mutations yield the two different phenotypes. Comparable phenotypic differences were also observed for the two yeast mutants, C^{R}_{321} and C^{R}_{323} (27). Thus, it seems likely that different base changes in this region might result in a variety of CAP resistance phenotypes.

Sequence Homology of Large rRNA Genes

Comparison of the nucleotide sequences of the large rRNA genes in the vicinity of the yeast CAP-r mutants previously revealed a high degree of homology between the *E. coli* DNA (28), yeast mtDNA (5), mouse mtDNA (29), human mtDNA (1) and paramecium mtDNA (S. Jaffrey, personal communication). Two regions of high homology, one of 13 nucleotides and the other of 10 nucleotides, were observed surrounding the yeast CAP-r mutants (11).

More recently, the sequence in this region for the Physarum polycephalum nuclear coded large rRNA gene (30) and for the corn chloroplast 23S rRNA gene (31) have been reported. The 3 mtDNA sequences and the corn chloroplast DNA sequence are identical in these two conserved regions. However, these sequences differ slightly from those of E. coli and Physarum. The 3 mtDNA rRNA sequences are compared to those of E. coli and Physarum in Figure 3.

In the 13 nucleotide box on the left of Figure 3, the E. coli sequence differs from the Physarum sequence by 3 nucleotides and from the three mtDNAs by 2 nucleotides. The Physarum sequence differs from the three mtDNA sequences by 3 nucleotides as well. For the right hand 10 nucleotide box E. coli and the 3 mtDNAs share complete homology while Physarum differs from these sequences by 2 bases. These observations would be consistent with large rRNA of the mtDNA and chloroplast DNA being more closely related to the E. coli rRNA gene than to that of the Physarum nucleus.

It has been observed that the large rRNA gene of both S. cerevisiae ω+ strain mtDNA and Physarum nuclear DNA have an intron inserted at the same location in the 13 nucleotide conserved box. This result suggests that either there may be constraints on the possible locations of introns

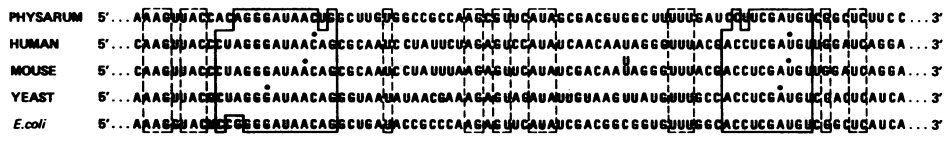

Figure 3: Sequence Homologies at the 3' End of Different rRNAs. The rRNA sequences presented were deduced from the corresponding DNA sequences. The Physarum nuclear 28S rRNA sequence (30) corresponds to nucleotide 149 to 165 and to 1157 to 1223 (nucleotide 166 to 1156 represents the intron present in the gene but absent at the RNA level). The human 16S rRNA mtDNA sequence (1) corresponds to nucleotide 2920 to 3003, the mouse LA9 mtDNA sequence (29) corresponds to the nucleotide 2486 to 2570, the yeast 21S rRNA mtDNA sequence (5) corresponds to nucleotide -77 to +7 of the ω- strain and the E. coli 23S rRNA sequence (28) corresponds to nucleotide 2433 to 2516.
The two largest homologous sequences observed between the three mtDNA rRNAs (human, mouse, and yeast) are fully boxed (13 nucleotides on the left and 10 nucleotides on the right). The corresponding homologous regions of the Physarum and E. coli sequences are included. The dotted boxes show additional complete sequence homology between the five organisms. Dots above nucleotides indicate the position where mutations have been found in CAP-r variants (see Figure 4).

in this gene or that the mtDNAs and Physarum nuclear DNA had a common ancestor with an intron in this position.

Sequence Comparisons of Different CAP-r Mutants

In addition to the nucleotide changes reported for the yeast CAP-r mutants C^R_{321} and C^R_{323} (5) and the two human CAP-r mutants described here, we have previously described the nucleotide change associated with a mouse CAP-r mutant, 501-1 (11). Kearsey and Craig (32) have also described nucleotide changes for an independent HeLa CAP-r mutant, MC63, and a mouse 3T3 mutant, 3T3 CAP-r. The location of the associated nucleotide changes are indicated in Figure 3 by dots and the specific mutations shown in Figure 4. A most striking correlation is seen for 3 of the 5 mammalian CAP-r mutants. HeLa mutants 296-1 and MC63 as well as mouse mutant 501-1 all have exactly the same nucleotide change, resulting in a U to C transition in the 10 nucleotide conserved box of the large rRNA. This change is one nucleotide 3' to the A to C transition found for yeast mutant C^R_{321}. It seems extremely unlikely that chance alone could explain the appearance of these nucleotide changes in this highly conserved region in 3 independent CAP-r mutants. Rather, these changes must be associated with the CAP resistance phenotype. It is further significant that all 3 of the CAP-r mutants have acquired a C in this location in the rRNA. The probability of a C being substituted in 3 independent trials by chance alone is less than 4 $((1/3)^3)$. Consequently, it is tempting to suggest that this base may alter the

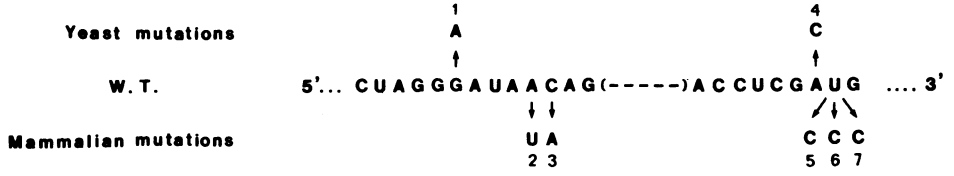

Figure 4: Comparison of the rRNA Base Pair Mutations Associated to CAP-r Phenotypes. Only the wild type (W.T.) sequence of the two longer stretches of homology (13 nucleotides and 10 nucleotides) present in the 3' end of all three mtDNA large rRNAs (human, mouse, and yeast) is presented in this figure. These two rRNA regions correspond to nucleotide 2929 to 2941 (left part of the sequence) and to nucleotide 2984 to 2992 (right part of the sequence) of the human mtDNA sequence (see also Figure 3). The first line indicates the yeast CAP-r mutants (1) C^R323 G to A and (4) C^R321, A to C. The third line indicates the mammalian mutants: (2) mouse 3T3, A to U, (3) human HT102W, C to A, (5) mouse 501-1, U to C, (6) human 296-1, U to C and (7) human MC63, U to C (5, 11, 32 and this work).

stereochemical interaction of the drug with the ribosome.

The remaining three CAP-r mutations are all located in the 13 nucleotide conserved box. The human HT102W mutation is due to a C to A transversion. The mouse 3T3 CAP-r mutation is immediately adjacent to that of HT102W and is an A to U transversion. Finally, the yeast mutation, C^{R}_{323}, is 4 nucleotides 5' from the 3T3 CAP-r mutation and is a G to A transition. Since these mutations are dispersed over 6 nucleotides, these results suggest that more nucleotides of this region could be involved in the CAP binding site. Alternatively, additional constraints may be acting on the sequence of the right-hand 10 nucleotide box with the result that only a limited number of CAP-r mutants can be expressed.

One of the major phenotypic differences between cytosol 80S ribosomes and bacterial and mitochondrial ribosomes is their resistance to CAP. Though generally used as a major phenotypic trait to argue for the similarity between organelle genomes and bacteria, the molecular basis of this difference remains unknown. The sequence of the CAP-r mutants in the 13 nucleotide conserved region could suggest an explanation. The Physarum 28S nuclear rRNA gene differs from the mtDNA 16S rRNA gene and the E. coli 23S rRNA gene by an A to U transversion in the 13 nucleotide box one nucleotide 3' to the CAP-r mutation of HT102W (Figure 3). This fact raises the intriguing possibility that this sequence change could have rendered the progenitors of the 80S ribosome CAP-r. Comparison with more eukaryotic nuclear rRNA sequences should help to confirm this hypothesis. Since the Archaebacteria have also been found to be CAP-r (33), it will be most interesting to examine the nucleotide sequence of their large rRNA genes in this region.

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