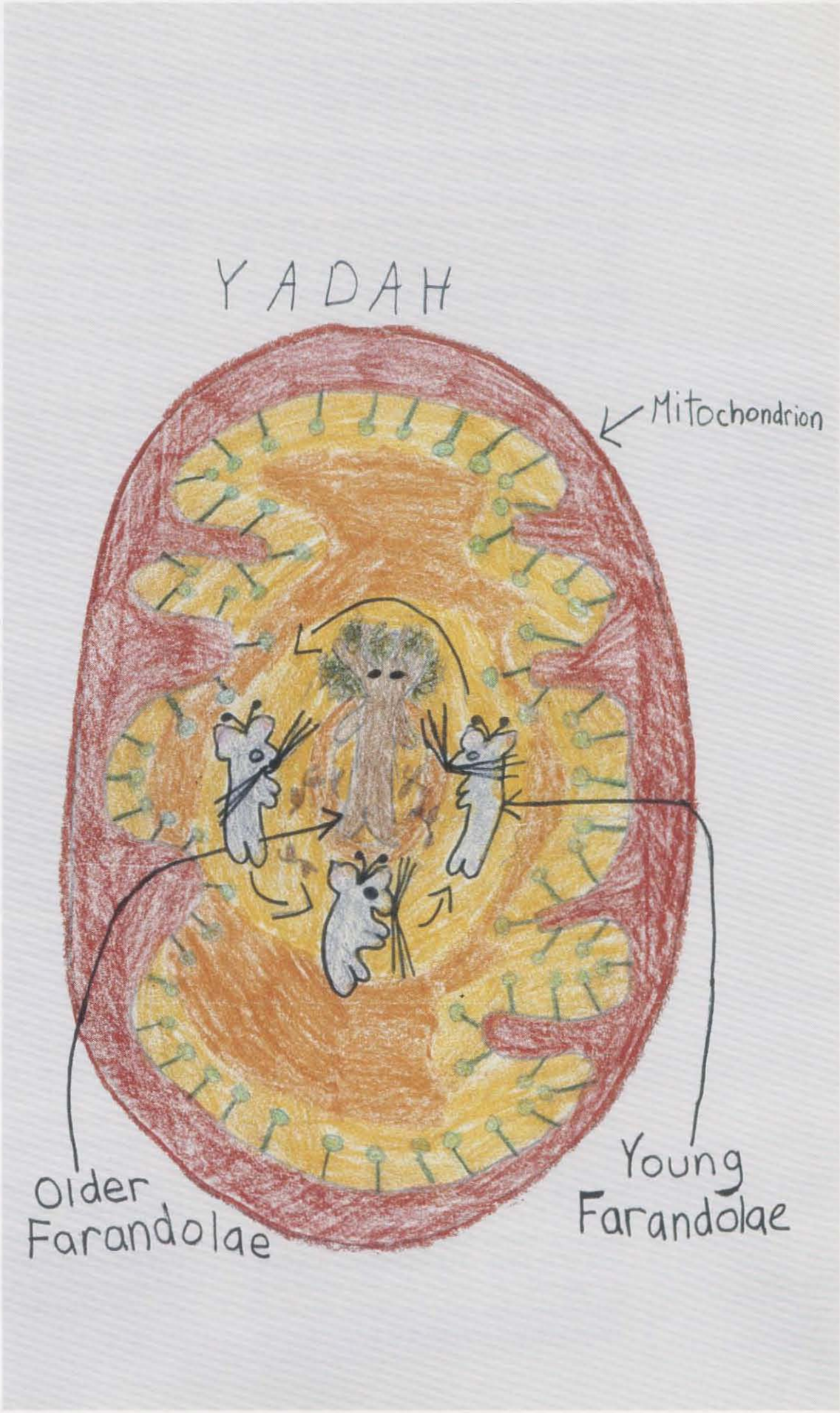

YADAH
Mitochondrion
Older Farandolae
Young Farandolae

Mitochondrial DNA: The Second Genetic System

Prospects are promising that, with all the basic information now available on the human mitochondrial genome, its role in human disease and aging will soon be understood.

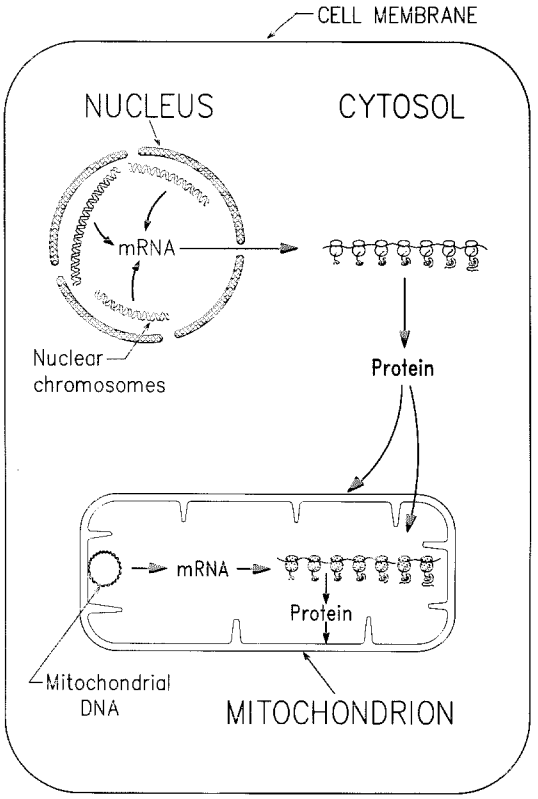
by Giuseppe Attardi

Mitochondria do not really contain microscopic, mouse-like creatures called farandolae, as imagined in Madeleine L'Engle's science fiction book for children, "A Wind in the Door." (Yadah is the name of a particular mitochondrion in the book.) Farandolae, the book explains, are "genetically independent of their mitochondria. . . . And if anything happens to the farandolae in the mitochondrion, the mitochondrion gets sick. And probably dies." A number of diseases recently identified are, in fact, the result of mutations in mitochondrial DNA. Drawing by Laura Attardi, age 10.

All animal and plant cells and other nucleated cells contain, besides the main genetic system localized in the nucleus, a second genetic system sequestered within the mitochondria. These are the powerhouses of the cell, specialized for the production of energy from respiration. The evolutionary origin of mitochondria merges with the origin of the present-day nucleated cells. It is generally accepted that mitochondria are, in fact, descendants of primitive aerobic bacteria, which were engulfed by the progenitors of contemporary nucleated cells about 1.5 billion years ago. These early progenitors were not capable of aerobic metabolism, but acquired the capacity to utilize atmospheric oxygen for energy production by incorporating organisms that could. During the long evolution that followed their acquisition by the nucleated cells, the primitive bacteria lost the capacity of autonomous multiplication and became dependent to an increasing extent on the host cell for all their functions. This loss of autonomy by the intracellular bacteria was the consequence of the transfer to the nucleus of the major part of the genes of the primitive bacterial chromosome.

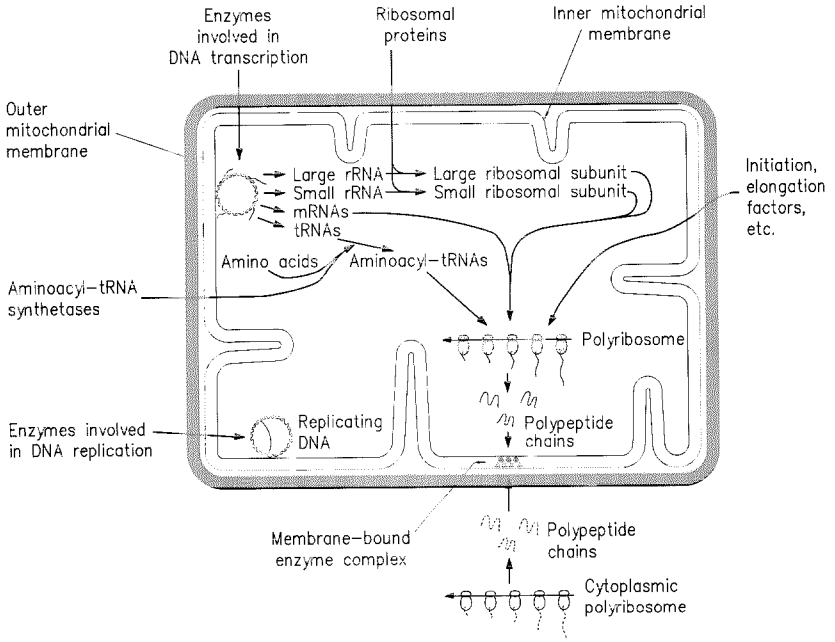
The DNA sequestered in mitochondria is the residue of those bacteria's genetic material. The dimensions of the DNA are very small—equivalent to about one three-thousandth of the smallest human chromosome. The mitochondria themselves have maintained some similarities in size and shape to the primitive bacteria. Of the two mitochondrial membranes, the external one derives from the membrane of the nucleated cell that engulfed the bacterium, whereas the internal one derives from the bacterial membrane and maintains some of its chemical characteristics.

Every present-day nucleated cell contains a large number of mitochondria, varying in man between a few such organelles and several hundred, depending upon the type of cell. Each mitochondrion contains several identical or near-identical copies of mitochondrial DNA, and, accordingly, each cell contains from a few dozen to a few thousand molecules of mitochondrial DNA. This variability in the number of mitochondria reflects the energy needs of the various cell types. Thus, in brown fat, which is a tissue whose mitochondria are specialized for heat production from respiration, each cell's cytoplasm is literally packed with mitochondria. Adult humans have very little brown fat; it is, however, abundant in children, and heat production by brown fat mitochondria is essential for newborn infants' survival. In some cell types mitochondria are uniformly distributed in the cytoplasm, while in others they are located in close proximity to other structures or organelles that require a high level of energy to perform their function. Thus, in heart muscle, mitochondria are tightly packed in linear arrays in the narrow spaces separating myofibrils—the structures specialized for muscular contraction, which depend for their activity on adenosine triphosphate (ATP), the product of chemical energy generated in mitochondria. Another striking example of the close association of mitochondria with structures requiring a high supply of energy is provided by spermatozoa. In these cells the long tail contains longitudinal contractile fibrils, which ensure the



Right: The formation of functional mitochondria within a nucleated cell requires the participation of a large number of proteins encoded in nuclear genes, synthesized in the cytosol, and then imported into the mitochondria. But also necessary are a few proteins that are encoded in the mitochondrial DNA itself and synthesized in the mitochondria.

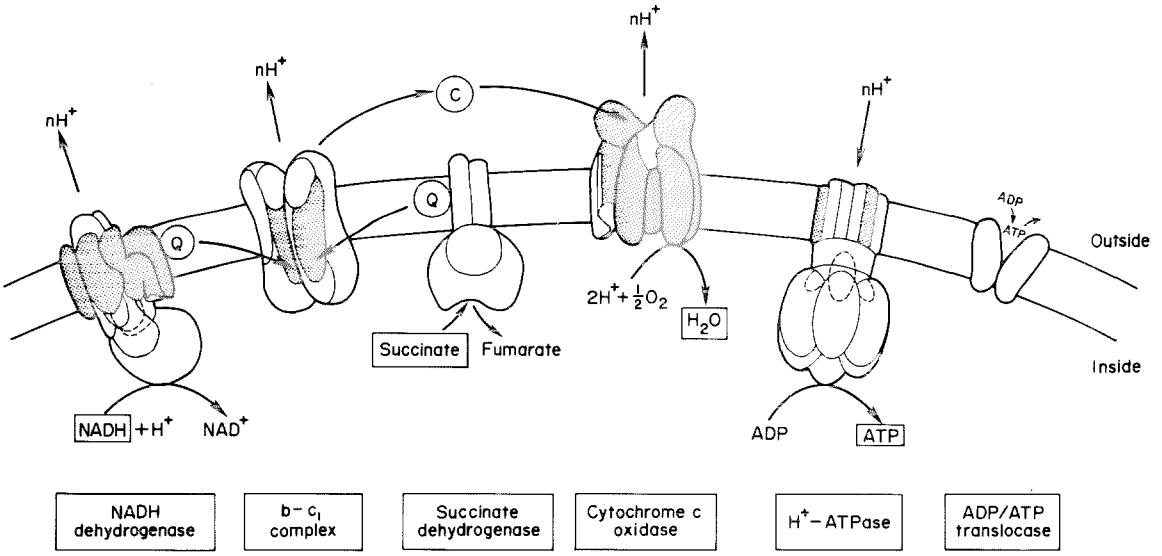
Below: This diagram of a mitochondrion shows the route by which the information in mitochondrial DNA is translated into the proteins that, along with the proteins imported from the cell's cytosol, form the enzyme complexes of the inner mitochondrial membrane.



sperm motility and which are surrounded by tightly packed mitochondria aligned to form a spiral.

In the course of their evolution from primitive bacteria, mitochondria became completely dependent on nuclear genes for their growth and function. Most mitochondrial proteins, including those necessary for the replication and expression of mitochondrial DNA, are encoded in nuclear genes, as shown in the illustration above. The genes are transcribed into RNA copies carrying genetic messages. These messenger RNAs (mRNAs) are transported into the soluble fraction of the cytoplasm, or cytosol, where they are translated into proteins by the protein-synthesizing apparatus; these proteins are subsequently imported into the mitochondria. The small number of genes of the primitive bacterial chromosome that constitute the mitochondrial DNA encode proteins that play an essential role in the mitochondrion's energy-producing functions. These genes are also transcribed into mRNAs, which are then translated into proteins by a mitochondria-specific protein-synthesizing apparatus. The RNA components of this protein-synthesizing machinery are also encoded in mitochondrial DNA.

The diagram at left describes what is known about the role of animal mitochondrial DNA in the formation of mitochondria. Mitochondrial DNA is replicated through the activity of enzymes encoded in nuclear genes and synthesized in the cytosol. Other enzymes encoded in the nucleus transcribe the DNA into RNA copies. Mitochondrial DNA codes for two ribo-



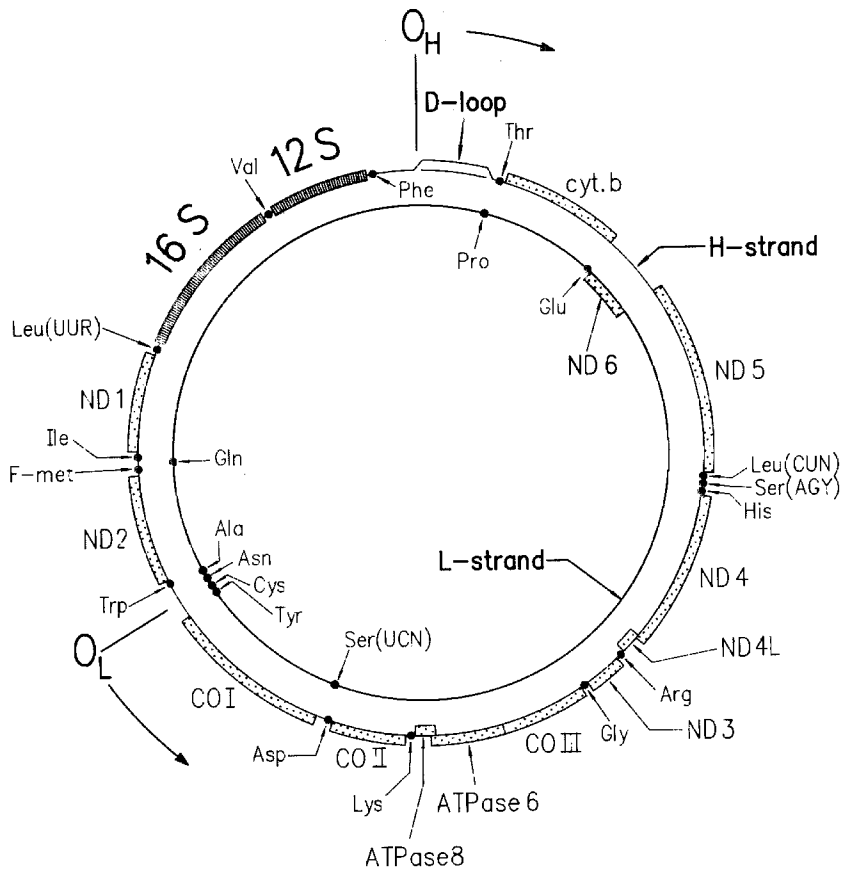
The genetic origins of important components of the mitochondrion can be seen in this drawing of the enzymes of the inner mitochondrial membrane, which preside over the production of energy. These include four respiratory enzymes, along with H^+-ATPase and ADP/ATP translocase. The enzyme subunits that are encoded in mitochondrial DNA are represented by shading, while those encoded in the cell nucleus are unshaded.

somal RNA species, or rRNAs, which are specific structural components of mitochondrial ribosomes, that is, the machines specialized for protein synthesis within the mitochondria. The two rRNA species become associated with a large set of proteins encoded in the nucleus, and synthesized in the cytosol, to form the large and small subunits of the mitochondrial ribosomes. Mitochondrial DNA also codes for 13 mRNAs, which specify an equivalent number of proteins. Finally, mitochondrial DNA encodes 22 different transfer RNAs, or tRNAs. These are small RNA molecules specific for different amino acids, which have the function of translating the genetic messages contained in the DNA sequence into the amino acid sequence of proteins. Each species of tRNA becomes linked to its corresponding amino acid through the activity of specific enzymes that are encoded in the nucleus (aminoacyl-tRNA synthetases). The mRNAs bind to the ribosomal subunits to form structures called polyribosomes that look like strings of beads. These, with the help of aminoacyl-tRNAs and of specific initiation and elongation factors, decipher the messages contained in the mRNAs and synthesize the corresponding proteins. These proteins then become associated with proteins that have been imported from the cytosol into the mitochondria and form the large enzymatic complexes of the inner mitochondrial membrane.

That enzymatic apparatus of the inner mitochondrial membrane is shown in close-up in schematic form above. This apparatus presides over the production of energy and includes four respiratory enzymes, which transfer in series the electrons derived from the oxidation of respiratory substrates to oxygen, with concomitant production of water. The energy producing apparatus also includes the ATP synthetase, also called mitochondrial proton-ATPase, which synthesizes ATP by utilizing the energy produced by respiration. And finally, it includes a protein that regulates the traffic of ATP and of its precursor ADP (adenosine diphosphate) across the inner mitochondrial membrane. In the diagram the subunits of the enzyme complexes of this membrane that are encoded in the nucleus are not shaded to distinguish them from those that are encoded in mitochondrial DNA, which are shaded. You can see that three respiratory enzymes and the ATP synthetase claim their genetic origin from both genomes.

The genetic map of human mitochondrial DNA on the following page shows the genes transcribed from the two strands of DNA. This information has emerged from studies that we started at Caltech about 20 years ago. This work culminated 10 years later in the determination of the complete sequence of human mitochondrial DNA in Frederick Sanger's laboratory in Cambridge University in England, and four years ago in the complete functional identification of the proteins encoded in mitochondrial DNA, carried out in our laboratory, in collaboration with Russell Doolittle at UC San Diego.

The arrangement of the genes in human mitochondrial DNA, and probably in mitochondrial DNA from all vertebrate cells, exhibits characteristics of compactness and economy that



Representing work begun at Caltech 20 years ago, this complete map of human mitochondrial DNA shows the positions of all the genes. Most of the genes are transcribed from the H-strand (outside), including those coding for the 2 rRNAs (hatched bars), 12 proteins (dotted bars), and 14 tRNAs (black dots). The L (inside) strand includes 8 genes for tRNAs (black dots) and 1 gene for a protein (dotted bar). The map illustrates the compactness of mitochondrial DNA, as opposed to nuclear DNA, in which long stretches of noncoding sequences separate the genes. Here the genes lie right next to each other, and a nontranscribed segment in one strand corresponds to a transcribed segment in the other.

have no parallel in the living world, except in viruses. In fact, the genes transcribed from one or the other of the two strands saturate the length of mitochondrial DNA almost completely. The majority of the genes are transcribed from one of the two strands, which is designated as the heavy (H) strand because of its relative density in a solution of cesium chloride. These genes include those for the 2 rRNA species, 12 genes coding for proteins, and 14 genes coding for tRNAs. The genes transcribed from the other strand, designated as the light (L) strand, include 8 genes for tRNA and 1 gene encoding a protein. Note that a nontranscribed segment in one of the two strands corresponds to a transcribed segment in the other strand. In the nuclear chromosomes the genes are separated by long noncoding segments, and many genes are discontinuous, with intervening sequences interrupting the coding sequences. By contrast, in mitochondrial DNA, the genes, all continuous, are immediately adjacent to each other and in some cases even overlapping, and there is an almost complete absence of noncoding stretches. Mitochondrial DNA of vertebrate cells, in the course of the evolution of these cells from the more primitive nucleated cells, has undergone an extreme reduction of its dimensions by elimination of intergenic spacers. This contraction in size has also been accompanied by a reduction and structural simplification of the individual genes themselves. Note that the arrangement of the genes in the genetic map is very characteristic: the genes coding for the rRNA species and those coding for proteins are separated with an almost absolute regularity by tRNA genes.

It is not surprising that a unique mode of DNA transcription into RNA has evolved to match the extremely compact and economical gene organization of the mammalian mitochondrial DNA. In fact, in contrast to the nuclear genes, which are copied into RNA molecules individually, mitochondrial genes are transcribed from each strand in the form of giant molecules corresponding to the entire length of the DNA, with each comprising many genes. These giant transcripts must then be cut by specific enzymes to produce the various species of rRNAs, tRNAs, and mRNAs.

A characteristic property of the human—and of mammalian in general—mitochondrial DNA is its great tendency to mutate, thus changing its nucleotide sequence. This tendency to mutate is about 10-fold higher than in nuclear genes. Apart from mutations occurring as a result of replication mistakes, there are those produced by direct action of chemical agents on DNA. Cellu-

lar DNA, in general, is known to suffer oxidative damage from oxygen derivatives produced by aerobic metabolism. Bruce Ames at UC Berkeley has shown that this damage is about 15 times greater in mitochondrial DNA than in nuclear DNA, and damage by alkylation (the addition of a hydrocarbon chain) is also much more frequent in mitochondrial DNA. DNA repair systems are very inefficient in mitochondria, and, in addition, mitochondrial DNA is not protected by histones or similar proteins, as nuclear DNA is. The sequence variation that is continuously produced in mitochondrial DNA, when it affects the DNA of the germ cell line, may be transmitted to the progeny. This transmission occurs exclusively through the maternal lineage—only the egg contributes its mitochondrial DNA to the zygote at the time of fertilization. Therefore, every individual inherits his or her mitochondrial DNA exclusively from the mother, and the mother in turn from her mother, and so on. Today, a powerful technology is available to investigate the sequence variation of mitochondrial DNA among individuals. Thus, it has been established that, between two individuals randomly chosen, mitochondrial DNA differs on average in about 50 of its 16,560 nucleotide pairs, that is, in approximately 0.3 percent of its nucleotides.

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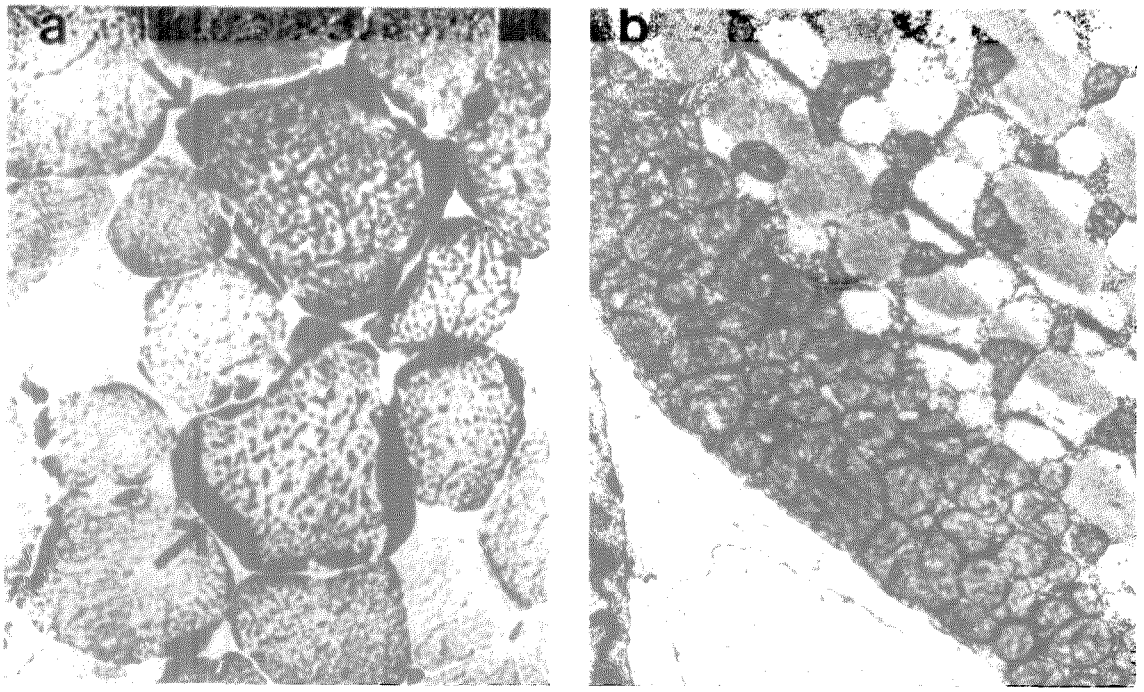
The large variation existing between mitochondrial DNA sequences of different individuals has provided a powerful tool for studying the genetic relatedness of human populations and thus for investigating the evolution of man. Furthermore, the exclusive maternal inheritance of mitochondrial DNA allows the tracing of the genetic differences between individuals through maternal lineages in populations. By comparing the mitochondrial DNA sequences of a large number of individuals from different geographic populations, an evolutionary tree has been constructed (by Alan Wilson and his collaborators at UC Berkeley) that relates the different mitochondrial DNA types to one another and to an ancestral mitochondrial DNA type. This ancestral type is postulated to have belonged to a woman who lived in Africa about 200,000 years ago. By a similar analysis of mitochondrial DNA variation in Amerindian populations from North, Central, and South America, Douglas Wallace at Emory University has recently shown that the mitochondrial DNAs of American Indians must have derived from at least four primary maternal lineages of Asian origin.

About one-fifth of the nucleotide differences existing between mitochondrial DNAs of different individuals produces corresponding differences in the amino acid sequence of the proteins encoded in mitochondrial DNA. It is very likely that at least a part of this variation affects the properties of these proteins and has functional consequences. There is already good evidence from pathological situations that the sequence variation of mitochondrial genes plays a significant role in determining differences in the capacity to produce energy, especially in the tissues that have high energy requirements, such as the brain, the skeletal muscles, the heart, the retina, the kidney, and the liver.

Superimposed upon the mitochondrial DNA sequence variation between individuals that we inherit from our mothers is a mitochondrial DNA heterogeneity that is continuously produced in our tissues during our lives as a consequence of mutations. It is, in fact, to be expected that mitochondrial DNA mutations resulting from replication errors, and possibly from damage by oxidation or alkylation, accumulate progressively during the life of an individual. There is already clear evidence that mutations resulting from deletions or insertions of short DNA segments in mitochondrial DNA are much more abundant in senescent mice than in young mice. So it's a plausible idea that the progressive damage in mitochondrial DNA that occurs during aging contributes to the decrease in respiratory capacity of an individual's tissues. Researchers in Australia have demonstrated that such a decrease occurs in skeletal muscles as humans age; it presumably also takes place in other tissues.

Besides this aging-related, general deterioration of mitochondrial DNA, some specific mutations occurring in mitochondrial DNA, either inherited or produced during the life of an individual, can cause clear damage to the organism, thereby producing specific diseases. A heterogeneous group of diseases, called mitochondrial diseases and characterized by mitochondrial dysfunction, affects either singly or in combination the nervous system, the skeletal muscles, the heart, the retina, the kidney, and the liver—all organs that have high energetic needs and so depend heavily on the respiratory functions of mitochondria. For several of these diseases, an association with specific mitochondrial DNA mutations has been clearly demonstrated. One of them, designated MERRF (myoclonic epilepsy and ragged red fiber syndrome), is characterized by epilepsy, dementia, cerebellar disturbances, and defects of the skeletal and heart muscle. In this syndrome, as well as in other mitochondrial muscular diseases, muscle fibers of affected individuals exhibit a characteristic accumulation



The photograph at right (a) shows a cross section of muscle fibers from a patient with a muscular mitochondrial disease. Accumulations of mitochondria (stained darker) are apparent at the periphery of the ragged red fibers (arrows). In the higher magnification of an electron microscope (b) the large accumulation of mitochondria can be seen more clearly at the edge of a cross section of a single fiber. (Courtesy of Salvatore DiMauro, Columbia University.)

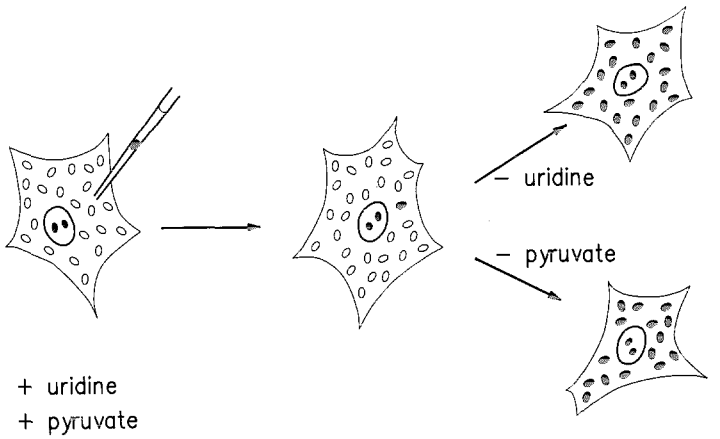
Some specific mutations occurring in mitochondrial DNA, either inherited or produced during the lifetime of an individual, can cause clear damage to the organism.

of mitochondria at the periphery, under the cell membrane. The left-hand photograph (a) above shows a cross section of muscle fibers from an individual affected by a mitochondrial muscular disease. The black material (stained red in the original preparation) at the periphery of the ragged red fibers represents accumulations of mitochondria. These are more easily recognizable in the view at higher magnification on the right (b)—an electron-microscope picture of a portion of a transverse section of a muscle fiber. You can see the enormous accumulation of mitochondria at the periphery of the fibers, which has resulted from a proliferation of defective mitochondria. This proliferation is an attempt on the part of the sick fibers to compensate for the functional defect of the mitochondria by producing more of them. Wallace and his collaborators have recently identified the mutation of mitochondrial DNA that produces the MERRF syndrome as a single nucleotide change in the tRNA specific for lysine, one of the amino acids.

Another mutation of mitochondrial DNA, which produces a well-characterized disease, had previously been identified. Leber's hereditary optic neuropathy, which is transmitted through the maternal lineage, affects mostly males and produces a rapid bilateral loss of central vision due to optic nerve atrophy. In most, if not all, of the families affected by this disease, the mutation, which changes a single amino acid, occurs at a specific site in a mitochondrial gene encoding a subunit of NADH dehydrogenase, the first respiratory enzyme.

Another type of disease-causing mutation that affects mitochondrial DNA is not inherited, but appears sporadically. These mutations consist of deletions that have removed a portion (between 8 and 75 percent) of the mitochondrial DNA. These deletions, first discovered by investigators at the University of London, do not involve the two origins of replication of the mitochondrial genome, therefore preserving the replicating capacity of the shortened molecules. Such deletions have been found in patients affected by a variety of mitochondrial diseases, such as Kearns-Sayre syndrome, characterized by paralysis of external eye muscles, retina degeneration, and cerebellar symptoms, and Pearson's disease, characterized by bone-marrow and pancreas alterations. The identification of patients affected by mitochondrial diseases clearly associated with mitochondrial DNA mutations has increased rapidly in the two years since the first molecular description of such diseases. With the increasing availability of molecular assays for such diseases and the growing awareness on the part of physicians of the possible mitochondrial genetic origin of syndromes affecting the nervous, muscular, and other systems, we expect the number of pathological forms associated with mitochondrial DNA mutations to continue to increase in the coming years.

Prospects are promising that genetic manipulations of the human mitochondrial genome can find applications in mitochondrial diseases. In our laboratory, we are developing new technologies aimed at transferring mitochondria from one cell to another, at replacing completely the mito-



In recent developments in the Caltech lab, human cell lines have been isolated whose mitochondria have been depleted of mitochondrial DNA. Such cells are deficient in respiratory function and dependent on uridine and pyruvate for growth, but injecting the cells with a single mitochondrion with functional DNA repopulates all the mitochondria with DNA and "cures" their deficiencies.

chondrial DNA complement of a cell with foreign mitochondrial DNA, and at introducing DNA directly into the mitochondria of a living cell. We are following two main approaches. First, graduate student Michael King has isolated human cell lines whose mitochondria have been completely depleted of mitochondrial DNA by long-term exposure to ethidium bromide, an inhibitor of mitochondrial DNA replication. These cell lines are, as expected, respiratory deficient, and derive their energy exclusively from fermentation of glucose. Furthermore, these cells have developed metabolic defects due to the lack of a respiratory chain. In particular, two metabolites, uridine and pyruvate, must be added to the nutritive medium for the cells to grow.

The drawing above shows how these cells lacking mitochondrial DNA (designated ρ^0) can be injected with single mitochondria containing functional mitochondrial DNA, and how this DNA can then repopulate the entire complement of mitochondria. As a result, the injected cell reacquires respiratory competence and the capacity to grow in the absence of uridine and/or pyruvate. Another approach more frequently applied for introducing mitochondria into the ρ^0 human cells involves removing the nuclei of the chosen mitochondrial donor cells, and then fusing the enucleated cells with the ρ^0 cells. Many "transmitochondrial" cell lines containing nuclei from ρ^0 cells and mitochondria derived from human cells from other sources have already been constructed in our laboratory by King and others. This type of experimental strategy will be very useful for introducing into ρ^0 cells mitochondria derived from patients affected by mitochondrial diseases. This approach has already allowed Anne Chomyn, senior research associate, to determine that the defect associated with a mitochondrial myopathy could be transferred with the injected mitochondria, and thus to establish that the mutation underlying the defect was of mitochondrial origin and to determine its nature.

We are also working on another type of genetic manipulation aimed at the direct introduction of intact mitochondrial DNA molecules or fragments of them into the mitochondria of human cells, by means of a so-called "biological gun." With this instrument, developed by John Sanford at Cornell, metallic microcarriers—bullets less than a micron in size and coated with the desired DNA—are fired directly into the cells by the force of gun powder. This gun has already been applied successfully to the transformation of chloroplasts in the unicellular alga *Chlamydomonas*, and of mitochondria in yeast. On the basis of others' experience with these organisms, we hope that DNA introduced with the biological gun into human cells will repopulate the entire mitochondrial complement of the cells and confer new properties on them. Such an experimental strategy, if successful, would open the way to the correction of mitochondrial DNA mutations by the introduction of functional genes into the defective cells.

In view of this recent work, I believe it is reasonable to expect that the new approaches for the genetic manipulation of human cells will be very useful for the diagnosis, the genetic and molecular analysis, and, eventually, the therapeutic treatment of mitochondrial diseases. □

In 1989 Giuseppe Attardi received the $70,000 Antonio Feltrinelli International Prize for Medicine, presented every five years by the Accademia Nazionale dei Lincei, founded in 1603 in Rome. This article was adapted from his acceptance speech at the ceremonies in Rome.

Attardi is the Grace C. Steele Professor of Molecular Biology. He earned his MD degree from the University of Padua in 1947 and joined the Caltech faculty in 1963, becoming full professor in 1967. In 1984 he was elected to the National Academy of Sciences.

His daughter's drawing on page 12 was suggested by Attardi as an illustration even though it was inspired by science fiction. He considers the book, written in 1973, quite prescient in its discussion of mitochondrial diseases, of which little was known before the past couple of years.