STUDIES OF HUMAN MITOCHONDRIA

I. Steady-state levels and metabolic properties of the mitochondrial tRNAs

II. Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA

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Abstract

The steady-state levels and metabolic properties of mitochondrial tRNAs have been analyzed in HeLa cells and correlated with the function of the tRNAs for organelle-specific protein synthesis. DNA excess hybridization experiments utilizing separated strands of mitochondrial DNA (mtDNA) and purified tRNA samples from exponential cells long-term labeled with [^{32}P] orthophosphate have revealed a steady-state level of 6×10^5 tRNA molecules per cell, with three-fourths being encoded in the heavy (H)-strand and one-fourth in the light (L)-strand. Hybridization of the tRNAs with a panel of M13 clones of human mtDNA containing, in most cases, single tRNA genes and a quantitation of two-dimensional electrophoretic fractionations of the tRNAs have shown that the steady-state levels of $tRNA^F$ and $tRNA^V$ are two to three times higher than the average level of the other H-strand-encoded tRNAs and three to four times higher than the average level of the L-strand-encoded tRNAs. Similar experiments carried out with tRNAs from cells labeled with very short pulses of [5-^{3}H] uridine have indicated that the rates of formation of the individual tRNA species are proportional to their steady state amounts. Therefore, the 15-fold to 60-fold higher rate of transcription of the $tRNA^V$ and $tRNA^F$ genes (transcribed with the rDNA transcription unit) relative to the other H-strand tRNA genes (transcribed with the whole H-strand transcription unit) and the 13-fold to 20-fold higher rate of transcription of the L-strand tRNA genes relative to the H-strand tRNA genes (other than $tRNA^V$ and $tRNA^F$ genes) are not reflected in the rates of formation of the corresponding tRNAs. The available data indicate that the majority of $tRNA^V$ and $tRNA^F$ transcribed from the rDNA transcription unit are degraded as they are excised from the primary transcripts. It also seems likely that the majority of the L-strand-encoded tRNAs are degraded before they are excised from the short-lived polycistronic transcripts. Furthermore, a role of the

aminoacyl tRNA synthetases in stabilizing the different tRNA species at relatively uniform levels is suggested. A comparison of the steady-state levels of the individual tRNAs with the corresponding codon usage for protein synthesis, as determined from the DNA sequence and the rates of synthesis of the various polypeptides, has not revealed any significant correlation between the two parameters.

In other experiments, isolated human mitochondria containing a mitochondrial DNA (mtDNA)-coded chloramphenicol resistance marker were injected at an average dose of less than one into sensitive human cells partially depleted of their mtDNA by ethidium bromide treatment. Under selective conditions, the mitochondria became established in the recipient cells with a frequency greater than 2 to 3×10^{-3}. A rapid and, in some cases, complete replacement of the resident mtDNA by the exogenous mtDNA took place in the transformants, as shown by multiple mtDNA and nuclear DNA polymorphisms. Intracellular mtDNA selection played a crucial role in this replacement, with significant implications for mitochondrial genetics.

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CHAPTER 1

Gene Expression in Human Mitochondria

Mitochondria are present in the cytoplasm of all eukaryotic cells, where they are primarily responsible for cellular respiration and the production of ATP. In mammalian cells, each mitochondrion contains a variable number of circular DNA molecules. Since these DNA molecules code for many proteins essential for mitochondrial function, as well as for the ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs) needed in translation, the mitochondria represent a genetic system that is distinct from the nuclear genome.

Genetic Organization.

The complete DNA sequence of the mitochondrial genome from four species of mammals -- human (Anderson *et al.*, 1981), mouse (Bibb *et al.*, 1981), cow (Anderson *et al.*, 1982), and rat (G. Gadaleta, personal communication) -- has been determined. Analysis of these sequences has revealed that, in addition to their similarities in size, these genomes have identical gene content and genetic organization. The genetic and transcriptional map of the human mitochondrial genome is shown in Figure 1. As is evident from this figure, the human, and the mammalian genomes in general, exhibit an extreme economy in their genetic organization. With the exception of approximately 7% of the genome around the heavy (H)-strand origin of replication, the mitochondrial DNA (mtDNA) is completely saturated with RNA and protein coding sequences. Two rRNA genes, 14 tRNA genes, and 12 protein coding genes are transcribed from the H-strand, and 8 tRNA genes and 1 protein coding gene are transcribed from the light (L)-strand. The tRNA genes are arranged in such a way that they are nearly always contiguous to the rRNA genes and the protein coding genes, with the intergenic spacing between these genes being, in most cases, represented by no, or very few nucleotides. Thus, the protein coding genes begin immediately, or within a few nucleotide intervals, with the initiation codon of the reading frame. In addition,

most reading frames lack a termination codon and end with either a T or a TA following the last sense codon. When processing occurs at the 3'-ends of the individual mRNAs (usually immediately preceding the 5'-end of a tRNA, see below), these mRNAs are polyadenylated creating a TAA termination codon.

RNA Synthesis and Processing.

Studies of transcription of mtDNA in HeLa cell mitochondria have shown that both strands are transcribed over their entire length (Aloni and Attardi, 1971; Murphy ***et al.***, 1975). Three initation sites for transcription have been found, one for transcription of the L-strand and two for transcription of the H-strand (Montoya ***et al.***, 1982; Yoza and Bogenhagen, 1984). The L-strand transcripts are polycistronic RNA molecules that begin in the displacement loop, near the H-strand origin of replication, and extend through the entire length of the L-strand. They are synthesized at a two- to three-fold higher rate than the H-strand transcripts (Cantatore and Attardi, 1980). One class of H-strand transcripts is represented by polycistronic RNA molecules that initiate near the 3'-end of the 12S rRNA gene and extend through the full length of the H-strand. These molecules are destined to produce the majority of the tRNAs and mRNAs. The other class of H-strand transcripts starts approximately 20 nucleotides upstream of the $tRNA^F$ gene, continues through the 12S rRNA, $tRNA^V$, and 16S rRNA genes, and terminates at the 3'-end of the 16S rRNA gene. They are transcribed at a rate 15 to 60 times higher than the larger H-strand transcripts and are responsible for the bulk of the rRNA formation (Montoya ***et al.***, 1983).

The transcription of the mtDNA in the form of large polycistronic RNA molecules and the presence of tRNA genes flanking nearly every rRNA and protein coding gene have led to the hypothesis that the tRNA sequences in the polycistronic transcripts function as signals for processing. The precise

endonucleolytic cleavages at the 5'- and the 3'-ends of the tRNA sequences, excising the tRNAs, release the rRNAs and the mRNAs. The tRNAs are further modified by the addition of CCA to their 3'-end, and they and the rRNAs also undergo some base modifications, most notably methylation. Furthermore, the rRNAs have one or more A residues added to their 3'-ends, whereas the mRNAs are modified by the addition of a long poly(A) tract (~55 residues) to their 3'-end. The sequence analysis of the 5'- and the 3'-ends of the rRNAs and most of the mRNAs from HeLa cell mitochondria (Crews and Attardi, 1980; Montoya *et al.*, 1981; Ojala *et al.*, 1981), and their alignment with the mtDNA sequence (Anderson *et al.*, 1981), have confirmed the above picture of mitochondrial RNA processing. The H- and L-strand transcripts are aligned with the mitochondrial genetic map in Figure 1.

Genetic Function.

The mRNAs for the mtDNA-encoded polypeptides are translated within the mitochondria on organelle-specific ribosomes. The mtDNA of mammalian cells encodes the two rRNAs and all the tRNAs required for translation, whereas all the proteins of the translation apparatus are nuclear DNA encoded. One of the unusual features of the mitochondrial genetic system is that it uses a genetic code that differs from the so-called "universal code." In mammalian mitochondria, UGA codes for tryptophan instead of functioning as a termination codon, AUA codes for methionine instead of isoleucine, and AGA and AGG are termination rather than arginine codons (Anderson *et al.*, 1981). In addition, AUG, AUA, and in some cases, AUU and AUC, function as initiation codons, and both AUG and AUA can code for internal methionines. Thus, by the use of a two out of three base interaction between codon and anticodon in the four codon families (genetic code boxes with four codons for one amino acid) and by the use of only one

$tRNA^M$ species for both initiation and elongation, 22 tRNA species are sufficient for translation of the mitochondrial mRNAs.

Each of the mitochondrial translation products has been assigned to one of the protein coding sequences of the mtDNA, and the function of these polypeptides has been determined. In animal cells, all of the mitochondrially-encoded proteins have been found to be components of the multisubunit enzyme complexes of the mitochondrial respiratory chain and the coupling proton translocating ATPase. Seven of the 13 polypeptides encoded in the mtDNA are components of the first enzyme complex of the mitochondrial respiratory chain, the rotenone-sensitive NADH-ubiquinone oxidoreductase (Chomyn *et al.*, 1985, 1986). No mtDNA-encoded polypeptide has been found to be associated with complex II, the succinate-ubiquinone oxidoreductase. At least one of the two *b* hemes of the ubiquinol-cytochrome *c* oxidoreductase (complex III) is associated with one of the mtDNA-encoded polypeptides, apocytochrome *b* (Hatefi, 1985). The three largest subunits of ferrocytochrome *c*-oxygen oxidoreductase (complex IV), COI, COII, and COIII, are also synthesized in mitochondria (Hare *et al.*, 1980). Finally, subunit 6 and subunit 8 of the ATP synthase (complex V, the so-called proton translocating ATPase) are mitochondrial translation products (Mariottini *et al.*, 1983; Chomyn *et al.*, 1983; Macreadie *et al.*, 1983). With the exception of apocytochrome *b*, little information is currently available concerning the specific function of the mitochondrially-encoded polypeptides within these multisubunit enzyme complexes of the inner membrane. The location in the mtDNA genetic map of the DNA and RNA sequences coding for these proteins is shown in Figure 1.

Regulation of Mitochondrial Gene Expression.

The mapping and sequencing of the mitochondrial RNAs, their alignment with the mtDNA sequence, and the identification of the transcriptional initiation sites have provided a clear understanding of mtDNA transcription in HeLa cells. The analysis of the metabolic properties of the mtDNA transcripts (Cantatore and Attardi, 1980; Gelfand and Attardi, 1981; Attardi *et al.*, 1982; Montoya *et al.*, 1983) and the correlation of these properties with the different transcription events (Montoya *et al.*, 1983) suggest the mechanisms by which the steady-state levels of the mRNAs and rRNAs are regulated. The longer half-life of the rRNAs (2.5 to 3.5 hr), as compared to the half-lives of the various mRNA species (varying between 25 and 90 min), combines with their higher rate of synthesis (15- to 60-fold that of the H-strand-encoded mRNAs) to produce their 25- to 250-fold higher steady-state levels over those of the various mRNA species. In addition, the L-strand is transcribed at a rate two- to three-fold higher than the combined transcription rates of the two H-strand transcription units, but, because of the very short half-lives of these transcripts, they do not accumulate to any significant extent. Thus, transcriptional control combines with posttranscriptional control to regulate the steady-state levels of the rRNAs and mRNAs.

The presence of tRNA genes within each of the three transcription units, which are transcribed at very different rates, led to the question of how tRNA formation is regulated in HeLa cells. We have examined the steady-state levels and metabolic properties of the mitochondrial tRNAs. The steady-state levels of $tRNA^F$ and $tRNA^V$, the two tRNAs encoded in the rRNA transcription unit, are only two to three times higher than the average levels of the other tRNAs transcribed from the H-strand. Pulse labeling experiments indicate that the majority of newly synthesized $tRNA^F$ and $tRNA^V$ do not become mature tRNAs and are rapidly degraded. The tRNA species transcribed from the L-strand are

represented *in HeLa* cell mitochondria at an average level which is approximately 70% of the average level of the H-strand encoded tRNA species. The role of posttranscriptional control in the maintenance of the steady-state levels of the mitochondrial tRNAs and the possible mechanisms by which this occurs are discussed in Chapter 2. The role that the steady-state levels of the tRNAs play in mitochondrial translation is likewise discussed in this chapter. This study has completed the *in vivo* analysis of the steady-state levels and metabolic properties of the three major classes of RNA-rRNA, tRNA, and mRNA, synthesized in HeLa cell mitochondria.

Current experiments are focusing on the molecular mechanisms regulating the different rates of transcription from the three transcriptional initiation sites. In addition, how the initiation of transcription at the H-strand initiation site for rRNA synthesis is coupled to its termination at the 3'-end of the 16S rRNA gene, whereas initiation at the other H-strand initiation site is not associated with termination at this site, is being investigated. Another very active area of research is the role that the nucleus and cytoplasm have in regulating mitochondrial gene expression. Most investigators are using *in vitro* systems to address these questions. Several soluble systems have been developed (Chang and Clayton, 1984; Bogenhagen *et al.*, 1984; Shuey and Attardi, 1985), as well as one that uses isolated mitochondria (Gaines and Attardi, 1984). Unfortunately, no transcription from the downstream H-strand promoter has been detected in any of the soluble systems. Analysis of a series of deletion mutations (Chang and Clayton, 1984; Bogenhagen *et al.*, 1984) and point mutations (Hixson and Clayton, 1985) have defined the sequences required for accurate initiation of transcription *in vitro* from the L-strand promoter (P_L) and the ribosomal H-strand promoter (P_{HR}). Fractionation of these systems indicates that the same components that are required for initiation of transcription of P_L are also

required for initiation from P_{HR}. Furthermore, a minimum of two factors are involved in transcriptional initiation, a nonselective or weakly-selective RNA polymerase and a mitochondrial transcription factor that confers promoter selectivity on the polymerase (Fisher and Clayton, 1985). The binding of this transcription factor to wild-type promoter-containing DNA fragments as well as to a series of mutant promoter-containing DNA fragments has been well characterized (Fisher *et al.*, 1987). All these experiments point towards the conclusion that because P_L is intrinsically a stronger promoter than P_{HR} *in vitro*, the L-strand is transcribed at a higher rate than the H-strand *in vivo*. Although transcriptional termination at the 3'-end of the 16S rRNA has been observed *in vitro* (Christianson and Clayton, 1986), no correlation between initiation and termination has been observed.

Current studies examining the regulation of mitochondrial gene expression *in vivo* are focusing on the role of translational control. The initial results of these experiments indicate that the various mitochondrial polypeptides are synthesized at very different rates (A. Chomyn, personal communication). Up to a ten-fold variation in the individual rates of synthesis are seen. In addition, the polypeptides that are present in the same enzyme complex, appear to be synthesized at similar rates. The relative abundance of the individual mRNA species does not seem to correlate with the rates of synthesis of the corresponding polypeptides. Further experiments exploring the role of translational control in mitochondrial gene expression and the possible mechanisms by which it occurs should prove interesting. In particular, the development of a soluble *in vitro* translation system should advance our understanding of the mechanisms of translational control considerably.

Future Prospects.

In the past ten years, a variety of methods of DNA-mediated nuclear transformation have been developed which have greatly facilitated investigations on the mechanisms and regulation of nuclear gene expression in eukaryotic cells. In contrast, in spite of the numerous attempts, published (Nagley *et al.*, 1985) and unpublished, no success has as yet been reported in DNA-mediated transformation of mitochondria. This failure has considerably hindered the *in vivo* dissection of the mechanisms of replication and expression of the mitochondrial genome and the development of mitochondrial genetics of mammalian cells.

The difficulty of defining the appropriate conditions inside the cell which would allow the penetration of exogenous DNA through the double mitochondrial membrane is probably responsible for the failure mentioned above. A reasonable approach to this problem would therefore be to manipulate mitochondria *in vitro*, and then to introduce the genetically altered mitochondria into an appropriate recipient cell. As a preliminary step in such an approach, the conditions for introducing isolated mitochondria in a viable form into a cell would have to be devised. We have developed a procedure, whereby isolated mitochondria containing mtDNA with a selectable marker are microinjected into and become established in appropriate recipient cells under selective conditions, leading to a rapid, and in some cases apparently complete replacement of the resident mtDNA. The dynamics of mtDNA replacement has provided an insight into the nature of the selection mechanisms involved. These experiments and a discussion of their implications for mitochondrial genetics, in general, are presented in Chapter 3.

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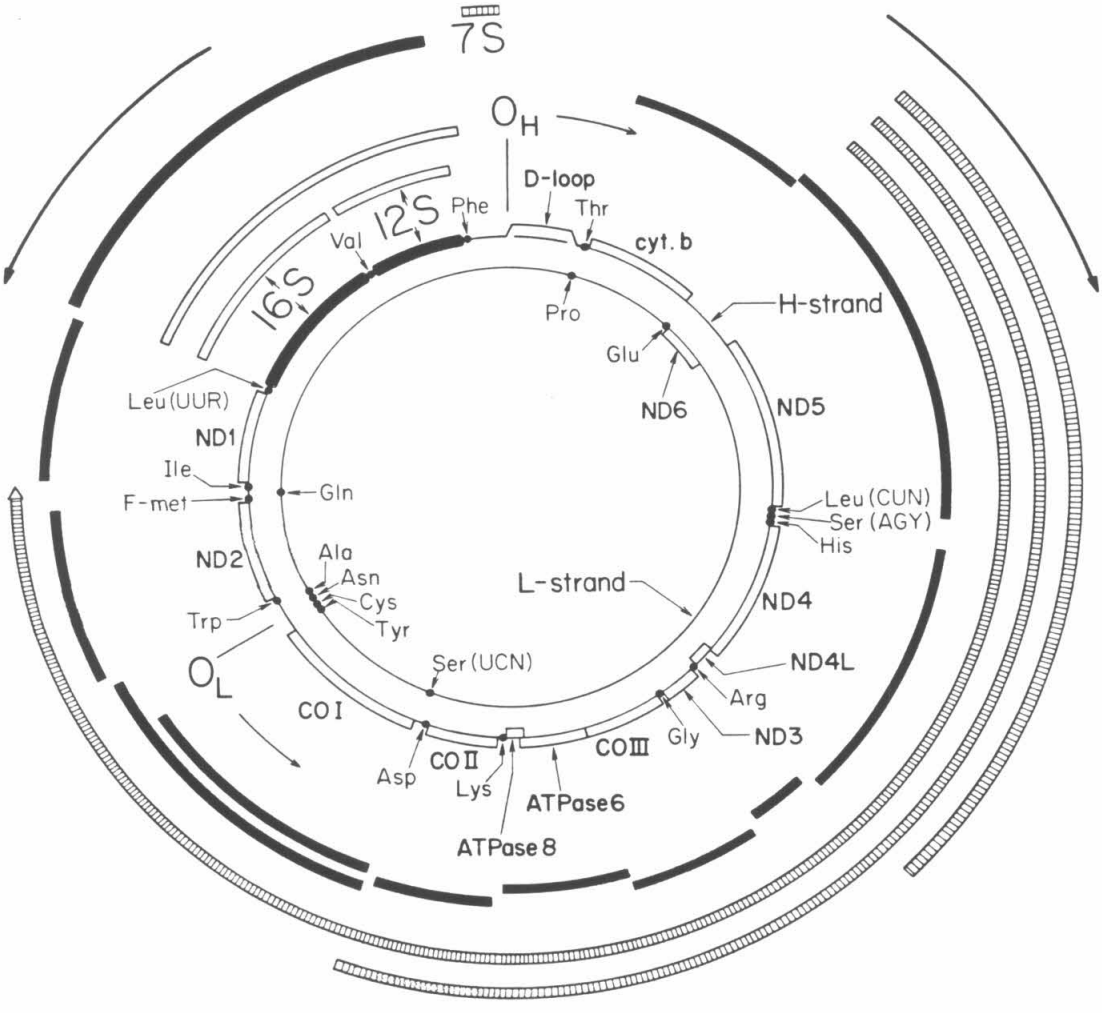
Figure 1. Genetic and transcriptional maps of the HeLa cell mitochondrial genome. The two inner circles show the positions of the two rRNA genes, the reading frames and tRNA genes, as derived from the mtDNA sequence (Anderson *et al.*, 1981). Mapping positions of the oligo(dT)-cellulose-bound and nonbound H-strand transcripts are indicated, respectively, by black and white bars, those of the oligo(dT)-cellulose-bound L-strand transcripts by hatched bars. Left and right arrows indicate the direction of H- and L-strand transcription, respectively. The vertical arrow, marked O_H, and the rightward arrow at the top indicate the location of the origin and the direction of H-strand synthesis; the arrow marked O_L indicates the origin of L-strand synthesis. (COI, COII, and COIII) Subunits I, II, and III of cytochrome *c* oxidase; (ND1, 2, 3, 4, 4L, 5, and 6) NADH dehydrogenase subunits 1, 2, 3, 4, 4L, 5, and 6; (CYTb) apocytochrome *b*; (H^+-ATPase 6 and 8) subunits 6 and 8 of H^+-ATPase.


7S
O_H
D-loop
Phe
Thr
cyt. b
12S
16S
Val
H-strand
Pro
Glu
ND6
ND5
Leu(UUR)
ND1
Ile
F-met
Gln
Leu(CUN)
Ser(AGY)
His
ND2
Ala
Asn
Cys
Tyr
L-strand
ND4
Trp
O_L
Ser(UCN)
ND4L
Arg
COI
COII
COIII
Gly
ND3
Asp
Lys
ATPase6
ATPase 8

CHAPTER 2

Steady-State Levels and Metabolic Properties of the Mitochondrial tRNAs and Their Relationship to Translation in HeLa Cell Mitochondria

Introduction

The analysis of mitochondrial DNA (mtDNA) transcription in HeLa cells has revealed the presence of three transcription units, one in the light (L) strand and two in the heavy (H) strand (Montoya *et al.*, 1982; Yoza and Bogenhagen, 1984). The product of one of the H-strand transcription units is a polycistronic RNA molecule corresponding to nearly the entire length of the H-strand and destined to produce the majority of the transfer RNAs (tRNAs) and messenger RNAs (mRNAs). The other H-strand unit is transcribed at a rate 15 to 60 times higher than the former and includes only the rDNA region. It is responsible for the vast majority of the rRNA formation (Montoya *et al.*, 1983). This transcription unit also codes for two tRNAs, $tRNA^F$ and $tRNA^V$. The L-strand transcription unit is transcribed at an overall rate which is two to three times higher than the combined rates of the H-strand transcription units (Cantatore and Attardi, 1980). Eight tRNA genes are included in this transcription unit. Thus, the human, and in general mammalian mitochondrial tRNA genes are located in three different transcription units, that are transcribed at three very different rates.

In order to analyze the regulation of tRNA formation by the three transcription units, we have examined the steady-state levels and metabolic properties of the tRNAs of HeLa cell mitochondria. We have found that the steady-state levels of $tRNA^F$ and $tRNA^V$ are only two to three times higher than the average of the levels of the other tRNAs transcribed from the H-strand. In addition, pulse labeling experiments have indicated that the majority of newly synthesized $tRNA^F$ and $tRNA^V$ do not become mature tRNAs and are rapidly degraded. The tRNA species transcribed from the L-strand are represented in HeLa cell mitochondria at an average level which is approximately 70% of the average level of the H-strand encoded tRNA species. These results point to the importance of posttranscriptional control in the maintenance of the steady-state

levels of the mitochondrial tRNAs. The possible mechanisms whereby this post-transcriptional control of the abundance of the tRNAs is achieved in human mitochondria are discussed.

2. Materials and Methods

(a) *Cell culture and labeling conditions*

Cell culture and labeling conditions are described by Attardi and Montoya (1983). Briefly, the S3 clonal strain of HeLa cells was grown in suspension in Dulbecco's modified Eagle's phosphate medium (EP) supplemented with 5% calf serum. Long-term labeling of cells with [^{32}P] orthophosphate was carried out in EP with 10^{-3} M cold phosphate and 5% dialyzed calf serum using 4.4 μCi [^{32}P] orthophosphate/ml for 24 or 48 hr. With an initial cell concentration of less than $2x10^5$ cells/ml, cell growth was exponential for the duration of the experiment. Pulse labeling of cells was performed in EP with 5% dialyzed calf serum using 75 μCi [5-^{3}H] uridine/ml for the indicated times. Pulse labeling of long-term [^{32}P]-labeled cells was carried out in EP with 10^{-3} M cold phosphate and 5% dialyzed calf serum in the presence of 4.4 μCi [^{32}P] orthophosphate/ml and 75 μCi [5-^{3}H] uridine/ml. The specific activity of the newly synthesized nucleic acids would be $2.78x10^4$ dpm [^{32}P]/μg of nucleic acid under these labeling conditions.

(b) *Mitochondrial tRNA isolation*

Cells were washed twice in 0.13 M NaCl, 0.005 M KCl, and 0.001 M $MgSO_4$, then once in 0.025 M Tris, pH 7.4, 0.13 M NaCl, 0.005 M KCl, and 0.7 mM Na_2HPO_4. The cells were resuspended in six cell volumes of 0.01 M Tris, pH 6.7, 0.01 M KCl, and 10^{-4} M EDTA. After 2 min, the cells were homogenized with a motor-driven Teflon pestle homogenizer until ~60% of the cells were broken. The homogenate was brought to 0.25 M sucrose and centrifuged at 1,100 × g for 3 min, then at 1,000 × g for 2 min. The supernatant was spun at 12,000 × g for 10 min, the pellet was resuspended well in ~10 cell volumes of 0.01 M Tris, pH 7.0, and 0.25 M sucrose, and the suspension centrifuged at 1,100 × g for 2 min. The supernatant was brought to 0.04 M EDTA, incubated on ice for 20 min and then

centrifuged at 12,000 × g for 10 min. The pellet, resuspended in 10 cell volumes of 0.01 M Tris, pH 7.0, 0.25 M sucrose, 0.01 M EDTA, was incubated on ice for 15 min, and then centrifuged at 12,000 × g for 10 min. The pellet, resuspended in 1 cell volume of 0.01 M Tris, pH 7.4, 0.15 M NaCl, 0.001 M EDTA, 100 μg pronase/ml, was incubated on ice for 5 min, then was brought to 1% SDS and incubated for an additional 30 min at room temperature. The solution was extracted twice with phenol, then ethanol precipitated. The nucleic acids, dissolved in 0.01 M Tris, pH 7.4, 0.15 M NaCl, 0.001 M EDTA, 0.5% SDS, were layered on 15-30% sucrose gradients in the same buffer. Approximately 5-6 OD_{260} units were layered on each 35 ml gradient and centrifuged in a SW27 rotor at 95,000 × g for 12 to 20 h. The gradients were fractionated from the top, and the fractions corresponding to the top 10 ml were pooled and ethanol precipitated. This material was used for two-dimensional analysis of the tRNAs. For hybridization analysis, the same material was electrophoresed on a 5% polyacrylamide, 7 M urea gel. The tRNA region was cut out, and the nucleic acids, isolated by the crush and soak method followed by ethanol precipitation. Residual acrylamide and other contaminants were removed by dissolving the precipitated nucleic acids in 0.2 M NaCl in TE (0.01 M Tris, pH 7.5, 0.001 M EDTA) and passing them through a 0.5 ml Sephadex A-25 column. After thoroughly washing the column, the tRNAs were eluted with 1 M NaCl, 1% SDS in TE, at 60°C. If the material was to be used only for DNA excess hybridizations, this step was omitted. The tRNAs were ethanol precipitated and resuspended in TE. Mitochondrial 12S and 16S rRNAs were isolated by electrophoresis of the appropriate fractions of the sucrose gradients on 1.4% agarose, 5 mM CH_3HgOH gels, and by cutting and eluting the appropriate bands.

(c) *mtDNA and M13 clones of mtDNA*

mtDNA and separated strands of mtDNA were isolated as described previously (Aloni and Attardi, 1971). M13 clones of mtDNA were obtained by insertion of various restriction fragments of mtDNA into M13mp8 and M13mp18 (Messing and Vieira, 1982; Yanisch-Perron *et al.*, 1985). In most cases, the opposite orientation to that of the first set of clones was obtained by directional subcloning into M13mp9 and M13mp19. A list of clones used in this study is given in Table 1, and their location with respect to the mitochondrial genetic map is shown in Figure 1. Single-stranded phage DNA was isolated from CsCl-banded phage as described by Strauss *et al.* (1986).

(d) *RNA-DNA hybridizations*

Separated strands of mtDNA and single-stranded phage DNA from M13 clones of mtDNA were hybridized with labeled RNA in solution. For DNA excess hybridizations, DNA (generally 5-10 μg of phage DNA or 0.1 to 10 μg mtDNA) and RNA samples in TE were heated in Eppendorf tubes at 95°C for 5 min, quickly cooled in ice water and brought to 0.02 M Tris, pH 7.5, 0.4 M NaCl, 0.002 M EDTA in a final volume of 20 to 50 μl. The tubes were incubated under water at 68°C for 16 to 24 hr. There was no detectable change in volume during incubation. The samples were then quickly cooled, diluted with 0.5 ml 2 to 4X SSC (1X = 0.15 M NaCl, 0.015 M Na_3citrate) and incubated with 2 to 30 μg RNAse A and 2 to 30 units RNAse T_1 (both enzymes having been pretreated at 90°C for 15 min) for 30 to 60 min at room temperature. The samples were brought to 10% trichloroacetic acid, 1% pyrophosphate, 1% NaH_2PO_4, and 200 μg BSA/ml, incubated on ice 30 min and filtered through Millipore HA filters (0.45 μm). The background for these hybridizations was determined by hybridizing equivalent amounts of mitochondrial tRNA with an excess of M13mp8 or of M13 clones of

mtDNA not containing a tRNA gene(s). RNA excess hybridizations with separated strands of mtDNA were performed as described by Cantatore and Attardi (1980).

(e) *Two-dimensional gel analysis*

Two-dimensional gel analysis was carried out as described by deBruijn *et al.* (1980). [^{32}P]-labeled tRNAs were visualized by exposure of wet gels to X-ray film. In the case of [^{3}H]-labeled tRNAs, the gels were fixed in 50% methanol, 10% acetic acid, rinsed, then soaked in AutoFluor (National Diagnostics) with 10% glycerol for 30 min, dried at 80°C under vacuum and exposed at -70°C.

3. Results

(a) *Isolation of mitochondrial tRNAs*

Previous procedures developed in this laboratory for the isolation of mitochondrial nucleic acids in pure form have depended upon the use of micrococcal nuclease to degrade the extra-mitochondrial nucleic acids (Attardi & Montoya, 1983). These procedures proved unsuitable for the isolation of mitochondrial tRNAs because of the large amounts of small nucleic acid fragments formed by this nuclease. Therefore in the present work, in order to minimize the contamination of mitochondria by cytoplasmic RNAs and nuclear nucleic acids, the cells were gently homogenized, and the mitochondria, isolated by differential centrifugation, were washed extensively in isotonic solutions containing high concentrations of EDTA. The latter treatment was expected to disrupt the cytoplasmic ribosomes (Attardi *et al.*, 1969), and thus to minimize any cytoplasmic tRNA contamination.

The mitochondrial RNAs of interest were size-selected by sucrose gradient sedimentation. The low molecular weight species were then fractionated by

polyacrylamide gel electrophoresis, as shown in Fig. 2. Lane 1 is an autoradiograph of the low molecular weight RNAs long-term [^{32}P]-labeled *in vivo*, after electrophoresis on a 5% polyacrylamide, 7 M urea gel. Indicated is the region of the gel from which the material was eluted for hybridization analysis. A sample of the same low molecular weight RNAs was electrophoresed on a 20% polyacrylamide, 7 M urea gel, and lane 2 of Fig. 2 shows an autoradiograph of a portion of this gel. In the lower part of this lane, one can see a group of closely migrating bands representing the mitochondrial tRNA species, with sizes ranging from 59 to 75 nucleotides (Anderson *et al.*, 1981). One can also see near the top of this lane, migrating as a doublet band, a small amount of cytoplasmic 5S rRNA. Cytoplasmic tRNAs are generally larger than the mitochondrial tRNAs, and would migrate in an area of the gel extending from a position well above the mitochondrial tRNAs to the area including the top several mitochondrial tRNA species (data not shown; Roe *et al.*, 1981). No bands are detectable in the portion of the gel immediately above the mitochondrial tRNAs, indicating the purity of the mitochondrial tRNA preparation.

The purity of the mitochondrial tRNAs is further demonstrated by the results of hybridization of *in vivo* labeled tRNAs with mtDNA. As shown in Fig. 3(a), when an excess of H-strand or L-strand mtDNA was annealed with the [^{32}P]-labeled mitochondrial tRNA preparation, more than 70% of the labeled material hybridized with the mtDNA. The remaining 30% may represent mitochondrial tRNAs for which the hybridization conditions were not optimal. The tRNAs which are transcribed from the H-strand have on the average only a 35% (G+C) content, with four having less than 30% (G+C), while the L-strand-encoded tRNAs average a 43% (G+C) content. However, the hybridization conditions utilized in the present work (68°C in 0.4 M salt) were optimal for tRNAs of higher (G+C) content (~44%). These conditions were chosen to favor the

melting of the secondary structure of the tRNAs, but they may have been too stringent for the hybridization of the tRNAs with the lowest (G+C) content with the mtDNA. In another study of tRNAs from HeLa cell mitochondria, using a similar procedure to isolate mitochondria but a different methodology to estimate the purity of the tRNAs, it was estimated that less than 10% of the tRNAs isolated represented cytoplasmic tRNA species (Lynch and Attardi, 1976).

The results of mitochondrial tRNA excess hybridizations with H- and L-strands of mtDNA are shown in Figure 3(b). These data demonstrate that there is very little, if any, contamination of the tRNAs by degradation products of other mtDNA-coded RNAs. This result is confirmed by the absence of any hybridization of the tRNAs with M13 clones of mtDNA not containing tRNA genes. The levels of hybridization shown in Figure 3(b) indicate an approximate gene copy number of 12 for the tRNA genes transcribed from the H-strand and 6 for those transcribed from the L-strand. These levels may be below the expected values (14 and 8 genes, respectively, for the H- and L-strand (Anderson *et al.*, 1981)) for the same reason mentioned for the DNA-excess hybridizations, i.e., non-optimal hybridization conditions for all the tRNA species. Furthermore, as will be discussed below, the human mitochondrial tRNAs have a high metabolic stability (half-life >24 hr). Therefore, assuming a complete metabolic stability, after a 48 hr labeling with [^{32}P] orthophosphate (as used in these experiments), the specific activity of the tRNAs would be 20% lower than if there was a complete turnover of the tRNA population (based on a five-fold increase in cell mass during this 48 hr labeling). When this correction is applied to the tRNA saturation values, numbers of 15 tRNA genes transcribed from the H-strand and 7.5 tRNA genes transcribed from the L-strand can be calculated.

Figure 4(a) is an autoradiograph of a two-dimensional separation of the mitochondrial tRNAs long-term labeled with [^{32}P] orthophosphate. The tRNAs

were separated in the first dimension on a 20% polyacrylamide, 7 M urea gel and in the second dimension on a 20% polyacrylamide, 3 M urea gel. Greater than 20 distinct species are distinguishable in this pattern. Ten tRNAs have been unambiguously identified by hybridization analysis of the eluted species with M13 clones of mtDNA containing tRNA genes, and are designated in Figure 4(a). Two additional tRNAs have been tentatively identified based upon their unique migration properties and are marked with asterisks. In particular, $tRNA^{S}_{AGY}$, the smallest mitochondrial tRNA with 59 nucleotides, was recognized from its rapid mobility during elctrophoresis in the first dimension, a separation based primarily on molecular weight. $tRNA^{Q}$, one of the larger tRNAs with 72 nucleotides, was identified by its low mobility during electrophoresis in both the first and second dimension. The low mobility of this tRNA in the second dimension is a result of its strong secondary structure (49% (G+C)), which resists denaturation in 3 M urea. Two other species have been identified as belonging to the L-strand encoded four-tRNA cluster consisting of the $tRNA^{A}$, $tRNA^{N}$, $tRNA^{C}$ and $tRNA^{Y}$ genes. They are labeled X1 and X2. Species which have not been positively identified and presumably represent unidentified mitochondrial tRNAs, are labeled 1 to 5. Several tRNAs are represented by multiple species ($tRNA^{S}_{UCN}$, $tRNA^{Y}$, $tRNA^{T}$, and $tRNA^{R}$). This was seen previously for mitochondrial tRNAs isolated from bovine liver and was attributed to varying degrees of completion of the CCA addition at their 3' terminus (Roe *et al.*, 1981).

(b) *Steady-state levels of mitochondrial tRNAs*

The steady-state level of the mitochondrial tRNAs was determined in experiments in which the mitochondrial tRNAs, labeled for 24 or 48 hr *in vivo* with [^{32}P] orthophosphate, were hybridized with an excess of H- or L-strands of total mtDNA. A value of 6.0×10^{5} molecules/cell, representing the maximum

recovery from more than 10 independent experiments, was obtained using tRNAs labeled for 48 hr. The average ratio of tRNAs transcribed from the H-strand to those transcribed from the L-strand was 3.0, corresponding to 4.5×10^5 molecules of H-strand-encoded tRNAs and 1.5×10^5 molecules of L-strand-encoded tRNAs. The steady-state level of $tRNA^V$, 8.4×10^4 molecules/cell, was calculated from the average fraction of the total tRNAs (0.14) represented by the corresponding spot in several two-dimensional gel fractionations, multiplied by the total number of tRNA molecules/cell and corrected for its length. Hybridization analysis gave a similar result. For each individual tRNA, its relative proportion with respect to $tRNA^V$ was determined in different experiments by DNA excess hybridization of M13 clones of mtDNA containing tRNA genes with the total tRNAs or by quantitation of two-dimensional fractionations of the tRNAs. From the average proportion of individual tRNAs relative to $tRNA^V$, the number of tRNA molecules/cell of each species was calculated (Table 2). Again, corrections for the size variations of the tRNAs were made. Direct comparison of the steady-state levels of the same tRNA species determined by the two methods described above usually yielded very similar results ($tRNA^L_{UUR}$, $tRNA^I$ and $tRNA^M$, $tRNA^S_{UCN}$, $tRNA^G$, and $tRNA^T$). The largest discrepancies occurred with $tRNA^R$ and with $tRNA^F$ and $tRNA^K$, which two-dimensional analysis gave a value of 50%, and respectively, 25% higher than the value determined by hybridization analysis. The most likely explanation of this result for $tRNA^R$ is the low (G+C) content of the $tRNA^R$ gene (23%) and the suboptimal conditions used for hybridization. On the other hand, $tRNA^F$ and $tRNA^K$ have reasonably high (G+C) contents (41% and 33%, respectively). The higher levels for these tRNAs determined by the two-dimensional analysis may indicate the presence of another tRNA species in the corresponding region of the gel. The values in Table 2 are minimum values because no corrections have been made for losses that occur

during the isolation of the tRNAs. In addition, the assumption has been made that the tRNAs are uniformly labeled. If the tRNAs have a half-life longer than the period of labeling (48 hours), up to 20% of the tRNAs would not be labeled.

Analysis of the steady-state amounts of the tRNA species indicates that the levels of $tRNA^F$ ($4.9x10^4$ molecules/cell) and $tRNA^V$ ($8.4x10^4$ molecules/cell), transcribed from the rRNA transcription unit, are 1.9, and respectively, 3.2 times higher than the average value for the other 12 tRNA species transcribed from the H-strand ($2.6x10^4$ molecules/cell). Among these 12 tRNA species there is only a small variation in their steady-state levels, $tRNA^M$ being the most abundant ($3.1x10^4$ molecules/cell), and $tRNA^T$, the least abundant ($1.8x10^4$ molecules/cell). The tRNAs transcribed from the L-strand occur at an average level ($1.9x10^4$ molecules/cell) which is approximately 70% of the average level of the tRNAs transcribed from the H-strand (excluding $tRNA^F$ and $tRNA^V$). Among the tRNAs analyzed, species X1 ($tRNA^A$ or $tRNA^N$ or $tRNA^C$ or $tRNA^Y$) is the most abundant, at $2.2x10^4$ molecules/cell.

(c) *Metabolic Properties of the Mitochondrial tRNAs*

Considering the very different transcriptional rates of the mitochondrial tRNA genes and the relatively uniform steady-state levels of the mature tRNAs, instability of the transcripts undoubtedly plays an important role in the regulation of the steady-state levels of the tRNAs. To investigate at which step this regulation occurs, HeLa cells, long-term labeled with [^{32}P] orthophosphate, were exposed to [5-^{3}H] uridine in the presence of [^{32}P] orthophosphate for short periods of time. The mitochondrial tRNAs were isolated and their [^{3}H] to [^{32}P] ratios were determined by hybridizing them with M13 clones of mtDNA or H- and L-strands of mtDNA. The results of several pulse-labeling experiments are shown in Fig. 5 for the total tRNAs hybridized with the H- and L-strands (panel *a*), as well

as for several individual tRNAs representative of the three transcription units (panel *c*). Figure 5 also shows the kinetics of accumulation of [5-^{3}H] uridine in the total tRNAs hybridized with the separated strands (panel *b*) and in the same individual tRNAs analyzed in panel *c* (panel *d*). As can be seen, the [^{3}H] to [^{32}P] ratios for the tRNAs transcribed from the H- and the L-strands are quite similar. This similarity extends to the individual tRNA species, irrespective of which transcription unit they are transcribed. The [^{3}H) to [^{32}P] ratios, which reflect the turnover rate of the tRNAs, thus demonstrate a very similar half-life for the various species of tRNA. The turnover rate of the tRNAs is less than that seen in the mRNAs or even in the more stable rRNAs (data not shown; Montoya *et al.*, 1983).

The shapes of the curves measuring the [5-^{3}H] uridine labeling of mitochondrial tRNAs (Fig. 5*d*) and the comparison with previous analyses of [5-^{3}H] uridine incorporation into mitochondrial RNA (Attardi and Attardi, 1971; Montoya *et al.*, 1983) strongly suggest that the data for the 10 min pulse are abnormally high, for unknown reasons. It is clear from Fig. 5(d) that the accumulation of label in tRNA^{F} and tRNA^{V} is more rapid than in the other H-strand encoded tRNAs, this difference being particularly evident after the longer pulses. These differences in [^{3}H] labeling are indicative of the higher rate of synthesis of these two tRNAs, and correlate well with their higher steady-state levels. However, it is clear that the rate of labeling of tRNA^{F} and tRNA^{V} is not 15 to 60 times higher than that of the other H-strand-encoded tRNAs, as expected from the relative rates of transcription of the two transcription units from which the two sets of tRNAs derive. In addition, there is no evidence of saturation of labeling of the tRNA^{F} and tRNA^{V} which would be indicative of a rapid turnover rate of the tRNAs.

The points discussed above are also applicable with respect to the tRNAs transcribed from the L-strand. The accumulation of [5-^{3}H] uridine in these tRNAs is marked slower than in tRNA^F and tRNA^V and, in general, also slightly slower than in the other tRNAs transcribed from the H-strand. Thus, there is no evidence of a rapid rate of synthesis, or of a rapid turnover of the tRNAs transcribed from the L-strand that can be correlated with the high rate of transcription of the L-strand and the metabolic instability of the L-strand transcripts (Aloni and Attardi, 1971; Cantatore and Attardi, 1980).

The results described above indicate that, in HeLa cells, all mitochondrial tRNAs irrespective of which transcription unit they derive from, appear to have very similar rates of synthesis and rates of turnover. This conclusion is further substantiated by the analysis shown in Fig. 4(b). This figure is a fluorograph of a two-dimensional gel separation of the mitochondrial tRNAs pulse labeled for 8 min with [5-^{3}H] uridine. In spite of small variations in the relative migration of the spots, which are common in this type of fractionation, the overall pattern of the tRNAs in this gel is very similar to the pattern of the tRNAs long-term labeled with [^{32}P] orthophosphate (Fig. 4a). No additional species seem to be present. As would be expected from the [^{3}H] to [^{32}P] ratios determined in the hybridization experiments, the different [^{3}H]-labeled tRNA species exhibit relative levels of labeling that are quite similar to their steady state levels.

Discussion

Regulation of the steady-state levels of the mitochondrial tRNAs

This is the first analysis of the steady-state levels of tRNAs from mitochondria of any organism. This study has demonstrated that the levels of the different mitochondrial tRNA species in exponentially growing mammalian cells are relatively uniform. This is a remarkable conclusion considering the widely

varying rates at which the corresponding tRNA genes are transcribed. Thus, while transcriptional control plays an important role in the regulation of the steady-state levels of the rRNAs and mRNAs (Gelfand and Attardi, 1981; Attardi *et al.*, 1982), it is clear that posttranscriptional mechanisms must be the predominant means by which the steady-state levels of the tRNAs are regulated.

In mammalian mtDNAs, the mitochondrial tRNAs are transcribed as part of larger polycistronic RNA molecules in which tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and protein-coding sequences. In these polycistronic molecules the tRNA structures are believed to act as recognition signals for the processing enzymes, which make precise endonucleolytic cleavages in the primary transcripts yielding the mature rRNAs, mRNAs and tRNAs. Several enzymatic activities are required for the excision and maturation of the tRNAs. In particular, two endoribonucleases are needed, one to make a cleavage precisely on the 5'-side of each tRNA sequence, and another to make a cleavage on the 3'-side. In addition, since the CCA of the tRNAs is not coded in the DNA, a nucleotidyl transferase is needed to add these nucleotides to the 3'-end of the tRNAs.

The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, starts approximately 20 nucleotides upstream of the $tRNA^F$ gene (Montoya *et al.*, 1983; Chang and Clayton, 1984; Bogenhagen *et al.*, 1984), continues through the 12S rRNA, $tRNA^V$, and 16S rRNA genes and terminates at the 3'-end of the 16S rRNA gene (Fig. 6). The corresponding transcript is processed to yield the mature rRNAs and, because of its very high rate of synthesis (Montoya *et al.*, 1983), is responsible for the bulk of the rRNA formation. Mapping and sequencing of the ends of the rRNAs (Crews and Attardi, 1980; Dubin *et al.*, 1982) and comparison with the DNA sequence (Anderson *et al.*, 1981) have indicated that the endonucleolytic cleavages which

precisely excise $tRNA^V$ also form the 3' end of the 12S rRNA, to which an oligo(A) stretch of variable length is attached and the mature 5'-end of 16S rRNA. CCA addition would complete the maturation of this tRNA. However, the analysis of the metabolic properties of $tRNA^V$ performed in the present work has shown that very little of the newly synthesized $tRNA^V$ becomes mature. Furthermore, there is no indication of a rapid turnover of preexisting $tRNA^V$ molecules. Therefore, the majority of $tRNA^V$ molecules must be specifically degraded as they are excised from the ribosomal RNA transcripts.

One can only speculate how the specific degradation of these nascent tRNA molecules occurs. Conceivably, CCA addition may be needed to stabilize the tRNAs as they are excised from the polycistronic transcripts. However, if CCA addition were a limiting factor in tRNA formation, and if $tRNA^V$ and the other tRNAs were equivalent substrates for the nucleotidyl transferase, a much larger fraction of the nascent $tRNA^V$ would be stabilized by CCA addition than is actually found. The same argument would also exclude any other general tRNA stabilizing factor, such as translation elongation factors, or other proteins that may associate with the tRNAs and help stabilize them. On the other hand, the existence of tRNA stabilizing factors that are specific for the individual species of tRNA and are present in limiting, approximately equivalent amounts for the various tRNAs could explain the results. In fact, a $tRNA^V$-specific factor of this class would prevent the accumulation of newly synthesized $tRNA^V$ and would keep its steady-state level fairly similar to that of the other tRNAs.

An analysis of the tRNA cycle in exponentially growing *Escherichia coli* has indicated that the tRNAs are associated with the corresponding aminoacyl tRNA synthetase, or elongation factor Tu-GTP (EFTu-GTP), or the ribosome (Gouy and Grantham, 1980). Almost no time is spent by the tRNA as a free molecule. The aminoacyl tRNA synthetases are the ideal candidates for being the limiting

factors which stabilize the newly synthesized tRNAs. In *E. coli* the cellular abundance of the different aminoacyl tRNA synthetases is quite similar, with 500 to 800 molecules of each species per genome, depending on the growth rate (Neidhardt *et al.*, 1977). This corresponds to approximately one synthetase for every 7 to 8 tRNA molecules. Under normal growth conditions, there is a strong positive correlation between the rate of synthesis of the aminoacyl tRNA synthetases and the growth rate of the bacteria (Neidhardt *et al.*, 1977). Thus in mitochondria, it is possible that the aminoacyl tRNA synthetases, which are nuclear DNA encoded, are under some general growth related regulation and are not subject to feedback control from the mitochondria. All the synthetases may be present in approximately equal, limiting amounts in the mitochondria. It would appear plausible that if the newly synthesized $tRNA^V$ is not quickly acylated by the valyl tRNA synthetase, it cannot associate with EFTu-GTP and may be rapidly degraded. No other tRNA specific proteins are known.

The endonucleolytic cleavage at the 3'-end of $tRNA^F$ also forms the mature 5'-end of the 12S rRNA. However, $tRNA^F$ must also undergo cleavage at its 5'-end for the removal of the short leader. There is evidence from *in vivo* and *in vitro* experiments (Yoza and Bogenhagen, 1984; Gaines and Attardi, 1984a, 1984b) that suggests that the removal of the leader is a late step in the formation of $tRNA^F$ and 12S rRNA. Thus, this cleavage may not be an obligatory step in the formation of the rRNAs and $tRNA^V$. This may explain the slightly lower rate of labeling and lower steady-state level of $tRNA^F$ as compared to $tRNA^V$. Furthermore, the fact that $tRNA^F$ is not a part of the large polycistronic transcript of the whole H-strand, whereas $tRNA^V$ is, may also contribute to the lower steady-state level of $tRNA^F$ relative to $tRNA^V$. Whether complete processing occurs or not, the evidence is clear that $tRNA^F$ is also specifically degraded as it is excised from the primary transcript.

The relatively low steady-state levels of tRNAs transcribed from the L-strand are also anomalous, when compared with the high rate of transcription of the corresponding genes. The L-strand is transcribed at a rate two- to three-fold higher than the combined transcription rates of the two H-strand transcription units, yet the tRNAs encoded in this strand are present in mitochondria at an average level which is only 70% of the average level of the tRNAs transcribed from the H-strand. It is known that the L-strand transcripts have a much shorter half-life than the H-strand transcripts and do not accumulate to any significant extent (Aloni and Attardi, 1971; Cantatore and Attardi, 1980). It seems a plausible hypothesis that the vast majority of these transcripts decay before any processing occurs, the balance between degradation and processing being governed by the relative activity of the enzymes involved. So, in contrast to the situation with $tRNA^V$ and $tRNA^F$, the tRNA sequences would be degraded before they are excised from the polycistronic RNAs. Thus, it appears possible that two very different mechanisms, degradation of polycistronic transcripts and the stabilizing effect of tRNA synthetases, are used in mammalian mitochondria to maintain the relatively uniform levels of the mitochondrial tRNA species.

Another aspect of the posttranscriptional regulation of the levels of the mature mitochondrial tRNAs is their relative stability. Previous work has shown that the longer half-life of the rRNAs (2.5 to 3.5 hr), as compared to the half-lives of the various mRNA species (varying between 25 and 90 min), combines with their higher rate of synthesis (15- to 60-fold that of the H-strand-encoded mRNAs) to produce their 25- to 250-fold higher steady-state levels over the various mRNA species (Gelfand and Attardi, 1981). As shown in Table 3, the tRNAs are nearly as abundant as the rRNAs (3.4×10^4 molecules/cell, Attardi *et al.*, 1982), on a per individual tRNA species basis. However, their rate of formation is much lower than that of the rRNAs, and approaches the transcription

rate of the large polycistronic transcript of the H-strand. In fact, the number of tRNA molecules synthesized in a cell in a 48 hr period based on a synthesis rate of the non-rDNA region of 1.3 x 10^5 nucleotides per min per cell (Attardi *et al.*, 1982), is 6.4 x 10^5 tRNA molecules, and is essentially the same as that determined experimentally in the present work. These calculations indicate that the tRNAs are very stable, with a half-life certainly greater than 24 hr. This is in agreement with previous observations (Knight, 1969; Zylber and Penman, 1969; Attardi and Attardi, 1971).

Mitochondrial tRNAs and translation

In prokaryotic and eukaryotic organisms, translation of mRNAs is a complex process. In mitochondria, the essential features of the protein-synthesizing machineries found in other organisms have been retained. The mtDNA of mammalian cells codes for all the RNAs required in translation, whereas all the proteins are nuclear DNA encoded. In the nucleo-cytoplasmic compartment, the rRNAs are under independent transcriptional control from the mRNAs and tRNAs, and it is mainly at this level that the relative amounts of the three main RNA classes are regulated. In mitochondria, although transcriptional control does play a role, posttranscriptional mechanisms appear to be of primary importance. Yet, as seen in Table 3, the relative amounts of ribosomes, mRNA molecules, and tRNA molecules found in the cytoplasmic and mitochondrial compartments of HeLa cells are nearly identical.

The levels of individual tRNAs also appear to be regulated in prokaryotic and eukaryotic organisms. A variety of organisms have been found to have strongly nonrandom usage of synonomous codons (for review, see Ikemura, 1985). In many of these organisms, whenever a codon is frequently used, the corresponding tRNA species level is also found to be high. This strong correlation

between the most frequently used synonomous codon and the most prevalent isoaccepting tRNA species has been shown to be true for *E. coli* (Ikemura, 1981), *Salmonella typhimurium* (Ikemura and Ozeki, 1983) and *Saccharomyces cerevisiae* (Ikemura, 1982). However, when this situation is examined in HeLa cell mitochondria, no such correlation is seen. There are two cases where there are two isoaccepting tRNAs (for serine and leucine), and in both cases there is a strong 5 to 7 fold bias for one set of codons over the other. However, the levels of the serine isoaccepting tRNAs are nearly identical. In the case of the leucine isoacceptors, the tRNA for the least prevalent set of codons (UUR) is one of the most abundant tRNAs. Furthermore, the evidence strongly suggests that the other leucine tRNA ($tRNA^{L}_{CUN}$, present in the $tRNA^{L}_{CUN}$, $tRNA^{S}_{AGY}$, $tRNA^{H}$ cluster) occurs at a lower level than $tRNA^{L}_{UUR}$.

A second correlation that has been found in many organisms is between the tRNA levels and the frequency of use of amino acids in cell proteins (see de Boer and Kastelein, 1986 for review). The mitochondria offers an excellent opportunity to examine the possible presence of this correlation, because not only have the levels of most individual tRNAs been determined, but the codon usage of all translated proteins and their relative rates of translation are also known. Figure 6 shows the steady-state level of the individual tRNA species versus the corresponding codon usage in the mitochondrially translated proteins. The codon usage has been corrected for the relative rates of individual polypeptide synthesis, estimated from the labeling of proteins following a 15 min [^{35}S]-methionine pulse (A. Chomyn, personal communication). The data were analyzed by linear regression, and the regression line for all the data points (solid line) and for all the data points except $tRNA^{F}$ and $tRNA^{V}$ (dashed line) are shown in the figure. A high positive slope value, indicative of a strong dependence of tRNA use on its abundance, is not seen in either line. A very low correlation coefficient (r) was

calculated for each regression line. For both lines, r was found to be between -0.1 and 0.1, indicating a very poor fit of the data with the regression lines (i.e., the data cannot be approximated at all by a straight line). Gouy and Grantham (1980) have calculated the average time for the individual translational elongation steps in *E. coli* at physiological concentrations of tRNAs and ribosomes. They find that the tRNA concentration is not limiting for the elongation rate, and that increases in the tRNA to ribosome ratio would not significantly increase the translation rate. In *E. coli*, there are approximately 10 tRNAs/ribosome. Thus, the lack of a strong correlation of tRNA levels with codon usage does not imply less efficient translation in mitochondria.

In the above analysis of the elongation rate, the tRNAs were considered to be present in equal proportions. When the relative steady-state levels of tRNAs have been examined in various species, at most a ten-fold variation between the levels of different tRNAs was seen (Ikemura, 1981, 1982; Ikemura and Ozeki, 1983). For the majority of the tRNAs, the variation was less than three-fold. This variation in the level of individual tRNAs is quite similar to that seen in HeLa cell mitochondria, and is not enough to significantly affect the translation rate. However, if an extreme imbalance is present, detrimental effects on translation would be expected. *In vivo*, an extreme imbalance in aminoacyl-tRNA species produced by amino acid starvation or by a selective inhibition of a specific aminoacylation causes increased misincorporation of amino acids and frameshifting (see review by Cozzone, 1980). Frameshifting has also been observed *in vitro* when individual purified tRNAs were added in excess to the translation reaction (Atkins *et al.*, 1979). Thus, limiting the amounts of $tRNA^F$ and $tRNA^V$ to levels similar to those of the other tRNAs has important implications for mitochondrial translation. If these tRNAs were present at levels more representative of the transcription rates of the corresponding genes, amino

acid misincorporation and frameshifting would be expected in the mitochondrial translation products. The same would be true for the L-strand-encoded tRNAs. Therefore, although one does not see a strong correlation between the steady-state levels of the tRNAs and codon usage in HeLa cell mitochondria, adjustments are made at the time of synthesis in the steady-state tRNA levels to optimize the rate and accuracy of mitochondrial translation.

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Table 1
Human mitochondrial DNA M13 clones

Name/ M13 Vector	Size (bp)	Location[1]	Sites used		Strand[2]
			mtDNA	Vector (if different)	
mp18.KS8.1	476	16134-41	KpnI/SacI		H
mp8.M9	739	1-740	MboI	BamHI	H
mp9.M9	739	1-740	MboI	BamHI	L
mp19.KS8.3	2537	41-2578	KpnI/SacI		H
mp18.KS8.3	2537	41-2578	KpnI/SacI		L
mp19.XS5.51	1247	1194-2441	XbaI/SphI		H
mp18.XS5.51	1247	1194-2441	XbaI/SphI		L
mp18.X9.2	1760	1194-2954	XbaI		H
mp18.X5.5	1760	1194-2954	XbaI		L
mp8.M4	1669	1227-2896	MboI	BamHI	H
mp9.M4	1669	1227-2896	MboI	BamHI	L
mp18.XS6.2	513	2441-2954	XbaI/SphI		H
mp19.XS6.2	513	2441-2954	XbaI/SphI		L
mp18.KR74	1544	2578-4122	KpnI/EcoRI		H
mp19.KR74	1544	2578-4122	KpnI/EcoRI		L
mp18.XB9.4	705	2954-3659	XbaI/BclI	XbaI/BamHI	H
mp8.M11	596	3063-3659	MboI	BamHI	H
mp9.M11	596	3063-3659	MboI	BamHI	L
mp18.BR54	463	3659-4122	BclI/EcoRI	BamHI/EcoRI	H
mp19.BR54	463	3659-4122	BclI/EcoRI	BamHI/EcoRI	L
mp8.HE1	1152	4122-5274	EcoRI		H
mp19.HE2	929	5275-6204	HindIII/EcoRI		H
mp8.HE2	929	5275-6204	HindIII/EcoRI		L
mp18.HE2	929	5275-6204	HindIII/EcoRI		L
mp18.XH5.2	1237	6204-7441	XbaI/HindIII		H
mp8.M7	754	6904-7658	MboI	BamHI	H
mp9.M7	754	6904-7658	MboI	BamHI	L
mp18.XB52	217	7441-7658	XbaI/BclI	XbaI/BamHI	H
mp19.XB52	217	7441-7658	XbaI/BclI	XbaI/BamHI	L
mp19.X5.1	846	7441-8287	XbaI		H
mp18.X5.1	846	7441-8287	XbaI		L
mp19.XB66	629	7658-8287	XbaI/BclI	XbaI/BamHI	H
mp18.XB66	629	7658-8287	XbaI/BclI	XbaI/BamHI	L
mp8.M8	733	7859-8592	MboI	BamHI	H
mp9.M8	733	7859-8592	MboI	BamHI	L
mp18.XB53	305	8287-8592	XbaI/BclI	XbaI/BamHI	H
mp19.XB53	305	8287-8592	XbaI/BclI	XbaI/BamHI	L
mp18.BS51	1056	8592-9648	BclI/SacI	BamHI/SacI	H
mp19.BS51	1056	8592-9648	BclI/SacI	BamHI/SacI	L
mp8.M5	1525	8729-10254	MboI	BamHI	H
mp9.M5	1525	8729-10254	MboI	BamHI	L

Name/ M13 Vector	Size (bp)	Location[1]	Sites used		Strand[2]
			mtDNA	Vector (if different)	
mp8.M3	1668	10254-11922	MboI	BamHI	H
mp9.M3	1668	10254-11922	MboI	BamHI	L
mp19.XH5.31	1424	10257-11681	XbaI/HindIII		H
mp18.XH5.31	1424	10257-11681	XbaI/HindIII		L
mp18.H78	890	11680-12570	HindIII		H
mp8.HE3	890	11680-12570	HindIII		L
mp18.BR55	719	11922-12641	BclI/EcoRI	BamHI/EcoRI	H
mp18.XK76[3]	1097	14956-16053	KpnI/XhoI	KpnI/SalI	H
mp19.XK76[3]	1097	14956-16053	KpnI/XhoI	KpnI/SalI	L
mp8.M6	979	15591-16569	MboI	BamHI	H

[1]Nucleotide numbering according to Anderson *et al.* (1981).

[2]Indicates that the plus strand of the phage DNA corresponds to the indicated strand of mtDNA.

[3]Deletes spontaneously at a very high frequency.

Table 2

Steady-state levels of the mitochondrial tRNAs

(molecules/cell × 10^{-4})

Heavy strand			Light Strand		
tRNA	2D[1]	Hybridization[2]	tRNA	2D[1]	Hybridization[2]
F		4.9	Q	1.0	
K		3.0	A,N,C,Y		6.3
F,K	10.0		X1	2.2	
V	8.4	8.4	X2	1.7	
L_{UUR}	2.8	2.9	S_{UCN}	2.1	1.6
I	2.6		P		1.7
M	3.1				
I,M		5.6	Unidentified		
W		2.9			
D		2.4	1	2.5	
G	2.6	2.8	2	2.9	
R	3.0	1.9	3	4.7	
S_{AGY}	2.0		4	3.5	
L_{CUN},S_{AGY},H		6.1	5	2.8	
T	1.7	2.0			

[1]Determined by quantitation of two-dimensional fractionation of mitochondrial tRNAs as described in the text.

[2]Determined by DNA excess hybridizations of total mitochondrial tRNAs with M13 clones of mtDNA as described in the text.

Table 3

Average Number of ribosomes, mRNA molecules and tRNA molecules in HeLa cell cytoplasm and mitochondria

	Cytoplasmic compartment	Mitochondrial compartment
Ribosomes per cell*	5.6×10^6	3.4×10^4
mRNA molecules per cell	6.4×10^5	7×10^3
tRNA molecules per cell	7.7×10^7	6×10^5
Ribosomes per mRNA molecule	9	5
tRNA molecules per ribosome	14	18
tRNA molecules per mRNA molecule	120	86

The amounts per cell of the three main classes of RNA were derived from data by Darnell (1968) and Murphy and Attardi (1977) for the cytoplasmic compartment, assuming a total RNA content of 30 pg/cell, and from data by Attardi *et al.* (1982) and this paper for the mitochondrial compartment.

*Estimated from rRNA content.

Fig. 1. Map position, with respect to the genetic map of the mitochondrial genome, of the M13 clones of human mtDNA isolated in the present work. The top portion of the Figure shows a linearized genetic map of the human mitochondrial genome (Anderson *et al.*, 1981; Chomyn *et al.*, 1985, 1986). The scale in kilobase pairs (kb) is based on the numbering system of Anderson *et al.* (1981). In the lower half of the Figure, aligned with the genetic map are shown the various fragments of mtDNA that have been cloned in M13 vectors, identified by their symbols (see Table 1). The plus strand of the M13 clones whose symbols appear above the line corresponds to the H-strand of mtDNA, while the plus strand of those whose symbols appears below the line corresponds to the L-strand of mtDNA. COI, COII, COIII: subunits I, II and III of cytochrome *c* oxidase; CYT b: apocytochrome *b*; ATPase 6 and ATPase 8: subunits 6 and 8 of H^+-ATPase, ND1, ND2, ND3, ND4, ND4L, ND5 and ND6: subunits of NADH dehydrogenase.

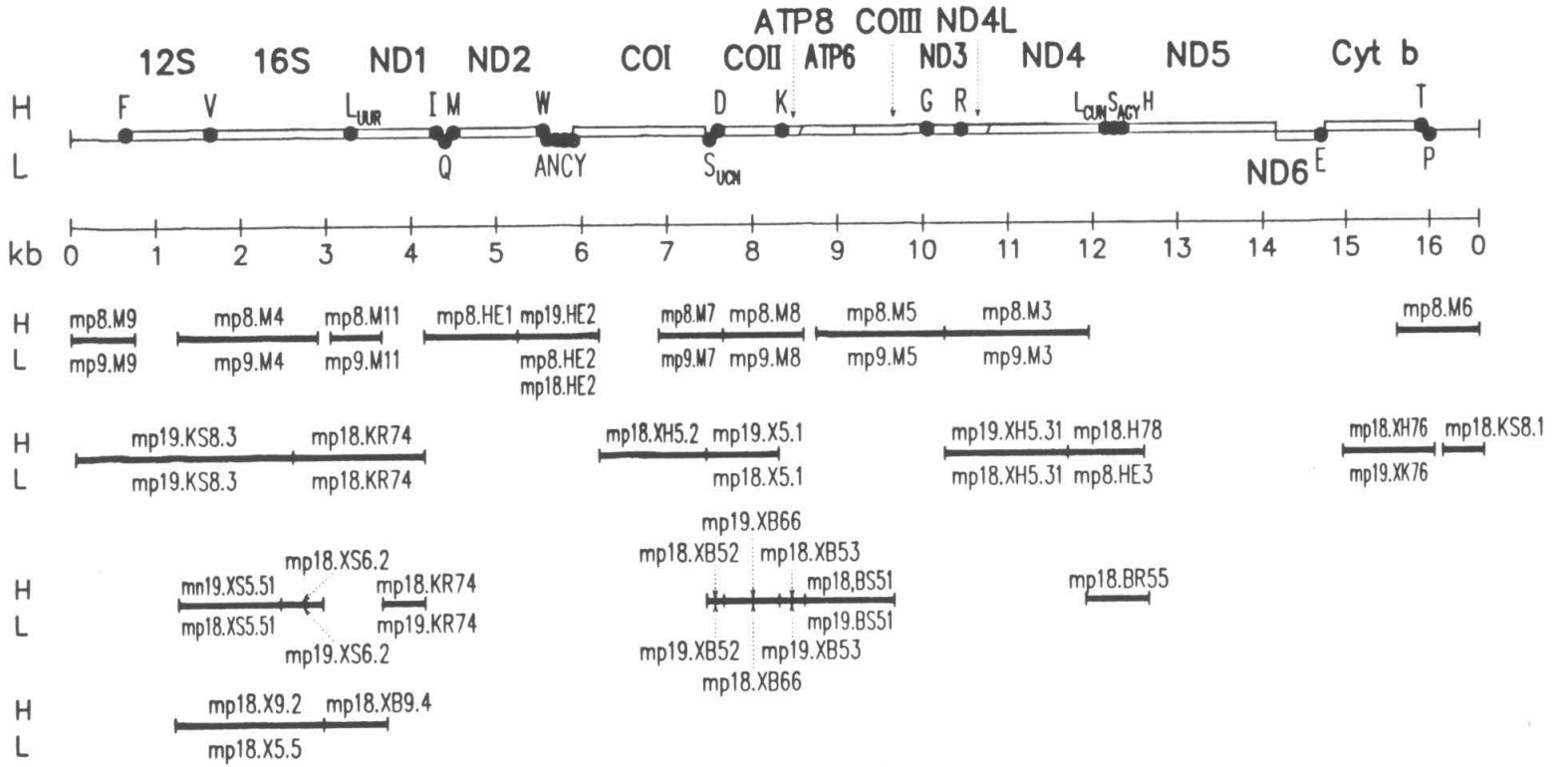

12S
16S
ND1
ND2
COI
COII
ATP8
COIII
ATP6
ND4L
ND3
ND4
ND5
Cyt b
H
L
F
V
L UUR
I M
W
D
K
G
R
L CUN S AGY H
T
Q
ANCY
S UCN
ND6
E
P
kb 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 0
mp8.M9
mp9.M9
mp8.M4
mp9.M4
mp8.M11
mp9.M11
mp8.HE1 mp19.HE2
mp8.HE2
mp18.HE2
mp8.M7 mp8.M8
mp9.M7 mp9.M8
mp8.M5
mp9.M5
mp8.M3
mp9.M3
mp8.M6
mp19.KS8.3
mp18.KR74
mp18.XH5.2 mp19.X5.1
mp18.X5.1
mp19.XH5.31 mp18.H78
mp18.XH5.31 mp8.HE3
mp18.XH76 mp18.KS8.1
mp19.XK76
mp18.XS6.2
mn19.XS5.51
mp18.KR74
mp18.XS5.51
mp19.KR74
mp19.XS6.2
mp19.XB66
mp18.XB52 mp18.XB53
mp18,BS51
mp19.BS51
mp19.XB52 mp19.XB53
mp18.XB66
mp18.BR55
mp18.X9.2 mp18.XB9.4
mp18.X5.5

Fig. 2. Autoradiographs of long-term labeled mitochondrial tRNAs electrophoresed on 5% or 20% polyacrylamide, 7 M urea gels. Low molecular weight RNAs, long-term labeled with [^{32}P] orthophosphate, were size selected by sucrose gradient centrifugation and electrophoresed on either 5% or 20% polyacrylamide, 7 M urea gels. The indicated region of the 5% polyacrylamide gel was cut, and the RNA eluted and subsequently used for hybridization analysis. 5S, cytoplasmic 5S rRNA.


5% 20%
5S
5S
mt
tRNA

Fig. 3. Hybridization of mitochondrial tRNAs with mtDNA. (a) An excess of H-strand (H) or L-strand (L) mtDNA was annealed with a constant amount of purified tRNAs, labeled with [^{32}P] orthophosphate for 48 hr. The percentage of the tRNA samples hybridized with each strand at varying DNA concentrations is plotted. (b) An excess of long-term (48 hr) [^{32}P]-labeled tRNAs were annealed with a constant amount of H-strand (H) or L-strand (L) mtDNA. The relative gene copy number per DNA molecule, at various ratios of tRNAs to mtDNA (weight to weight), is plotted. The relative gene copy number per DNA molecule was calculated from the radioactivity of the tRNA hybridized with the DNA, knowing the specific activity of the RNA (considered to be equal to that of the medium) and the amount of DNA in the hybridization reaction.

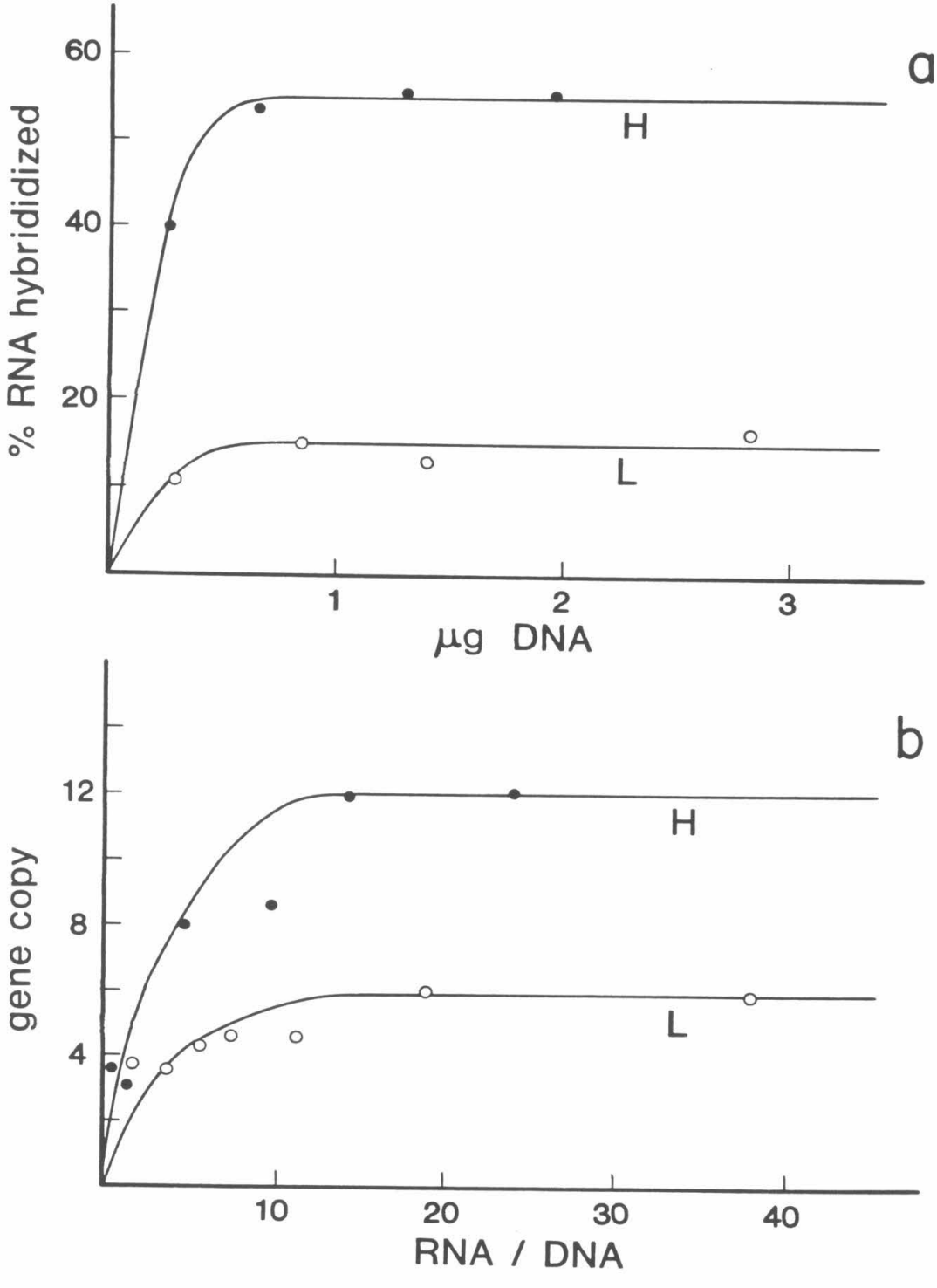

a
% RNA hybridized
60
40
20
H
L
1
2
3
µg DNA
b
gene copy
12
8
4
H
L
10
20
30
40
RNA / DNA

Fig. 4. Two-dimensional polyacrylamide gel fractionation of purified mitochondrial tRNAs. (a) Autoradiograph of long-term (24 hr) [^{32}P]-labeled tRNAs separated by two-dimensional electrophoresis, carried out as described by deBruijn *et al.* (1980). The mitochondrial tRNAs, indicated by their single letter amino acid code, were identified by hybridizing individual eluted tRNA spots with M13 clones of mtDNA. Species X1 and X2 are either $tRNA^A$, $tRNA^N$, $tRNA^C$, or $tRNA^Y$. Species marked with asterisks have been tentatively identified on the basis of their unique migrational properties (see text). Species 1 through 5 are mitochondrial tRNAs which have not been identified. (b) Fluorograph of mitochondrial tRNAs pulse labeled for 8 min with [5-^{3}H] uridine and electrophoresed as in (a). Inset: shorter exposure of the $tRNA^F$ and $tRNA^V$ region of the two-dimensional fractionation pattern.

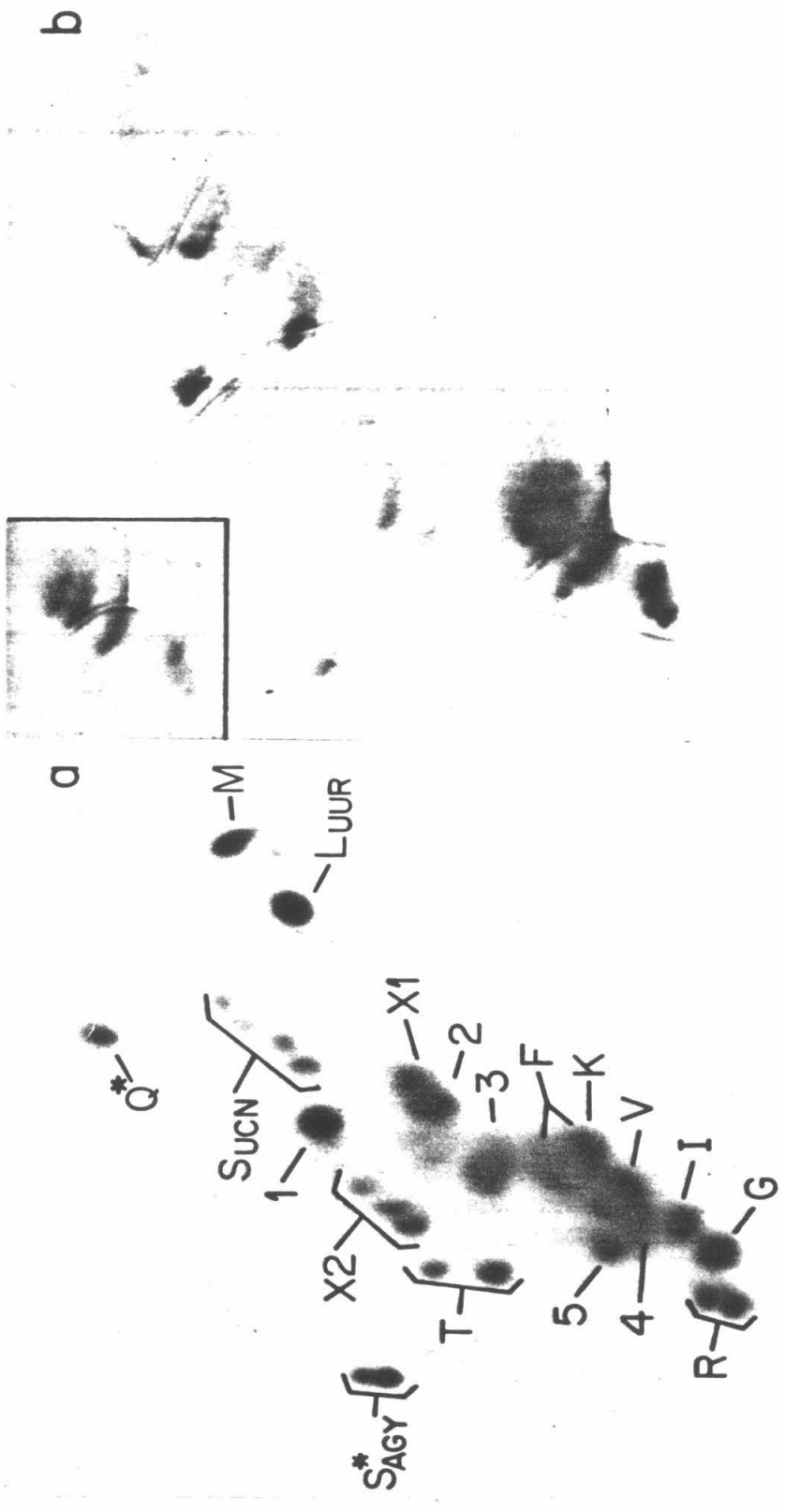

a
b
M
LUUR
Q*
SUCN
1
X2
X1
2
3
F
K
V
I
G
T
5
4
R
S*AGY

Fig. 5. [^3H] to [^{32}P] ratios and [^3H] accumulation in mitochondrial tRNAs isolated from long-term (24 hr) [^{32}P]-labeled HeLa cells exposed for 5, 8, 10, 12 or 15 min to [5-^3H] uridine and hybridized with H-strand and L-strand mtDNA or with individual M13 clones of mtDNA. The [^3H] to [^{32}P] ratios for each time point were determined by hybridizing samples of H- and L-strands of mtDNA (a) or M13 clones of mtDNA (c) with samples of total purified tRNA preparations. These values were then corrected for the different uridine contents of the individual tRNAs. The [^3H] accumulation data for each time point were derived by multiplying the [^3H] to [^{32}P] ratios determined for the total tRNAs encoded in the H- or L-strand (b) or for the individual tRNAs encoded in the M13 cloned mtDNA fragments (d) by the average steady-state levels of the corresponding tRNAs (Table 2). Results are expressed in arbitrary units.

a
b
c
d
H
L
F
V
R
S_UCN
G
ANCY
K
L_UUR
T
L_UUR, S_UCN
3H / 32P
3H Incorporation
Minutes

Fig. 6. Genetic and transcription maps of the human mitochondrial genome. The two inner circles show the positions of the two rRNA genes, the tRNA genes (black circles), and the reading frames. In the outer portion of the diagram, the identified functional RNA species other than tRNAs are represented by black bars [those deriving from the two H-strand transcription units, starting at H1 (rDNA) and H2 (total H-strand)] or cross-hatched bars (those deriving from the L-strand transcription unit). The white bars represent unstable, presumably non-functional by-products. COI, COII, COIII: subunits I, II and III of cytochrome *c* oxidase; CYT b: apocytochrome *b*; ATPase 6 and ATPase 8: subunits 6 and 8 of H^+-ATPase; ND1, ND2, ND3, ND4, ND4L, ND5 and ND6: subunits of NADH dehydrogenase; O_H, O_L: origin of H-strand and, respectively, L-strand synthesis (from Attardi, 1986).

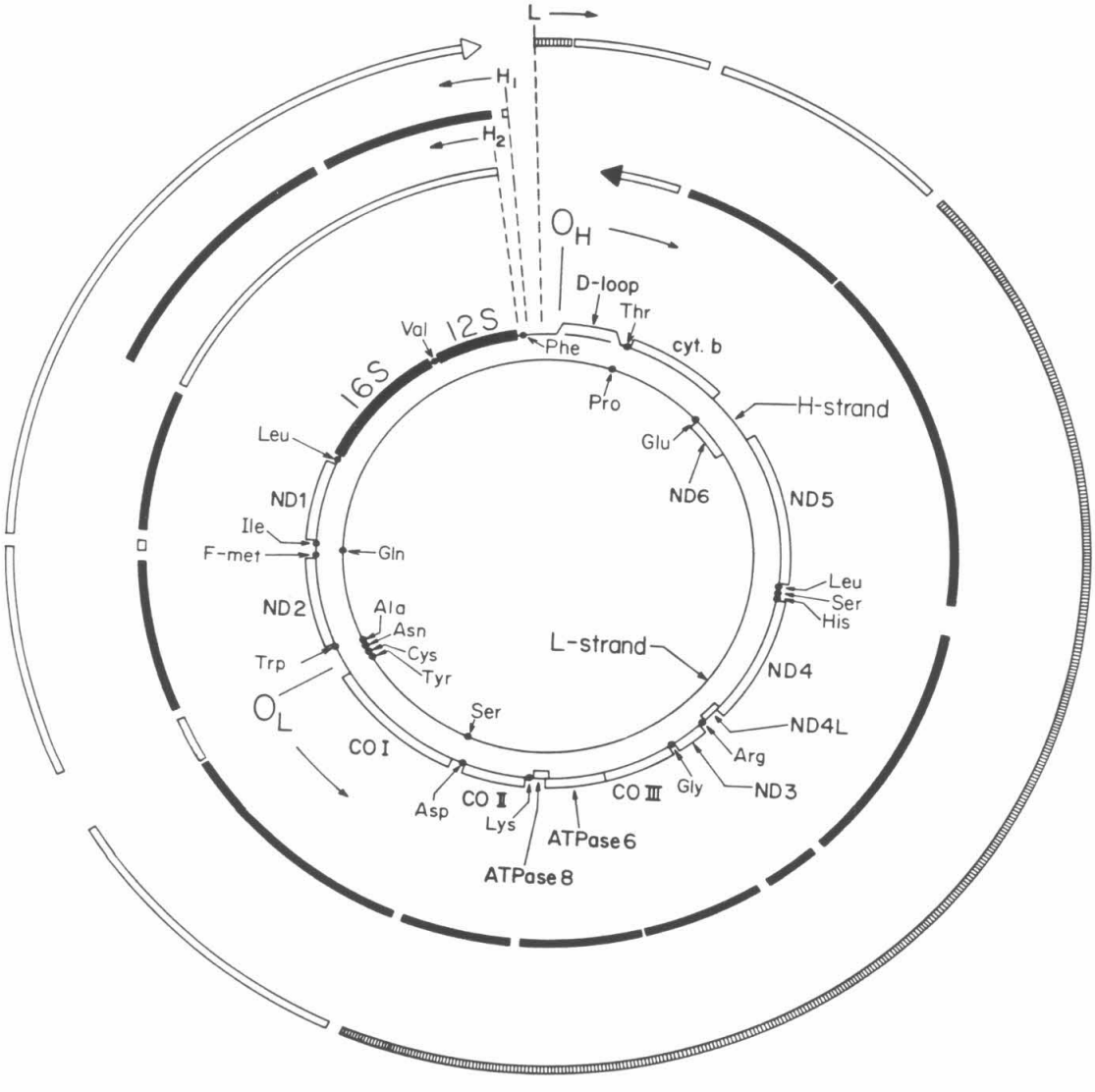
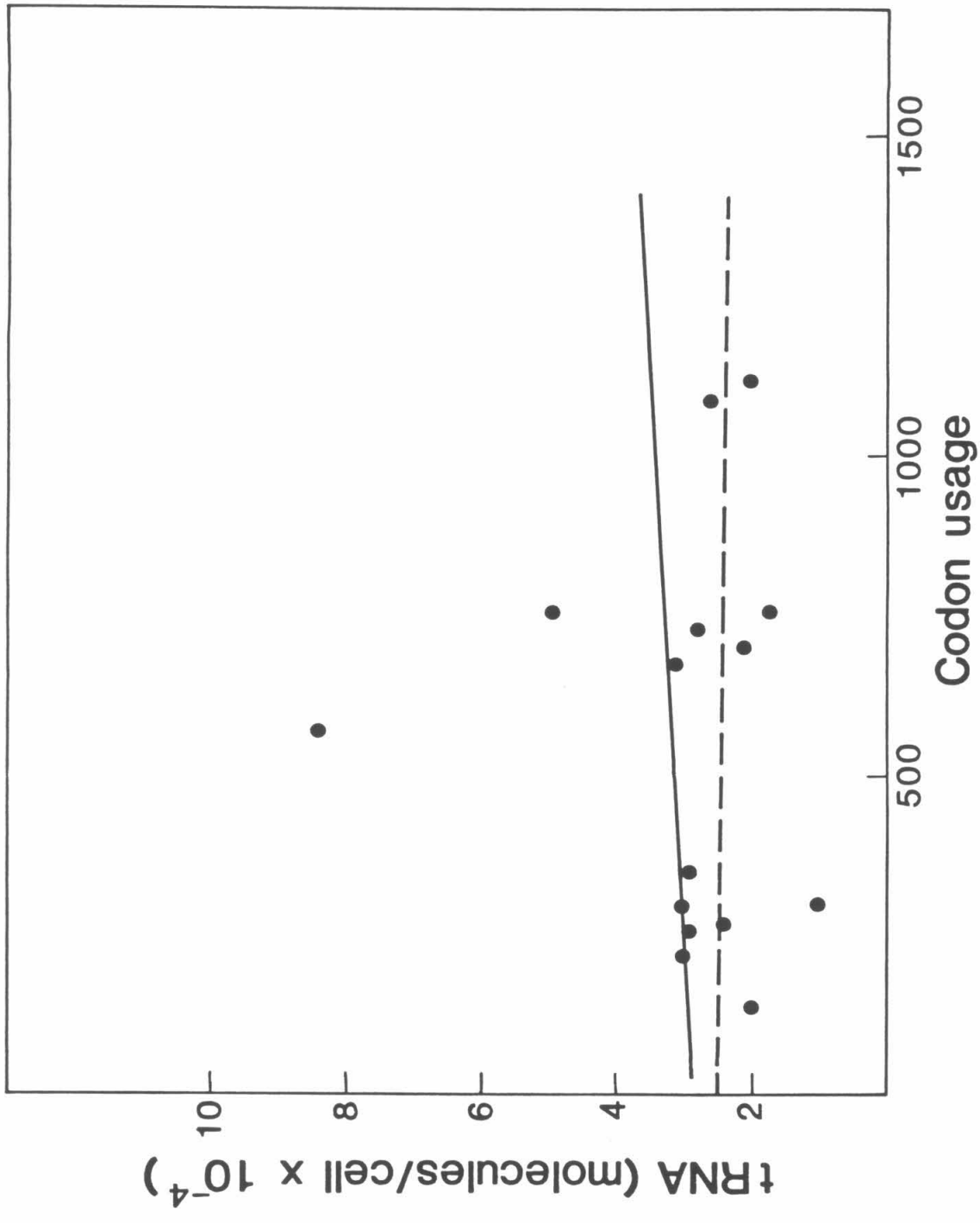

L
H1
H2
OH
D-loop
Thr
Val
12S
Phe
cyt. b
16S
Pro
H-strand
Leu
Glu
ND1
ND6
ND5
Ile
F-met
Gln
Leu
Ser
His
ND2
Ala
Asn
Cys
Tyr
L-strand
ND4
Trp
OL
Ser
ND4L
Arg
CO I
Asp
CO II
Lys
CO III
Gly
ND3
ATPase 6
ATPase 8

Fig. 7. Correlation of tRNA abundance and frequency of its usage. For each identified tRNA, its steady-state level is plotted against its relative codon usage. The codon usage was determined by multiplying the codon usage in each polypeptide (Anderson *et al.*, 1981) by the relative rate of polypeptide synthesis estimated from the labeling of proteins following a 15 min [^{35}S]-methionine pulse. Linear regression analysis of all the data points is shown by the solid line, and for all data points excluding $tRNA^F$ and $tRNA^V$, by the dashed line.


tRNA (molecules/cell x 10^-4)
2
4
6
8
10
500
1000
1500
Codon usage

CHAPTER 3

Injection of Mitochondria into Human Cells Leads to a Rapid Replacement of the Endogenous Mitochondrial DNA

Introduction

Despite numerous attempts, published (Nagley et al., 1985) and unpublished, no success has as yet been reported in DNA-mediated transformation of mitochondria. This failure has considerably hindered the *in vivo* dissection of the mechanisms of replication and expression of the mitochondrial genome and the development of mitochondrial genetics of mammalian cells. The lack of knowledge concerning the mechanisms which maintain the high copy number of mtDNA and the forces which operate in intracellular mtDNA selection has prevented a rational approach to transformation of mitochondria.

We have made significant progress towards this goal by introducing in a viable form genetically marked mitochondria into the cytoplasm of appropriate recipient cells and studying the fate of the exogenous mtDNA. In particular, in the present work, isolated human mitochondria containing a selectable marker in their mtDNA were injected into human cells partially depleted of their mtDNA and became established under selective conditions. Unexpectedly, a rapid, and in some cases, apparently complete replacement of the recipient cell mtDNA was observed in the transformants. The dynamics of mtDNA replacement has provided an insight into the nature of the selection mechanisms involved, with important implications for mitochondrial genetics in general.

Results

Characterization of the CAP23 mutation

CAP23, a VA_2B cell line chloramphenicol (CAP)-resistant derivative previously isolated in this laboratory (Mitchell et al., 1975) and shown to possess a cytoplasmically inherited mutation (Mitchell and Attardi, 1978), was chosen as the mitochondria donor for microinjection. All mutations associated with CAP resistance in mtDNA from mammalian cells and yeast have been located in one

region of the mitochondrial large rRNA gene near its 3'-end (Wallace, 1982). Therefore, in order to determine the sequence change in CAP23 mtDNA, a mtDNA fragment from this variant comprising the region of interest was cloned in M13 and sequenced by the dideoxynucleotide chain termination method.

The DNA sequence revealed a T to C transition at nucleotide 2,991 (Fig. 1*a*), one of three known sites of CAP^R mutations in mammalian cells (Wallace, 1982). This transition creates a recognition sequence for the restriction enzyme Mae II (Fig. 1*a*) making it possible to detect directly the mutated mtDNA molecules and to estimate their proportion relative to the wild-type molecules. In particular, the new MaeII site cleaves a 408 bp MaeII-PvuII fragment into a 339 bp and a 69 bp fragment (Fig. 1*b*), providing two convenient markers for the mutation. Analysis of mtDNA of the CAP23 cell line, which had been maintained for several years in the presence of 40 μg CAP per ml, revealed no detectable CAP^S molecules (<0.4%, as estimated by densitometric analysis of the autoradiograms shown in Fig. 1*c,d,e*).

Transformation of 143BTK$^-$ and HT1080-6TG cells

The first microinjection experiments were carried out using, as recipients, cells of the parental line of CAP23 (VA_2B) and of its 6-thioguanine (6TG)-resistant derivative TG6 (Mitchell and Attardi, 1978). In order to reduce the amount of mtDNA in these cells, and thus to increase the relative proportion of CAP^R mtDNA introduced, the recipient cell population had been treated with 30 ng ethidium bromide (EB)/ml for 3-4 days prior to microinjection. A similar treatment of VA_2B cells with 20 ng of the drug/ml had been previously shown to result in the reversible reduction of the mtDNA content per cell to about 10% of the normal (Wiseman and Attardi, 1978). A few CAP^R colonies developed among the injected cells. On the basis of their frequency (0.5 to 1×10^{-4}) and of the

presence among their mtDNA molecules of a small proportion (3 to 16%) of CAP^S molecules, these clones appeared to be true transformants.

The relatively low frequency of transformation observed with VA_2B and TG6 made the microinjection approach impractical with these cell lines. Furthermore, for the quick identification of the transformants, it was desirable to use recipient cells whose mitochondrial and nuclear DNA could be easily distinguished from the donor counterpart by restriction fragment length polymorphisms (RFLP's). Therefore, other human cell lines unrelated to CAP23 were tested. Among these, the 5-bromodeoxyuridine (BrdU)-resistant $143BTK^-$ and the 6TG-resistant HT1080-6TG (Croce, 1976) proved to be susceptible to high-frequency transformation by CAP23 mitochondria. The drug-resistance markers of these cell lines provided a convenient selection against any possible CAP23 cell in the mitochondrial preparation.

Figure 2 shows the results of experiments carried out to analyze the cloning efficiency and the mtDNA content per cell of 143B and HT1080 after different times of exposure of mass cultures to 50 ng EB/ml. In these experiments, uridine at 50 μg/ml was included in the medium during EB treatment, because of evidence indicating a protection by pyrimidines from the growth-inhibitory effects of EB (Morais et al., 1980; Grégoire et al., 1984). Both the 143B and the HT1080 cells grew at near-to-normal rates in the presence of 50 ng EB/ml for up to 5 days (data not shown). However, the plating efficiency of 143B declined appreciably, dropping to less than 10% of control values after 5 days EB treatment (Fig. 2*a*). The plating efficiency of HT1080 was only moderately affected (Fig. 2*b*). Treatment of 143B cells with EB produced a rapid decrease in the mtDNA content per cell during the first day (Fig. 2*a*); the rate of decrease then tapered off, and the mtDNA content per cell reached a minimum of 3% of the control level after 5 days of cell exposure to the drug. The rate of loss of

mtDNA in HT1980 cells, upon exposure to EB, was somewhat lower (Fig. 2*b*). After transfer to EB-free medium, the mtDNA content per cell of 143B cells treated for 3 days and HT1080 cells treated for 3 or 4 days with the drug remained relatively constant for the first five days, then rapidly increased to normal levels over a period of several days. No such recovery occurred in the presence of CAP (data not shown).

On the basis of the results described above and other results not shown, a pretreatment with 50 ng EB/ml for 3 days and, respectively, 4 days was chosen for the 143B and the HT1080 cell lines. Several microinjection experiments with CAP23 mitochondria were carried out with both cell lines, as detailed in Experimental Procedures and Table 1. After microinjection and one-day incubation in medium without EB, selection for transformants was carried out in Dulbecco's modified Eagle's (DME) medium with 0.9 mg glucose/ml and with 50 and, respectively, 100 μg CAP/ml for the 143B and HT1080 cells. Under these conditions, no spontaneous CAP^R mutant was seen among 4×10^6 cells plated of either cell line. Furthermore, no CAP^R colonies were observed when mitochondrial preparations used for microinjections were plated in large amounts in medium capable of supporting the growth of CAP23 cells. This excluded the possibility that any CAP^R colonies appearing among the injected cells were intact CAP23 cells contaminating the mitochondrial preparation.

Distinct colonies were observed in 19 of a total of 30 experimental plates of 143B cells. In most cases, these colonies were observed 14-17 days post-injection, and the plates that exhibited CAP^R colonies had, in general, more than one. Overall, a minimum of 19 CAP^R transformants, as subsequently confirmed by the DNA analysis (see below), were obtained from an estimated 9.6×10^3 injected 143B cells (Table 1). Dishes containing the presumptive 143B transformant cells were trypsinized and replated in selective medium. During

subsequent expansion of each transformant, the CAP concentration was increased to 100 μg/ml and the glucose concentration raised to the normal level of 4.5 mg/ml.

The HT1080 cells grew to confluence in the area of plating on the petri dishes, making it impossible to distinguish CAP^R colonies. Four weeks post-injection, all experimental plates were trypsinized and replated in selective medium with 200 μg CAP/ml. Within several days, 10 of 14 experimental plates exhibited CAP^R cell growth. Thus, overall, at least ten CAP^R transformants, as verified by the DNA analysis (see below), were obtained from 3.5×10^3 injected HT1080 cells (Table 1). During subsequent expansion of the transformant cell lines, the glucose concentration was raised to 4.5 mg/ml.

DNA analysis of the transformants

Total DNA for early mtDNA analysis was isolated 25-33 days post-injection from the majority of 143B transformants and 35-39 days post-injection for all HT1080 transformants. DNA for nuclear DNA and late mtDNA analysis was isolated several weeks later from representative transformants. All putative transformants revealed the presence in their mtDNA of the MaeII site associated with CAP resistance in CAP23. This is shown in Fig. 3c for 12 representative transformants of the 19 isolated from 143B, and in Fig. 4c for the 10 transformants isolated from HT1080. However, in order to avoid the ambiguities deriving from the possible selection of pre-existing CAP^R mutants in the recipient cell populations, two mtDNA RFLP's independent of the mutation conferring resistance were used to discriminate between CAP23 mtDNA and recipient cell mtDNA and to quantitate their relative amounts. In particular, HaeIII and HaeII polymorphisms proved to be convenient for this purpose. These RFLP's are described in the legends for Figs. 3 and 4.

All presumptive transformants showed both the HaeIII and HaeII markers characteristic of CAP23 mtDNA, but most of them also exhibited the markers characteristic of the mtDNA of the recipient cell line. HaeIII patterns for 12 transformants and HaeII patterns for two representative transformants (#63 and #66) derived from 143B are shown in Fig. 3*a* and 3*b*, respectively. HaeII patterns for the 10 transformants and HaeIII patterns for two representative transformants (#37 and #48) isolated from HT1080 are shown in Fig. 4*a* and 4*b*, respectively.

The proportions of the CAP23 mtDNA and recipient cell mtDNA varied in different transformants; however, it is clear from Fig. 3(*a*,*b*) and 4(*a*,*b*) that the CAP23 mtDNA was always predominant. In several transformants of HT1080 there was no detectable recipient cell mtDNA (#34,#37,#48,#49). The proportions of the CAP23 mtDNA and recipient cell mtDNA were estimated as described in the legend of Table 1, and the results are summarized in the Table. In general, HT1080 transformants exhibited at the time of analysis a substantially lower proportion of CAP^S mtDNA (avg. 2.6%) than did 143B transformants (avg. 21% in 14 analyzed). It seems unlikely that the somewhat longer interval post-injection before DNA was extracted from the HT1080 transformants was responsible for this observation, since the difference in the time of analysis was presumably compensated by the longer generation time of these transformants (see below). Rather, the greater selective pressure placed on the HT1080 transformants (100 μg and 200 μg CAP/ml vs. 50 μg CAP/ml) or their longer pretreatment with EB (4 days vs. 3 days) may have contributed to their different behavior. It should be noted that two 143B transformants examined at a later time post-injection [8 weeks, i.e., at the time of nuclear DNA analysis (see below)] also exhibited no detectable CAP^S mtDNA (estimated <1%, Table 1).

The data on mtDNA polymorphisms indicating the presence of both parental mtDNAs in most CAP^R 143B and HT1080 derivatives exclude the remote

possibility that these derived from occasional CAP23 cells contaminating the mitochondrial preparation which had also escaped killing by the selective medium, or, alternatively, from rare CAP-resistant mutants preexisting in the recipient cell population. Instead, the evidence strongly argues that these CAP^R derivatives were true mitochondrial transformants of the recipient cells. Further support for this conclusion has come from an examination of nuclear DNA polymorphism carried out on transformants that at the same time of analysis appeared to contain exclusively CAP23 mtDNA. As shown in Fig. 3*d* and Fig. 4*d*, transformants 63 and 66 of 143B and transformants 37 and 48 of HT1080 revealed only the pYNH24 variable number tandem repeat nuclear marker (Nakamura et al., 1987) characteristic of the respective recipient cell line.

Phenotypic properties of the transformants

Figures 5 and 6 illustrate the growth properties and drug-resistance phenotype of some representative transformants, as compared to those of the parental lines. In particular, transformants 63 and 66 isolated from 143B and transformants 37 and 48 isolated from HT1080 were chosen for this analysis. Transformants 63 and 66 were resistant to 100 μg BrdU/ml as the 143B recipient cell line, whereas the donor CAP23 cells were very sensitive to this drug (Fig. 5). Similarly, transformants 37 and 48 were resistant to 10 μg 6TG/ml as the HT1080 recipient cells, while the CAP23 cells were totally inhibited in their growth by this drug (Fig. 6).

As concerns the response to CAP, the 143B cells showed only a slight sensitivity of their growth to 100 μg/ml of the drug during the first few days of their exposure to CAP; however, after five days, the cells were all detached from the plate, in contrast to the culture not exposed to CAP, and, after subculturing, the cells were inhibited to a large extent in their growth by the same

concentration of the drug (Fig. 5b). Similarly, the HT1080 cells were only slightly sensitive to 200 μg CAP/ml in the early phase of the growth experiment, becoming almost completely inhibited by the drug after subculturing (Fig. 6b). By contrast, transformants 63 and 66 showed since the beginning of the experiment a nearly complete resistance to 100 μg CAP/ml, which persisted after subculturing (Fig. 5c and 5d); likewise, transformants 37 and 48 showed a high level of resistance to CAP, comparable to that of CAP23 cells, which also persisted after subculturing (Fig. 6c and 6d). The basis for the partial initial resistance of the 143B and HT1080 cell lines to CAP is not known. However, it should be noted that this resistance was lost upon pretreatment of these cells with EB (data not shown).

It is interesting that the generation time of the two 143B transformants analyzed, 63 and 66, in the absence of CAP (~17 hr), was very similar to that of the recipient 143B cells, and significantly shorter than that of the donor CAP23 cells (~22 hr). By contrast, the generation time of the two HT1080 transformants, 37 and 48, in the absence of CAP (20-22 hr), was significantly longer than that of the recipient HT1080 cells (~17 hr) and similar to that of the CAP23 cells. Since, in both types of transformants, the CAP23 mtDNA had completely or almost completely substituted the recipient cell mtDNA, it seems likely that subtle differences in the reciprocal compatibility of the nuclear and mitochondrial genomes played a crucial role in determining the growth rate of the two classes of transformants.

Discussion

The conclusion that the CAP^R derivatives of 143B and HT1080 isolated here represent true mitochondrial transformants is based on four lines of evidence: 1) all the transformants exhibit the same CAP^R mutation of mtDNA as CAP23, as shown by the presence of the MaeII polymorphism; 2) the great

majority or, in some cases, totality of the mtDNA of the transformants has polymorphic markers characteristic of the donor cells, while the totality of nuclear DNA in the same cells exhibit a polymorphic marker characteristic of the recipient cells; 3) the transformants appear with a frequency at least 10^4 higher than CAP^R derivatives in non-injected recipient cells; and 4) the drug-resistance phenotype of the transformants is clearly distinct from that of either the donor or the recipient cells. The apparent frequency of transformation to CAP resistance of the injected 143B and HT1080 cells was 1.9 and, respectively, 2.9×10^{-3}. However, it is likely that the real frequency of transformation was considerably higher, because only one transformant was counted in each positive plate. Although transmission of a mtDNA-dependent phenotype had been obtained previously by injection of mitochondria into the very large cells of *Paramecium* (Knowles, 1974) or into *Neurospora hyphae* (Diacumakos et al., 1965), no injection of mitochondria into mammalian cells in culture had been attempted so far. An acquisition of resistance to mitochondria-specific drugs by mouse cells following uptake by endocytosis of mitochondria from drug-resistant cells has been previously reported, but not documented in molecular terms (Clark and Shay, 1982), and its significance is uncertain. We designate the mammalian cell transformants obtained by microinjection of mitochondria "transmitochondrial cell lines."

Implications for mitochondrial genetics

The analysis of the quantitative behavior of the CAP^R mtDNA molecules introduced into the recipient cells has provided an insight into the selection mechanisms operating in the establishment of the transformant cell lines. In their most advanced stage reached so far, the HT1080 and 143B transformants contain no detectable parental mtDNA. One can estimate that the possible residual CAP^S

mtDNA in some of these transformants corresponds to less than 0.3% of the original complement, or fewer than 40 molecules per cell. The most striking observation, however, is that, in these cell lines, the substantially complete segregation of the CAP^R mtDNA was achieved in a relatively short time, i.e., 5 weeks. Considering the reduced growth rate of the injected cells in the initial phases of their selection and the total number of cells at the time of DNA isolation, this interval corresponds to at most 20-25 generations. From the reported values of tolerance of introduced volume by injected mammalian cells (Cooper et al., 1987, and John Izant, personal communication), we estimate that at most 10-15% of the cell volume was injected into cells which survived. From the mtDNA concentration in the mitochondrial preparations (Table 1) and the recipient cell volume (~4 pl for both cell lines) one can thus calculate that at most 5 to 35 mtDNA molecules, in different experiments (Table 1), were injected per cell. The mtDNA complement of CAP23 cells has been estimated to be ~19,000 (unpublished observations); if the mitochondria content of these cells is similar to that of HeLa cells (Posakony et al., 1977), the injected mtDNA molecules were presumably contained in one mitochondrion. Of the original mtDNA complement of $143BTK^-$ and HT1080-6TG cells [~18,000 and 13,000, respectively (unpublished data)], ~8 and 19% have been found to persist in 143B and, respectively, HT1080 cells after ethidium bromide treatment; thus, the introduced CAP^R mtDNA molecules would represent at most only 1 to 3% of the recipient cell mtDNA population at the time of injection. Therefore, one must explain how such a small fraction of the mtDNA population can segregate in apparently pure form in 20 to 25 generations.

In previous work on the isolation of CAP^R mutants and CAP^R hybrids and cybrids between CAP^R and CAP^S mammalian cell lines, the segregation of the CAP^R molecules was found to be a slow process and often incomplete (Kearsey

and Craig, 1981; Wallace, 1981, 1986; White and Bunn, 1984; Zuckerman et al., 1984). These observations led some investigators to conclude that intermitochondrial selection within a cell, based on selective replication of the CAP^R molecules, does not play a significant role in the evolution of the CAP^R cell lines (Kearsey and Craig, 1981; Wallace, 1981). It was argued that selection at the cellular level, which favors the cells with higher proportion of resistant genomes, combined with the expression of the CAP^R phenotytpe with as little as 11% of the mtDNA in a cell being CAP^R (Wallace, 1986), would be sufficient to account for the experimental observations. However, in the present experiments, it seems very unlikely that a selection at the cell population level by itself would lead to a complete segregation of the CAP^R mtDNA molecules in 20 to 25 cell generations. These data, therefore, strongly suggest that intracellular selection of CAP^R mtDNA molecules played a major role in the establishment of the transmitochondrial cell lines.

Although no mitochondrial protein synthesis product has been shown to be directly required for mtDNA replication, it is a plausible hypothesis that the alteration of the inner mitochondrial membrane structure and/or function resulting from the lack of mitochondrial protein synthesis products may affect the capacity of mtDNA to replicate. Very suggestive, in this respect, is the fact that the great majority of HeLa cell mtDNA molecules appear to be attached to the inner mitochondrial membrane (Albring et al., 1977). In *Saccharomyces cerevisiae*, "petite" formation concomitant with a drastic decrease in mtDNA content has been shown to occur after about 14 generations of growth in the presence of chloramphenicol or erythromycin (Williamson et al., 1971). Furthermore, inactivation of nuclear genes whose products are required for total or specific mitochondrial protein synthesis in *S. cerevisiae* has recently been shown to cause a rapid loss of wild-type mtDNA, strongly supporting the idea that

mitochondrial gene expression is required for the maintenance of mtDNA (Labouesse et al., 1985; Myers et al., 1985).

In the present work, it seems very likely that the decrease in mtDNA content of the recipient cells caused by their pretreatment with ethidium bromide played a major role in the rapid establishment of the injected mitochondria in these cell lines. The resulting increase in the input ratio of CAP^R to CAP^S mtDNA at the beginning of selection was, however, not the most important factor. In fact, in the microinjected cells, this ratio was still far lower than in the cybrids and hybrids previously analyzed. A plausible interpretation of the major effect of EB pretreatment of the cells is that the decrease in the amount of mtDNA below the normal level elicited a compensatory response in the recipient cell, which eliminated the normal constraints limiting the rate of mtDNA replication to an average of one replication cycle per mtDNA molecule per cell generation (Flory and Vinograd, 1973). Such a response, together with the selective replication of the CAP^R mtDNA molecules, resulted in their rapid amplification inside the injected cells. This amplification permitted the cells to rapidly reach a threshold level of functional mtDNA which allowed slow cell growth. At this point, a stringent selection at the cell population level, combined with the replicative advantage of the CAP^R mtDNA, produced its fast and complete segregation.

In the present experiments, a stringent selection was probably provided by the lack of pyrimidines in the growth medium due to the use of extensively dialyzed calf serum. It has been shown that uridine concentrations, as found in the serum of several mammalian species [2-10 μM (Karle et al., 1980)], can satisfy the cellular requirements for pyrimidines in cultured mouse cells in which ***de novo*** biosynthesis is blocked (Karle et al., 1984). Furthermore, previous work has shown that exogenous pyrimidines can protect avian cells from the growth inhibitory

effects of CAP or EB (Morais et al., 1980; Grégoire et al., 1984). This result has recently been extended to human cells treated with EB (unpublished observations). Thus, it would be expected that, in the presence of CAP, cells with a partial complement of functional (i.e., CAP^R) mitochondria would be more growth restricted by the lack of pyrimidines than cells with a full complement.

Since the selection of mutant mtDNA molecules at the mitochondrial level is most important in the early stages of establishment, this selection probably played a minor role in the hybrids and cybrids previously analyzed, where large numbers of CAP^R mitochondria were present from the beginning. Furthermore, it seems possible that the slow and incomplete loss of CAP^S mtDNA molecules in those experiments was due to the less stringent selection conditions applied because of the use of nondialyzed serum.

The analysis of the mtDNA polymorphisms of the transformants in the present work did not provide any evidence of recombination taking place between the CAP^R and CAP^S mtDNAs during the establishment of the new CAP^R cell lines, even though the examination of several polymorphic sites at widely separated positions in the mtDNA molecule increased the probability of detection of recombinant molecules. Therefore, one can conclude that, if there was any mtDNA recombination in these cell lines, this must have been a very rare event. This is in agreement with observations made on hybrids and cybrids (Hayashi et al., 1985; Zuckerman et al., 1984).

The methodology described in this paper can in principle be extended to the introduction into cells of mitochondria containing mtDNA with other selectable markers. The possibility of rebuilding the normal mtDNA complement of a cell from a few injected functional copies offers the opportunity of using this system for studying the factors, genetic and functional, that control the copy number of mtDNA and the mechanisms involved. More importantly, the possibility of

introducing with a high efficiency isolated mitochondria into cells opens the way for mtDNA-mediated transformation of cells by injection of mitochondria which have been manipulated *in vitro* in their genetic material.

Experimental Procedures

Cell lines and media

Dulbecco's modified Eagles medium (DME) with 4.5 mg glucose/ml was the basic medium for growing all cell lines in petri dishes. VA_2B cells were grown in DME supplemented with 10% calf serum and 3 µg 8-azaguanine (8AG)/ml; CAP23 cells in DME with 10% calf serum, 3 µg 8AG, and 40 µg chloramphenicol (CAP)/ml; VA_2B-TG6 cells in DME supplemented with 10% calf serum and 5 µg 6-thioguanine (6TG)/ml. Selection of CAP^R transformants was carried out in DME with 0.9 mg glucose/ml, 10% dialyzed calf serum, and 50 µg CAP/ml; in addition, 3 µg 8AG/ml and 5 µg 6TG/ml were added to the medium for VA_2B and, respectively, VA_2B-TG6 cells. $143BTK^-$ cells were grown in DME supplemented with 5% fetal calf serum and 100 µg 5-bromodeoxyuridine (BrdU)/ml. Selection of CAP^R transformants was carried out in DME with 0.9 mg glucose/ml, 5% dialyzed fetal calf serum, 100 µg BrdU/ml, and 50 µg CAP/ml. HT1080-6TG cells were grown in DME supplemented with 10% fetal calf serum and 10 µg 6TG/ml. CAP^R transformants were selected in DME with 0.9 mg glucose/ml, 10% dialyzed fetal calf serum, 10 µg 6TG/ml, and 100 µg CAP/ml.

CAP23 cells adapted for growth in spinner cultures were used for isolation of mitochondria. They were grown in Dulbecco's modified Eagle's phosphate medium supplemented with 10% calf serum, 3 µg 8AG/ml, and 40 µg CAP/ml.

Preparation of mitochondrial suspension

The mitochondrial suspension was prepared from 2-3 ml packed CAP23 cells, washed twice in 130 mM NaCl, 5 mM KCl, and 1 mM $MgSO_4$. The cells were resuspended in 18 ml 10 mM Tris, pH 7.0, 10 mM KCl, 1.5×10^{-4}M $MgSO_4$, allowed to swell 2 minutes, and then homogenized with a Teflon pestle homogenizer until approximately 60-70% of the cells were broken. The homogenate was brought to 0.25 M sucrose and centrifuged twice for 3 min at 1,500 × g. The supernatant was then centrifuged 10 min at 5,900 × g, and the pellet, resuspended in 3-4 ml 75 mM KCl, 10 mM phosphate buffer, pH 7.0, or 150 mM KCl, 10 mM phosphate buffer, pH 7.1, was centrifuged twice for 3.5 min at 3,700 × g. The supernatant was then used for microinjection.

Microinjection

VA_2B and VA_2B-TG6 transformants were obtained by injecting a mitochondrial suspension from CAP23 cells into cells which had been pretreated with 30 ng ethidium bromide (EB)/ml for 2-3 days. Samples of ~1,000 recipient cells in 0.1 ml DME with 10% calf serum and 30 ng EB/ml were plated within a 5-8 mm glass ring on 6 cm petri dishes. Twenty-four hours later, just prior to injection, the ring was removed and 6 ml of fresh medium without EB was added to each plate. 143BTK$^-$ and HT1080-6TG cells were prepared for microinjection in the same manner, except that they were pretreated for a total of 3 days and, respectively, 4 days with 50 ng EB/ml and 50 µg uridine/ml. The microinjection system was essentially as described by Capecchi (1980). Typical tip diameters were 1 µ or less. After injection, the cells were allowed to recover for 24 h in DME with 10% calf serum, and then exposed to the selective medium.

DNA analysis

Total DNA was prepared from cells by the method of Davis et al. (1980). Restriction digests of total DNA were electrophoresed as described in the Figure legends, and the DNA was electrophoretically transferred to Zeta-Probe membranes (BioRad), alkaline fixed, and hybridized according to the recommendations of the manufacturer. mtDNA probes cloned in M13 vectors were labeled by extension of the universal primer, as described by Sucov et al. (1987). Nuclear DNA probes and total mtDNA were labeled by extension of random hexanucleotide primers (Feinberg and Vogelstein, 1983).

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Table 1. Summary of microinjection experiments

Expt.	Cell line	Mitoch. preparation DNA[a] ng/ml	Mitoch. preparation Protein[b] mg/ml	No. of plates injected	No. of positive plates	No. of cells injected	Transform. frequency ($x10^{-3}$)	% CAP^S mtDNA[c]
1	HT1080	750	1.28	3	2	600	3.3	34 (<0.3, 39d) 37 (<0.3, 39d; <0.3, 68d)
2	HT1080	250	0.47	6	5	1,600	3.1	41 (1.7, 36d); 42 (1.1, 36d) 43 (5.9, 36d) 44 (4.3, 36d) 48 (<0.4, 36d; <0.3, 65d)
3	HT1080	200	0.55	5	3	1,300	2.3	49 (<0.4, 35d); 53 (10.9, 35d) 55 (0.5, 35d)
4	143B	1,200	0.90	5	3	1,800	1.7	57 (9.9, 29d); 60 (33.0, 33d) 63 (7.4, 29d; <1.0, 56d)
5	143B	1,000	0.93	5	4	1,800	2.2	66 (15.8, 28d; <1.0; 55d)
6	143B	1,500	1.03	5	5	1,900	2.6	73 (14.3, 25d); 74 (26.5, 25d) 78 (38.2, 25d); 79 (8.7, 29d) 80 (29.6, 25d)
7	143B	1,350	1.16	6	1	1,200[d]	0.8	
8	143B	950	1.26	4	2	1,200[d]	1.7	92 (29.5, 26d) 94 (11.2, 32d)
9	143B	700	4.40	5	4	1,700[d]	2.4	102 (37.3, 25d); 103 (20.9, 25d) 104 (12.2, 25d)

[a]Determined by dot blot analysis using known quantities of purified HeLa cell mtDNA as standards and probes.
[b]Determined by the method of Bradford (1976).
[c]In the HT1080 transformants analyzed, designated by their number, the CAP^S mtDNA was determined from the proportion of the 8,648 bp HaeII fragment relative to the sum of the 8,648, 2,200 and 1,900 bp HaeII fragments, as measured by densitometric analysis of appropriate exposures of the autoradiograms, and corrected for the length of the hybridized segments. In the 143B transformants, it was determined from the proportion of the 711 and 573 bp HaeIII fragments to the sum of the 1,284, 711 and 573 bp HaeIII fragments. An LKB Ultroscan XL laser densitometer was used. For each transformant, the time of analysis in days (d) is indicated. The underlined samples were used for both nuclear DNA (Figs. 3 and 4) and mtDNA analysis, the others only for mtDNA analysis.
[d]Estimated.

Fig. 1. Molecular characterization and quantitation of the CAP^R mutation in CAP23 mtDNA.

(*a*) The 529 bp PvuII-EcoRV fragment [2,653-3,181 (Anderson, et al., 1981)] of CAP23 mtDNA was cloned in M13mp18 and sequenced by the dideoxynucleotide chain-termination method (Strauss et al., 1986). A portion of the sequence containing the CAP^R mutation is aligned with the deduced sequence of VA_2B mtDNA. (*b*) The MaeII (M) and PvuII (P) sites in the mtDNA region surrounding the CAP^R mutation are shown to illustrate the new fragments produced by the mutation. The new MaeII site is denoted M^*. Nucleotide numbering is according to Anderson et al. (1981). (*c,d,e*) MaeII-PvuII digests of total DNA from VA_2B and CAP23 cells were electrophoresed in 5% polyacrylamide gels, electrophoretically transferred to a Zeta-Probe membrane (BioRad), and hybridized with the mtDNA probes mp18.KR74, the 1,544 bp KpnI-EcoRI fragment (2,578-4,122) inserted into the KpnI and EcoRI sites of M13mp18, and mp18.X5.1, the 846 bp XbaI fragment (7,441-8,287) inserted into the XbaI site of M13mp18. mp18.X5.1 hybridizes to the 63 and 58 bp MaeII fragments which serve as a reference for the identification of the 69 bp fragment.

a
----CGACCTCGATGTTGGATCA---- VA2-B
----CGACCTCGACGTTGGATCA---- CAP 23
Mae II
3000
b
P
M*
M
M
2.5
3
3.5
kb
16S rRNA
LI
ND1
360
408
339
69
c
VA2B
CAP23
408
360
339
d
VA2B
CAP23
408
360
339
e
60
63
50

Fig. 2. Cloning efficiency and mtDNA content of 143BTK$^-$ (a) and HT1080-6TG cells (b) during exposure to EB.

(*a*) Samples of 5×10^4 143BTK$^-$ cells were grown for different lengths of time in the presence of 50 ng EB/ml and 50 μg uridine/ml. Cells treated for 0, 72, 96, and 120 h with EB were plated in duplicate at 500 cells/plate, and colonies were counted approximately two weeks later. In a similar experiment, total DNA was extracted from cell samples treated for different times with EB, digested with HindIII, electrophoresed on 1% agarose gels, electroblotted, and hybridized with labeled HeLa cell mtDNA; DNA quantitation was carried out by densitometry of autoradiograms. (*b*) A series of experiments similar to that described above was carried out with HT1080-6TG cells. Analysis of mtDNA was carried out on EcoRI digests of the total DNA samples, as described in (*a*).

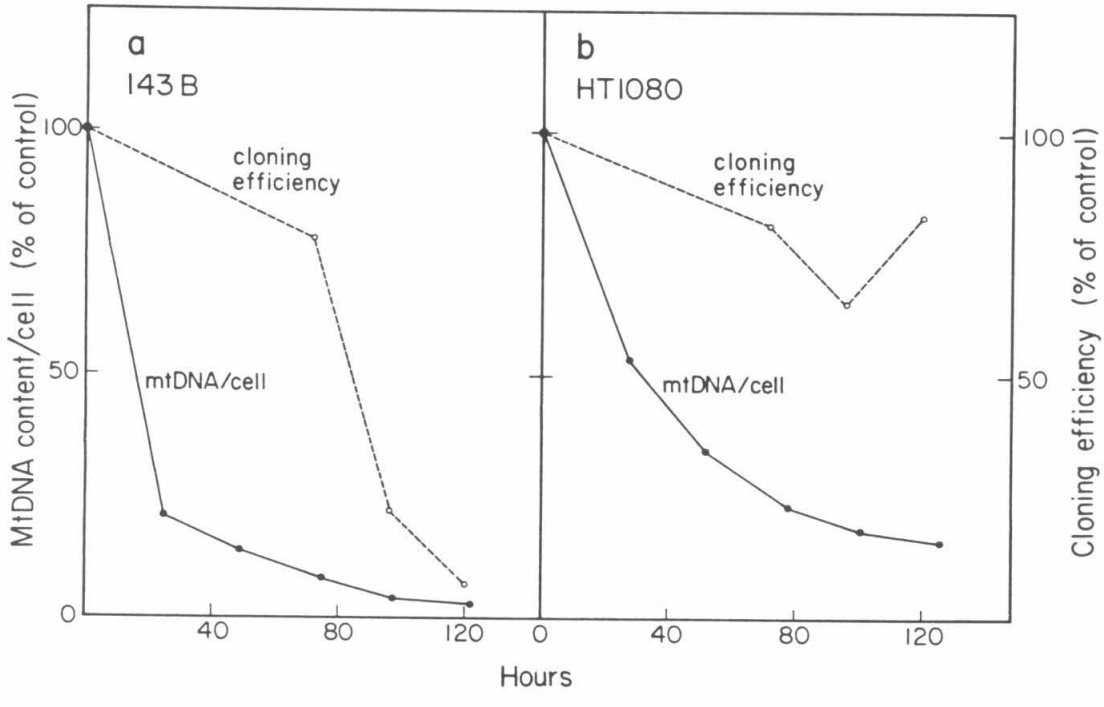

a
143B
b
HT1080
cloning efficiency
mtDNA/cell
cloning efficiency
mtDNA/cell
MtDNA content/cell (% of control)
Cloning efficiency (% of control)
100
50
0
40
80
120
0
40
80
120
Hours

Fig. 3. The 143BTK$^-$ transformants contain CAP23 mtDNA and 143BTK$^-$ nuclear DNA. Autoradiograms of blots of restriction digests of total DNA from various transformants of 143BTK$^-$ cells and from the parental 143BTK$^-$ and CAP23 cell lines, hybridized with mtDNA (*a,b,c*) and nuclear DNA (*d*) probes.

The 143BTK$^-$ transformants were obtained by injecting mitochondria into cells which had been pretreated for 3 days with 50 ng EB/ml, 50 μg uridine/ml. After expansion, total DNA was isolated from the transformants at the times indicated in Table 1, and samples of these DNAs as well as of the parental DNAs from approximately equivalent numbers of cells were digested with HaeIII (*a*), or HaeII (*b*), or MaeII (*c*), or MspI (*d*), and, after electrophoresis in 2% (*a*) or 1.2% (*b, d*) agarose or 5% polyacrylamide (*c*) gels, were electroblotted and hybridized. The probes used for (*a*) were mp8.M9 and mp8.M6, containing, respectively, the 739 bp (1-739) and 979 bp (15,591-16,569) Mbo 1 fragments inserted into the Bam HI site of M13mp8. The blot in (*b*) was hybridized with total mtDNA, that in (*c*) was hybridized with mp18.KR74 and mp18.X5.1, described in the legend of Fig. 1, and that in (*d*) was hybridized with the nuclear variable number tandem repeat marker pYNH24 (Nakamura et al., 1987). The numbers at the top of the gel indicate the various transformants. In (*a*) and (*b*), the black arrows indicate the polymorphic markers characteristic of CAP23 mtDNA, the white arrows, the markers characteristic of 143BTK$^-$ mtDNA. In *c*), the numbers indicate the sizes in bp of the MaeII fragments shown. The HaeIII restriction fragment length polymorphism (RFLP) used here is the absence of a HaeIII restriction site at 15,884 in CAP23 mtDNA, that is present in 143B. Common restriction sites are at 15,173, 16,457, 323, and 1,464. Thus, CAP23 exhibits a 1,284 bp fragment, while 143B has 711 and 573 bp fragments (a). The HaeII RFLP is the presence of a HaeII site at approximately 11,000 in CAP23, that is absent in 143B. Common restriction sites

are at 4,533, 9,506, and 13,181. This is seen as the loss of the 2,200 and 1,900 bp fragments and presence of a 4,125 bp fragment in 143B (b).

a
CAP23
60
79
92
94
102
103
104
143B
Hae III
CAP23
57
63
66
73
74
143B
b
Hae II
143B
CAP23
63
66
c
Mae II
69-
63-
58-
d
pYNH24

Fig. 4. The HT1080-6TG transformants contain CAP23 mtDNA and HT1080-6TG nuclear DNA. Autoradiograms of blots of restriction digests of total DNA from various transformants of HT1080-6TG and from the parental HT1080-6TG and CAP23 cell lines, hybridized with mtDNA (*a*,*b*,*c*) and nuclear DNA (*d*) probes.

HT1080-6TG transformants were obtained by injecting cells which had been pretreated for 4 days with 50 ng EB/ml, 50 μg uridine/ml. Conditions of electrophoresis, transfer and hybridization of restriction digests, probes and symbols are described in the legend of Fig. 3. HT1080 mtDNA, like 143B mtDNA, lacks a HaeII site at ~11,000 present in CAP23 mtDNA, and in addition lacks a HaeII site at 9053: thus, the 4,523, 2,200 and 1,900 bp fragments of CAP23 are converted in HT1080 to a 8,648 bp fragment (a). HT1080 mtDNA, like 143B mtDNA, has a HaeIII site at 15,884, absent in CAP23 mtDNA, which produces 711 and 573 bp fragments; in addition, the loss of a HaeIII site at 16,517 creates a 435 bp fragment as compared to 375 bp in CAP23 and 143B. The MaeII RFLP is described in the text.

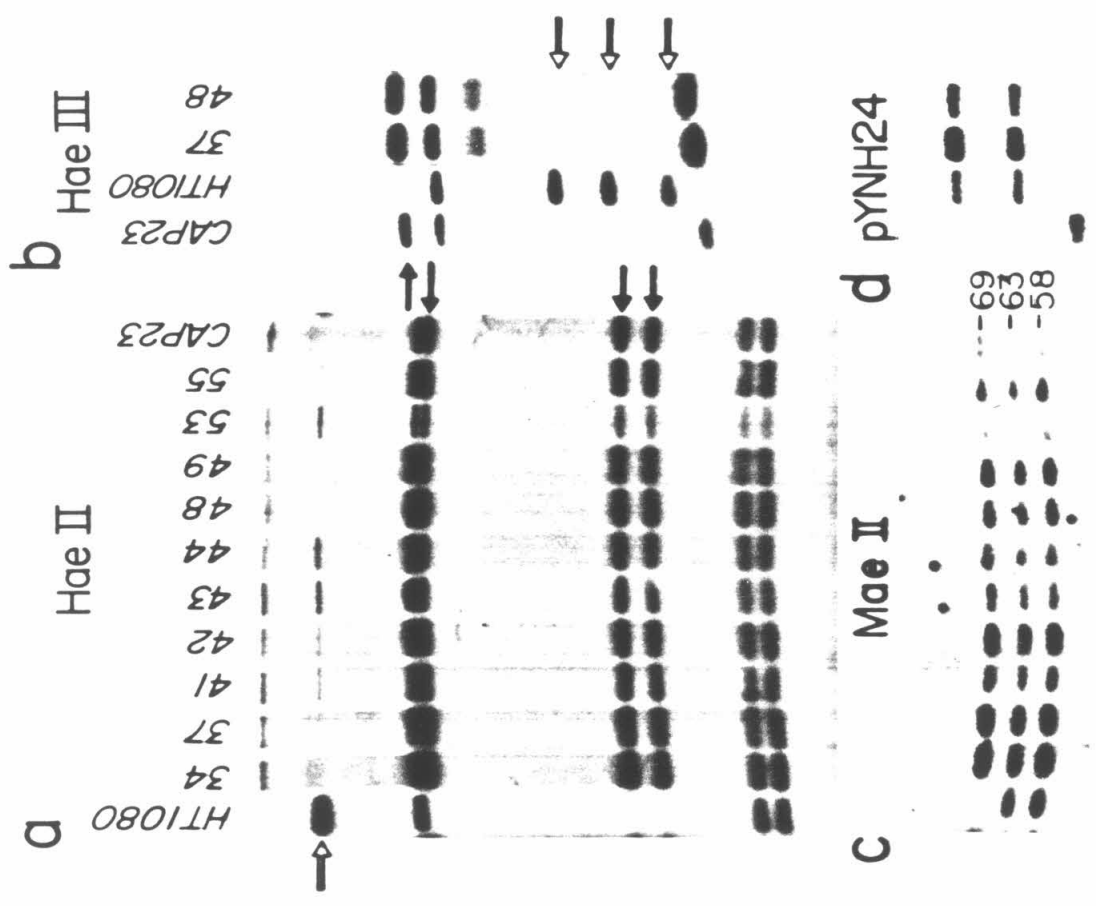

a
Hae II
HT1080
34
37
41
42
43
44
48
49
53
55
CAP23
b
Hae III
CAP23
HT1080
37
48
c
Mae II
d
pYNH24
-69
-63
-58

Fig. 5. Growth of CAP 23 cells *(a)*, 143 BTK$^-$ cells *(b)*, and transformants 63 *(c)* and 66 *(d)* in the presence or absence of CAP at 100 μg/ml, or BrdU at 100 μg/ml.

Multiple series of 10-cm plastic petri dishes were seeded each with a constant amount of cells in 10 ml normal DME medium with 5% dialyzed calf serum (CAP23), or 5% dialyzed fetal calf serum (143BTK$^-$, transformants 63 and 66), in the presence or absence of CAP or BrdU, as indicated. Prior to the growth experiment, transformants 63 and 66 had been maintained for 28 days post-injection in DME with 0.9 mg glucose/ml, supplemented with 5% dialyzed fetal calf serum, 100 μg BrdU/ml, and 50 μg CAP/ml, then for 13 days in normal DME with 5% dialyzed fetal calf serum, 100 μg BrdU/ml, and 50 μg CAP/ml, and then for 6 days in normal DME with 100 μg BrdU/ml and 100 μg CAP/ml. At various time intervals, cells from individual or duplicate plates were trypsinized and counted. In *b*, *c*, and *d*, at the time shown, cells from one or two plates of each series were trypsinized and replated on a new series of petri dishes.

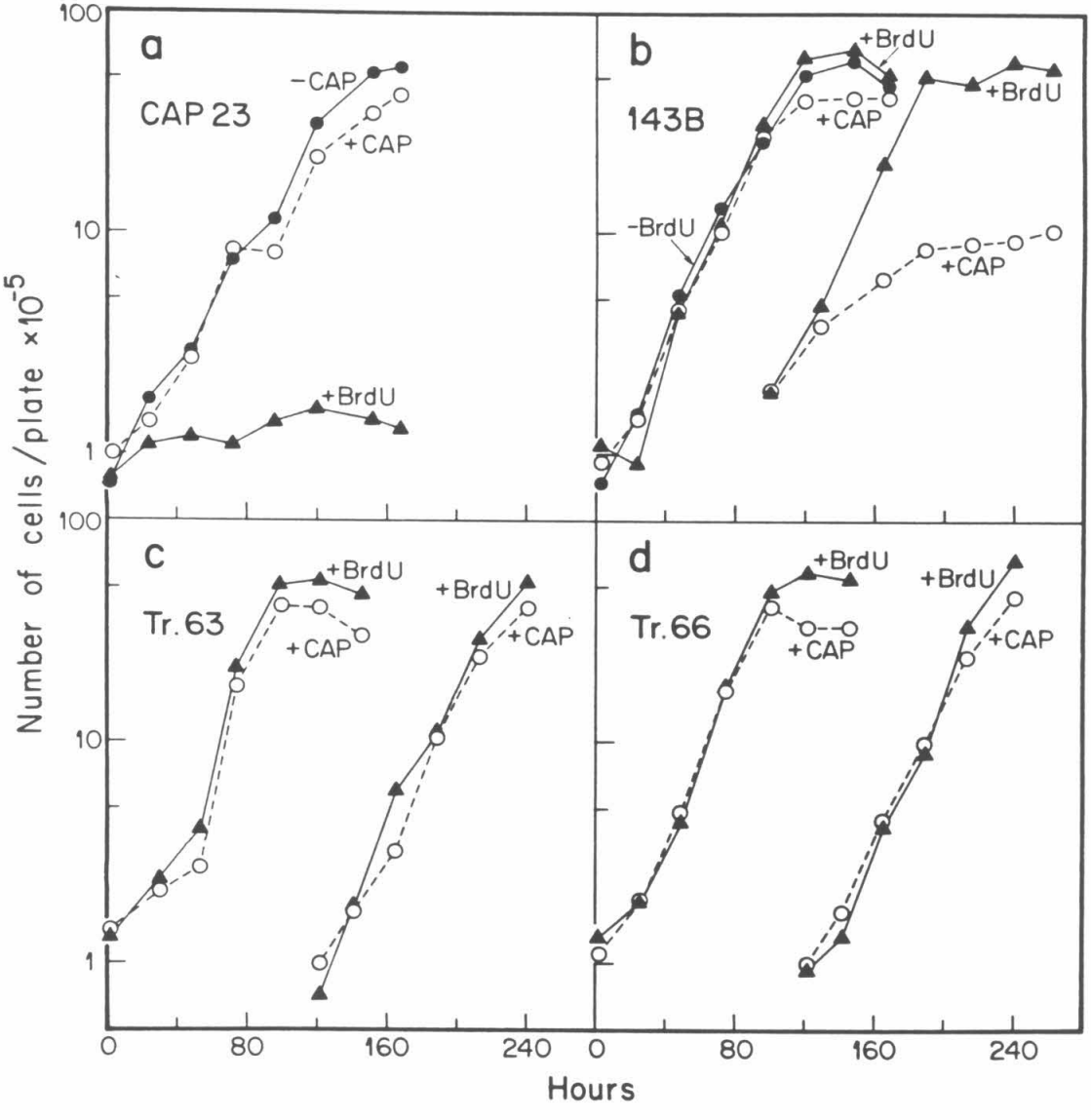
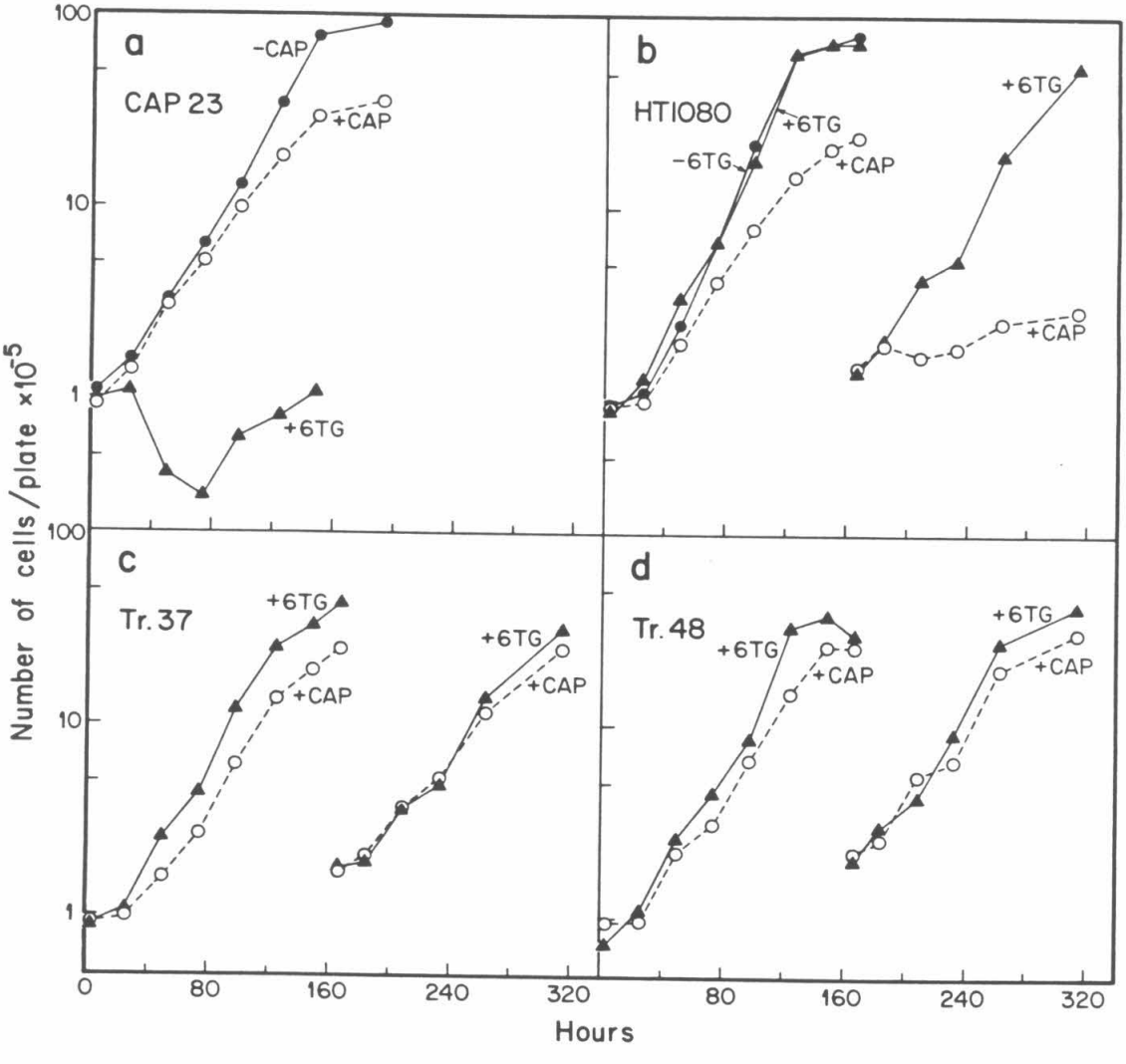

a
CAP 23
-CAP
+CAP
+BrdU
b
143B
+BrdU
+CAP
-BrdU
+BrdU
+CAP
c
Tr. 63
+BrdU
+CAP
+BrdU
+CAP
d
Tr. 66
+BrdU
+CAP
+BrdU
+CAP
Number of cells /plate ×10^-5
100
10
1
100
10
1
0
80
160
240
0
80
160
240
Hours

Fig. 6. Growth of CAP 23 cells *(a)*, HT1080-6TG cells *(b)*, and transformants 37 *(c)*, and 48 *(d)* in the presence or absence of CAP at 200 μg/ml or 6TG at 10 μg/ml.

Growth curves of the parental and transformed cell lines were determined as described in the legend of Fig. 5. Growth medium was normal DME, supplemented with 10% dialyzed calf serum (CAP 23) or 10% dialyzed fetal calf serum (HT1080-6TG, transformants 37 and 48). The transformant clones had been maintained for 32 and 29 days post-injection, resepectively, in DME with 0.9 mg glucose/ml, 10% dialyzed fetal calf serum, 10 μg 6TG/ml, and 100 μg CAP/ml, for 6 days in the same medium with 200 μg CAP/ml, and then for 27 days in normal DME with 10% dialyzed fetal calf serum, 10 μg 6TG/ml, and 200 μg CAP/ml.


a
CAP 23
-CAP
+CAP
+6TG
b
HT1080
+6TG
-6TG
+CAP
+6TG
+CAP
c
Tr. 37
+6TG
+CAP
+6TG
+CAP
d
Tr. 48
+6TG
+CAP
+6TG
+CAP
100
10
1
100
10
1
0
80
160
240
320
80
160
240
320
Hours
Number of cells/plate ×10⁻⁵