By crossing varieties of pea plants in his garden and observing the results, the nineteenth-century Austrian monk and botanist Gregor Johann Mendel formulated several principles of heredity and set the stage for modern genetics. Yet some of the intricacies of genetics as applied to human disease are just now beginning to be appreciated.

As is well known, a gene is a set of nucleotides on a chromosome that specifies a particular trait in an organism, such as eye color or the presence of a certain enzyme, and an allele is any of the alternative forms of that gene. Both chromosomes and alleles exist in pairs.

One of Mendel's principles states, in part, that only one member of a parent's pair of alleles for a particular trait is transmitted to the offspring. Thus, for any given trait only one of the two alleles from the mother and one of the two alleles from the father is passed on to the child to form a new allele pair that specifies the same trait. According to Mendel, it would not matter which parent contributed which allele.

Or so, until recently, everyone has thought. If the alleles contributed by each of the parents are exactly alike, the child is said to be homozygous for that gene; if the two alleles differ, the child is said to be heterozygous for the gene. Frequently, in the heterozygous situation, the expression of one allele of a gene is dominant over that of another, which is then called recessive. A recessive allele is expressed only when it is present in a double dose, that is, in a homozygous state.

Hence, alluding to the dominance or recessiveness of the responsible genetic allele(s), a trait or disease that has been inherited is usually termed dominant (either the homozygous or heterozygous situation) or recessive (always homozygous). The word autosomal is added as a prefix, as in autosomal dominant, if the gene is carried on one of the 22 pairs of autosomal (not x or y) chromosomes. Although inheritance is a little more complicated, for genes located on an x chromosome, there also is a recognizable Mendelian pattern.

Still another tenet of heredity, albeit promulgated well after Mendel's time, has been that all of the cell's genes are located on chromosomes in the nucleus. Genes were not thought to be located in other cellular organelles, such as mitochondria, out in the cytoplasm.

Non-Mendelian processes, however, are those that do not follow Mendel's classic laws. Many of these mechanisms have been discovered in the past few years, and they have intriguing implications for the understanding of certain previously inexplicable inherited diseases.

Mendel Never Knew: Nontraditional mechanisms of inheritance are only now being applied to human genetics, with great implications for the understanding of certain previously inexplicable inherited diseases.

Other mechanisms

The trouble with these tenets of inheritance is that, in light of new findings, they do not always hold true. Four recently recognized mechanisms of heredity-imprinting, uniparental disomy, mitochondrial DNA inheritance, and mosaicism—have shaken up concepts of inherited disease and shown that Mendelian laws do not explain everything. Although known and studied for some time in nonhuman organisms (see "An Essential Intruder - I" and "Mechanisms Mendel Never Knew" elsewhere in this Mosaic), these nontraditional mechanisms of inheritance are not only now being applied to human genetics, with great implications for the understanding of certain previously inexplicable inherited diseases.

The term applied to the first mechanism, genomic imprinting, refers to the astonishing fact that the functions of some genes differ depending on whether they come from the mother or from the father. In some way, possibly through a process called methylation (the attachment of methyl (CH3) groups to DNA), the DNA of certain alleles contributed by one parent but not the other is modified probably during or just after the formation of eggs and sperm, leading to nonexpression, or inactivation, of the alleles. These modifications are not mutations, however, and are potentially reversible. Genomic imprinting is thought to be the cause of deleterious effects that result from uniparental disomy, which occurs when an individual inherits both alleles of a gene (and probably both chromosomes on which those alleles reside) from one parent rather than from both. The term disomy comes from the word disome and refers to a chromosome set with paired members.

Mitochondrial inheritance is another non-Mendelian process in which differences in traits and gene functions are based on the gender of the transmitting parent, but the process differs from imprinting. Most of the time, only the mother's mitochondria—cellular organelles that contain their own mitochon-
Replicative segregation. After fertilization of oocyte by sperm, which contributes no mitochondria to offspring, mothers can pass on random proportions of mutant and wild-type mtDNA.
drial DNA (mtDNA)—are transmitted to the offspring. In addition, mtDNA operates under different rules than nuclear DNA—except, that is, when it is operating in conjunction with nuclear DNA.

Finally, mosaicism refers to the presence in an individual of two or more groups of cells that differ with respect to a single gene, parts of a chromosome, or even whole chromosomes. It results from an event that may take place early in development. If germline cells are affected, the change can even be inherited in Mendelian fashion. How frequently mosaicism occurs is unknown, but it is no longer correct to assume that all cells in the body have the same genetic alleles, or even that they have the same complement of genes.

Many geneticists and physicians have welcomed the new observations as explanations for some of the most unfathomable human diseases. Still, the number of human disorders in which they may play a role is not yet clear. One thing is certain, however: Inheritance is more complicated than has generally been realized.

**Imprinting and uniparental disomy**

Evidence for the role of imprinting and uniparental disomy in disease has been found in some pediatric cancers and chromosome-deletion syndromes, in association with certain recessive diseases, like cystic fibrosis, and in a deficiency of one of the blood proteins called complement. A number of other disorders, such as neurofibromatosis, myotonic dystrophy, and fragile-x syndrome, also may be included.

Human geneticists were jolted into recognizing the significance of imprinting and uniparental disomy when the molecular origins of Prader-Willi syndrome and Angelman syndrome, two ostensibly unrelated disorders, became known. Children with Prader-Willi syndrome, first described in 1965, are severely retarded; have jerky, repetitive, and lurching body movements; are prone to seizures; and are unable to speak coherently. Many Angelman children laugh loudly in short, uncontrollable bursts, sometimes at inappropriate moments. They also have characteristic facial appearances, with large mouths and red cheeks.

In the early 1980s, cytogeneticist David Ledbetter of Baylor University reported that many Prader-Willi patients were missing a piece (called a deletion) of one of their two chromosome 15s, specifically the area (or chromosomal address) termed 15q11q13. Five years later, other investigators made a startling discovery: patients with Angelman syndrome appeared to have the same exact deletion. Until that discovery, one genetic defect causing two different diseases was unheard of.

**Which parent?**

Yet the defect was not quite the same. In the mid-1980s, geneticists Tim Donlon and Marc Lalande, working in the laboratory of the late Samuel Latt of Children's Hospital in Boston (which is affiliated with Harvard University), generated cloned markers for this specific region on chromosome 15 and localized ten or so of them to the deletion area in Prader-Willi syndrome. That allowed them to define the extent of the deletion, which turned out to be very large, perhaps five million base pairs in size. Then, Robert Nicholls of the same laboratory, by using restriction enzymes that cut DNA mostly in the same places, which allows the parental origins of chromosomes to be identified, showed molecularly that the deletions in Prader-Willi syndrome are all on paternal chromosomes (something that also had earlier been demonstrated cytogenetically by Merlin Butler of Vanderbilt University).

Angelman syndrome was similarly studied by Joan H. M. Knoll, Nicholls, and others in Latt's laboratory. Using the same cloned markers, they found that the same area of chromosome 15 was deleted. But Knoll demonstrated that in this syndrome, in contrast to Prader-Willi syndrome, the deletion was on a maternally derived chromosome.

“What we had was the first suggestion that there might be genetic imprinting involved at this locus, because we had a deletion of apparently the same region resulting in very different phenotypes depending on whether they came from mom or dad,” Lalande says. “Others had earlier shown with transgenes in mice that genetic imprinting probably occurs, but this was the strongest evidence for it in humans.”

There was more to come. In about 25 percent of Prader-Willi patients, no deletions can be found on chromosome 15. Nicholls, Knoll, and others studied six such patients using DNA markers specific for the 15q11q13 subregion. In all six, they found there were two maternal specific gene mutations,” Nicholls told his colleagues who gathered at the American Society for Human Genetics meeting in November 1989. “This implies functional differences in alleles of

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the gene or genes from this region... that depend upon the sex of the transmitting parent. ... It is conceivable that the absence of a maternal contribution to the same 15q11.2-q13 region could result in the different disorder, Angelman syndrome, and that Angelman patients with normal chromosomes may have paternal disomy.”

The last part may be true for a small portion of the 25 percent of Angelman patients who have no detectable deletions. Nicholls, who is now at the University of Florida in Gainesville, says he and Knoll initially found no disomies (in fact, no obvious chromosomal problems at all) in at least ten such patients they studied. However, more recently, Sue Malcolm of the Institute of Child Health in London, as well as Nicholls and others, have found Angelman patients who inherited two copies of one chromosome 15 from their fathers.

In the uniparental disomy cases of Prader-Willi syndrome, Lalande and others speculate that fertilization of an egg with two maternal chromosome 15s results in a trisomy (three of the same chromosome) for chromosome 15. (Trisomies are frequently observed in cases of spontaneous abortion.) Then, however, the male chromosome is lost, resulting in an individual who appears perfectly normal, with two chromosome 15s, but who actually has Prader-Willi syndrome. “You could also argue that you get fertilization with a sperm that for some reason doesn’t have a chromosome 15, but we think this is unlikely,” says Lalande.

Nicholls explains that in the 75 percent of Prader-Willi patients who have large deletions, the breakpoints vary from patient to patient, but there must be common genes. Functions for some genes in this region of chromosome 15 are becoming better known, and because some of the same DNA sequences can be found on a part of mouse chromosome 7, in which imprinting appears to operate, Nicholls is developing a mouse model to study the factors involved in gene expression and imprinting. One of Nicholls’ colleagues in Florida, Dan Driscoll, is concentrating on the human aspect by looking, in both normal persons and patients with the two diseases, for evidence of methylation of the involved area of chromosome 15 in adult tissue, in several stages of fetal tissue, and during spermatogenesis and oogenesis.

In Prader-Willi patients without deletions, Nicholls has confirmed 15 cases of maternal disomy; another eight are not yet completely characterized. In most or all cases, the uniparental disomy involves the entire chromosome 15. Yet there are additional, if still mysterious, exceptions: In a few patients, neither deletions nor uniparental disomy seem to be present.

As for Angelman syndrome, Nicholls says that uniparental disomy may in fact be infrequent, and the disease occasionally may be inherited in Mendelian fashion. Lalande believes there will be multiple mechanisms involved; not just deletion and not just uniparental disomy will turn out to be involved.

How many genes are involved in Prader-Willi and Angelman syndromes? There are several hypotheses. Lalande believes that two genes control each syndrome and that expression of both genes is necessary for normal development. Imprinting, of course, would inactivate alleles, allowing the syndrome to occur. Alternatively, others believe that there is one key gene for each syndrome, or even multiple genes for each.

**Other examples**

Returning to uniparental disomy, there are other examples in which a double dose of a single parent’s chromosomes (or genes) is not equivalent to a single dose of each parent’s chromosomes. For example, J. Edward Spence and Arthur Beaudet of Baylor University, as well as Ruth Voss of Hadassah Hebrew University in Jerusalem, found two patients with cystic fibrosis (a recessive disease) who inherited two copies of one chromosome 7 (the site of the CF gene) from their mothers rather than a single copy of chromosome 7 from each parent. They also had intrauterine growth retardation with no apparent cause, leading the investigators to suspect that the maternal uniparental disomy was a factor in this, too.

Analysis of DNA markers showed that the fathers were clearly the real fathers of these children. Both parents are carriers in most cases of recessive disease. Yet there was no evidence here that the fathers were carriers. Instead, these were cases of an autosomal recessive disease in which both recessive alleles came from the same parent. The mother’s CF chromosome had doubled and the father’s normal chromosome 7, which, after fertilization, had formed a trisomy with the mother’s two chromosome 7s, was lost, helping the early embryo to survive.

“Uniparental disomy must be taken seriously,” says Judith G. Hall, a clinical geneticist/pediatrician at the University of British Columbia in Vancouver. “How often does it cause ‘recessive’ disease?”

“If you do the mathematics in trisomic conceptions,” she continues, “one out of three times you could end up with uniparental disomy, in which two of the same chromosomes come from one par-
ent. It could happen, "she says," with any chromosome."

Among other things, this has implications for genetic counseling. Ordinarily, in the case of a recessive disease in which both parents are carriers, the risk of having a second affected child is one in four. A situation like this throws everything off. Whether imprinting is involved in other syndromes produced by chromosomal trisomies or monosomies (in which there is only one of a specific chromosome) also merits scrutiny, says geneticist Terry Hassold of Emory University.

So far, studying DNA polymorphisms in chromosome 21s of both children with trisomy 21 (Down syndrome) and their parents, an international Down syndrome collaborative group has found that in about 95 percent of cases, the error is maternal. That is, nondisjunction (an error in which eggs or sperm end up with either none or more than one, instead of the usual single chromosome) occurs in the mother, so that two of the three chromosomes in the child are contributed by the mother and the third by the father. The same is always true for trisomy 16, which is the most common of human trisomies and which invariably ends in miscarriage. In Klinefelter syndrome, in which men have two or more x chromosomes (such as XXX), the extra x is maternal in origin 50 percent of the time.

Do the few Down syndrome patients with an extra paternal chromosome differ from those with an extra maternal chromosome? Clinical studies of both living patients and spontaneously aborted Down fetuses (about 80 percent of trisomy 21 fetuses miscarry) show no obvious differences.

Still, Hassold thinks Turner syndrome—in which females have a single x chromosome, are underdeveloped sexually, and have other anomalies—may be a better model of imprinting because only a single element is involved. Although no differences are apparent between patients with a paternally versus a maternally derived x chromosome, Hassold is studying the extra x for differences in methylation or other characteristics affecting gene expression.

Imprinting also may be involved in human disorders in which phenotype, age of onset, or severity varies depending on the gender of the parent transmitting the gene. The best examples are myotonic dystrophy, which is congenital and more severe when inherited from the mother, and Huntington disease, which in five percent to ten percent of cases first appear in childhood rather than in the fourth or fifth decade of life. In 80 percent to 90 percent of these cases, the mutant chromosome is inherited from the father.

Several geneticists have advanced theories about the variation in the age of onset of Huntington disease. For example, Charles Laird of the University of Washington in Seattle has drawn on observations in fruit flies to postulate that the disease is caused by what he calls a "dominant position-effect variegation," in which a chromosome alteration inactivates a nearby gene, called trans-inactivation.

In this case, he proposes that the chromosomal change inactivates both alleles at the Huntington locus and that this modifier of the Huntington gene may be carried on the x chromosome as a rare recessive allele. Different alleles of this modifier carried by the parents can influence the strength of inactivation of the Huntington locus on chromosome 4.

No direct evidence yet supports Laird’s theory of Huntington disease, but its plausibility is supported by recent findings that the appropriate genetic conditions occur in some cells and with some chromosomes. In addition, two human genes with sequences similar to a drosophila modifier gene have been reported to map to the human x chromosome. "This is a more subtle form of chromosomal imprinting—inactivation by a modifier of a locus—that has been seen very clearly in drosophila work and may have relevance to Huntington disease," Laird says. "And application of this combination of position effect variegation and parental effect may have value in explaining the non-Mendelian behavior of other diseases."

Cancers

Nor are cancers left out of the imprinting scenarios. In recent years, considerable evidence has been marshaled to indicate that genetic changes, particularly those that involve various types of inactivation of tumor-suppressor genes, underlie many cancers. Thus, the loss of a chromosome or one of its regions becomes a tool in understanding, identifying, and predicting cancers.

Much of this fits into the "two-hit" hypothesis developed 20 years ago by Alfred G. Knudson, Jr., of Fox Chase Cancer Center in Philadelphia. This theory holds that one event (a hit) that occurs during early development mutates an allele that normally has a cancer-suppressing function at one or more genetic loci in some cells. Later, a second hit knocks out the other allele, and cancer develops in those cells.

Carmen Sapienza of the Ludwig Institute for Cancer Research in Montreal has now neatly modified Knudson’s theory to accommodate an imprinting hypothesis that appears to explain genetic findings in certain pediatric tumors. Such cancers as Wilms’ tumor (affecting the kidneys), embryonal rhabdomyosarcoma (affecting muscles), osteosarcoma (affecting bone), and perhaps retinoblastoma (affecting the eyes) usually retain the paternally derived allele of the affected gene in the tumor tissue, having somehow lost the presumably normal maternal allele that balanced it. This is particularly true of sporadically occurring forms of these tumors.

While conceding that the first hit could be a mutation, Sapienza believes it could just as easily be an imprinting of the paternal allele which renders it inactive. A second hit knocking out the maternal allele would follow. As examples, he cites six out of six sporadic cases of embryonal rhabdomyosarcoma. He and colleagues found that the paternally derived allele on chromosome 11, but not the maternally derived allele, was present in the tumor tissue. Another report showed the same was true for two siblings with the disease. Other investigators have found that in the case of other sporadic tumors (16 out of 18 sporadic cases of Wilms’ tumor and nine out of ten sporadic cases of osteosarcomas) the paternally derived allele also remained in the tumor tissue.

Comparative DNA studies of families show that children with some familial pediatric cancers, particularly retinoblastoma (the gene for which is on chromosome 13), have the disease because they inherited a mutant tumor-suppressor gene from one or the other parent and then suffered a second hit. Yet, in other cancer families, such as two large ones in which Wilms’ tumor is inherited (always from males), no correlation exists between development of the cancer and inheritance of the specific chromosomal segment known to carry one of the genes (recently found on chromosome 11) for Wilms’ tumor.

It is in this second type of pediatric
tumor that Sapienza believes imprinting is operating, but through a modifier imprinting gene, located elsewhere in the genome, that commands that the tumor suppressor gene be imprinted, that is, inactivated. However, since only some people have the cancers and within that group only some cells of the body become cancerous, another factor must be involved. Here Sapienza hypothesizes that the imprint-command genes have different alleles that specify whether imprinting occurs and in how many cells a particular tumor-suppressor allele is imprinted. Examples of this are well documented in fruit flies. Other geneticists have postulated that imprinting also affects glomus body tumors, benign tumors of the head and neck. These tumors are inherited almost exclusively through the paternal line, whether or not the father himself is affected.

A number of other diseases may feature imprinting, according to Judith Hall. One way to find them is to focus on genetic disorders in which causative or associated genes map to areas of the human genome that are similar, in terms of DNA sequences, to regions in the mouse genome where certain genes are known to be imprinted.

**Human/mouse sequences**

An excellent example of such a relationship was recently reported in three articles in the May 31, 1991 issue of *Nature*: one by I. Henry of INSERM in Paris and others that focused on findings in a human disorder called Beckwith-Wiedemann syndrome; one by A. C. Ferguson-Smith of the AFRC Institute of Animal Physiology & Genetics Research in Cambridge and co-workers, which focused on observations in experimentally produced chimeric mice; and one by Melissa Little, Veronica Van Heyningen, and Nicholas Hastie of the MRC Human Genetics Unit in Edinburgh, Scotland, which tied the human and mouse information together.

Beckwith-Wiedemann syndrome involves fetal overgrowth, such as a huge tongue and kidneys, hypoglycemia, and, in a small percentage of patients, development of certain embryonal cancers, such as Wilms' tumor. Both the syn-

**Ragged red fibers.** Longitudinal section of patient's muscle (above) in work of Eric Shoubridge (below), shows hot spots, segmented consequence of overexpression, of mutant DNA caused when the ratio of normal to mutant mtDNA in a tissue changes as a random consequence of mitosis.
drome and the cancers have independently been associated with the 11p15.5 region of chromosome 11.

Henry and colleagues reported that in three out of eight patients with sporadically occurring Beckwith-Wiedemann syndrome there was uniparental (paternal) disomy for region 11p15.5. Furthermore, in 21 patients with the sporadic form of the syndrome, more genes in the 11p15.5 region were present in the homozygous condition than would be expected, suggesting that one chromosome probably had been duplicated. This was especially true for the genes coding for insulin and for insulin-like growth factor-2 (IGF-II), an important growth factor. This finding indicates that many of these 21 patients probably also had uniparental disomy.

Duplication of the paternally derived chromosome has already been found in Beckwith-Wiedemann cases that involve trisomy for chromosome region 11p15. And in Beckwith-Wiedemann patients who develop one of the embryonal cancers, if there is loss of an 11p15.5 allele in the tumors, it is always a maternal allele. All of this strongly suggests the presence of imprinting.

Mouse chromosome 7 and human chromosome region 11p15.5 are termed homologous because their DNA sequences are so similar. In addition, mouse chromosome 7 has several imprinted genes. Ferguson-Smith and colleagues reported in their article that when they made chimeric mouse embryos containing cells that were paternally disomic for part of chromosome 7, the embryos were abnormally large, just as in Beckwith-Wiedemann syndrome. Furthermore, only the paternal copy of the gene for IGF-II was expressed.

Thus, the genes involved in two similar conditions in mouse and man (one experimentally produced, of course) both seem to be imprinted and are located on homologous chromosomes. Both disorders involve uniparental paternal disomy and both possibly involve the same important growth factor. This suggests that a good place to start looking for imprinting in human disease is on human chromosomes homologous to mouse chromosomes where imprinting is already known to occur.

Disorders involving mitochondrial abnormalities have been recognized for about 30 years. But because of the non-Mendelian way in which mtDNA is transmitted, the large number of copies in each cell, and the poorly understood interaction between the mitochondrial and nuclear genomes, diseases involving mtDNA mutations have been identified and discussed by most geneticists only in the past few years.

**Mitochondrial inheritance**

In the early 1960s, neuropathologist Nicholas Gonatas of the University of Pennsylvania and the late neurologist Milton Shy used electron microscopy to describe abnormalities in skeletal muscle mitochondria of some patients. They related the changes to the patients' clinical symptoms, which included droopy eyelids, weak limbs, intolerance to exercise, difficulty moving the eyes (known as chronic progressive external opthalmoplegia, or CPEO), and often degenerative brain disease.

In 1963, W. King Engel, now of the University of Southern California, modified a cellular stain that showed mitochondrial changes in skeletal muscle biopsy samples of such patients. He called these oddly stained muscle fibers, which remain a hallmark of many of the known mitochondrial diseases, "ragged red fibers" (scraggly, dysfunctional fibers that stain red).

Also in the 1960s, several research groups began to describe biochemical defects in mitochondrial diseases. By the 1970s, a crude system of classification existed, explains neurologist Salvatore DiMauro of Columbia University College of Physicians and Surgeons in New York. A defect might occur in transport of substrates from the cytoplasm to the mitochondria, in oxidation of a substrate like pyruvate, or in oxidative phosphorylation, the process that produces adenosine triphosphate (ATP), the body's main energy source.

The year 1988 was a watershed year because specific mtDNA defects finally were linked to diseases. Ian Holt, Anita Harding, and John Morgan-Hughes of the Institute of Neurology, Queen Square, London, reported mtDNA deletions in nine out of 25 patients who had symptoms of mitochondrial myopathy (structurally abnormal mitochondria in skeletal muscle) and biochemical evidence of respiratory enzyme deficiencies. (Respiratory enzymes are those that catalyze terminal oxidation of a substance.) In addition, Douglas Wallace of Emory University showed that a point mutation in mtDNA was the cause of most cases of Leber's hereditary optic atrophy, a disease known to be maternally inherited and to result in optic nerve degeneration and cardiac dysrhythmias, among other problems. Diseases involving mtDNA mutations had arrived.

Currently, about 20 diseases resulting from either mtDNA defects, nuclear gene alterations that affect the general functioning of mitochondria or mtDNA, or other factors are known. Some are firmly classified, their origins clear; others are not. None is common.

To these must be added a list of so-called degenerative diseases such as Parkinson's disease and even normal aging, all of which are common and which some investigators believe are related to dysfunction of oxidative phosphorylation, which results in a decreased availability of ATP.

Oxidative phosphorylation involves the interconnected operation of five respiratory enzyme complexes bound to the mitochondrial inner membrane. Complexes I, II, III, and IV are involved in electron transport; complex V is ATP synthetase, the enzyme that finally assembles ATP, converting it from ADP. The process involves about 70 proteins, 13 of which are coded by mtDNA and the rest by nuclear genes.

**Quantitative factors**

Diseases involving mtDNA defects are at first bewildering, because their expression depends more on quantitative factors—presumably culminating in decreased amounts of ATP available to body tissues—than on the qualitative factors important in Mendelian inheritance.

These quantitative factors, worked out by Wallace and others, stem from the fact that each human cell has hundreds of mitochondria containing two to ten mtDNAs apiece, adding up to thousands of mtDNAs per cell. Within each cell, all of the mtDNA may be either normal or mutant, a situation called homoplasm, or mixtures of normal and mutant DNA may coexist, which is called heteroplasm. The tendency is toward homoplasm, which may favor normal over mutant mtDNA in rapidly dividing cells but not in quiescent tissue cells, where mutant mtDNAs can predominate.

During meiotic and mitotic replication
of cells, however, the mitochondria are randomly distributed to daughter cells, a process called replicative segregation. This means that not only can mothers transmit different proportions of mutant and normal (wild-type) mtDNA to different children—perhaps 50 percent wild-type and 50 percent mutant mtDNA to one child, and 10 percent wild-type and 90 percent mutant DNA to another—but the ratio of wild-type to mutant mtDNA can change in one person's tissues as a consequence of mitosis. Because of replicative segregation, some individuals may inherit sufficient mutant mtDNA to express the phenotype, while others may not. Hence, a mtDNA disease can appear to skip generations and be misidentified as a disorder involving instead an autosomal recessive mutation or even imprinting.

Another quantitative effect follows from replicative segregation and heteroplasmy. Once mutant mtDNAs account for a high enough proportion of the total mtDNA in a cell, the cell begins to express that phenotype. The effect of mtDNA mutations usually is to reduce the amount of ATP produced through oxidative phosphorylation (although some mutations are neutral in this regard), but since differing body tissues rely to varying extents on ATP, the body's reaction to mtDNA mutations is tissue specific. That is, the ATP available to each tissue must drop below a specific threshold before deterioration occurs. For example, it might take many fewer mutated mtDNAs to start the heart on a downward spiral than the liver. This is called the threshold effect.

A final quantitative factor is the high mutation rate of mtDNA—about ten times that of nuclear genes. This becomes especially important, says Wallace, in cells that no longer replicate, such as those of the brain, heart, skeletal muscle fiber, and kidneys. These cells may accumulate damage over the course of a lifetime, in contrast to blood cells, which are constantly replicating and in which there may be selection against mutant mitochondrial genomes.

Deletions

Deletions were the first mtDNA mutations associated with diseases. All nine patients with deletions described by Holt and co-workers in 1988 had CPEO, with or without limb weakness.

Soon after, several other groups found deletions in similar patients. The largest such study, in 1989, was conducted by Carlos Moraes, Salvatore DiMauro, and colleagues at Columbia University and illustrates the complexity of mtDNA diseases. The group found deletions in 32 out of 123 mitochondrial-disease patients whose muscle mtDNA was analyzed; all 32 had CPEO and ragged red fibers. Some also had a spectrum of other problems, including retinitis pigmentosa, heart block, central-nervous-system dysfunction, and what is called Kearns-Sayre syndrome, or KSS, a multisystem disorder that can result in death. None had affected relatives.

The deletions varied in size, but a common one was found in numerous patients. All such deletions are now known to remove a gene for at least one of the mitochondrial transfer RNAs, or tRNAs, which add specific amino acids to growing polypeptide chains during translation of RNA into protein. This suggests that a deleterious effect on all mitochondrial protein synthesis is the cause of the disease, now sometimes called KS/CPEO syndrome. Heteroplasmy also prevails in the affected tissue; homoplasmy for the deleted mtDNA would doubtless be fatal.

The same deletions were present in the patients' other tissues, such as liver and skin cells, although the percentage of deleted mtDNA varied considerably depending on the tissue. The fact that none of the 32 patients had affected relatives indicates that the mutations probably occurred spontaneously in the egg or zygote and then proliferated. The tissues affected and, thus, the severity of the disease depended on the threshold effect and on the stage of development at which the deletions occurred.

In another 21 patients with similar symptoms, no deletions could be found, although half had affected relatives. These patients may have a small, or point, mutation in mtDNA, or even a nuclear DNA mutation that has the same clinical effect. Either could be inherited. Molecular geneticist Eric Schon of the Columbia University group is searching the mtDNA of such patients for defects.

Some patients from families in which at least several members suffer from CPEO have multiple deletions in their mtDNA. These deletions differ among the affected family members, and their disease is inherited not maternally but in an autosomal-dominant fashion. DiMauro's group has found that a number of these families seem to have the same pattern of deletions. A nuclear gene that predisposes the mtDNA to undergo deletions may be behind this, somehow mysteriously establishing communication with the mtDNA.

A newly recognized form of KS/CPEO syndrome begins as a severe anemia in affected infants, who require many

Closing in. At Harvard-affiliated Children's Hospital in Boston, Marc Lalande (center) and colleagues Joan Knoll (left) and Joe Wagstaff track genetic footprints.
transfusions and have liver, pancreatic, and other problems. At first, it appeared that such infants could be saved by heroic supportive therapy. Later, however, Anita Harding reported that one infant who had at first improved developed KS syndrome; the same thing apparently occurred in two other cases as well. The infants were found to have a mtDNA deletion in their blood cells essentially identical to that uncovered in many KS/CPEO patients.

Evidently, the disease first affects the infants' bone marrow, because numerous deleted mtDNAs were present in the marrow. Gradually, the deleted mtDNAs are selected out in rapidly dividing blood cells, and improvement is possible. However, the mutation is also present in muscle as well as the central nervous system, where cells do not divide, and the mutant mtDNA cannot be diluted. Deterioration eventually ensues.

A puzzling aspect of the deletion mutants has been an often poor match between the genetic defect and the biochemical deficiency. Eric Shoubridge and George Karpati of the Montreal Neurological Institute also noticed that mtDNA heteroplasmy in affected skeletal muscles of patients involved normal numbers of wild-type mtDNA along with deleted mtDNAs. Did they coexist in the same cell, or the same mitochondrion? Could the distribution explain the poor fit between biochemical and genetic defects? Why are only some parts of skeletal muscle fibers red? They found that ragged red fibers do contain normal levels of wild-type mtDNA but also a huge number of mutant mtDNAs. The same parts of the muscle show low levels of the affected enzyme.

Effectively, Shoubridge says, abnormal mtDNAs are functionally dominant over normal levels of wild-type mtDNAs in ragged red areas. This explains why the biochemistry does not always match the deletion; it all depends on what part of the muscle is sampled. Shoubridge is attempting to learn why mutant mtDNAs proliferate so much in these areas and how many of them are required to distinguish wild-type expression.

Duplication of a short part of the mtDNA genome has also been reported by Joanna Poulton of Oxford University. The patients have CPEO and diabetes.

**Point mutations**

So far, four diseases are known to be caused by point mutations, either missense mutations that alter an amino acid or protein synthesis mutations that change tRNA genes. Emory's Wallace believes many more will be found. All four diseases can be diagnosed by molecular genetic techniques; the mutation occurs at a site cut by restriction enzymes.

The mutation found in most but not all cases of Leber's optic atrophy changes an amino acid in a complex I subunit gene. Some patients are homoplasmic for the mutation; other patients (and, in some cases, their unaffected relatives) are heteroplasmic. The disease is the same in all those affected, but age of onset and severity vary, unlike KS/CPEO syndrome, in which those affected can have a whole range of manifestations. Wallace believes that other genetic or environmental factors influence the disease's expression.

According to Holt and colleagues, another missense point mutation causes a neurological syndrome that includes retinitis pigmentosa. This syndrome is referred to as neuropathy, ataxia, and retinitis pigmentosa, or NARP.

The two remaining diseases known to be caused by point mutations—in certain tRNAs—are named, awkwardly but descriptively, myoclonic epilepsy and ragged-red fiber disease (MERRF) and mitochondrial myopathy, encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS).

The biochemical and genetic bases of MERRF were described by Wallace and co-workers in 1988 and 1990. The worst symptoms of MERRF, found only in those most severely affected, are dementia and an uncontrolled, debilitating muscle jerking. The mutation, which exists in the heteroplasmic state, affects a tRNA gene, which, in turn, causes deficiencies in complexes I and IV. Most people in the maternal lineage are affected to some extent, although some have only EEG or visual-evoked response abnormalities. Because the mutation has a less severe effect than some other mtDNA mutations, at least 75 percent of the mtDNAs must be mutant before any clinical symptoms appear. Interestingly, the two affected respiratory enzyme complexes have a high number of subunits that are coded by mtDNA as opposed to nuclear DNA. According to Harding, however, not everyone with MERRF has the point mutation.

The mutation that causes MELAS was reported at the end of 1990 by Yu-ichi Goto and co-workers at the National Institute of Neuroscience, National Center for Neurology and Psychiatry in Tokyo. In this disease, which is, in some cases, maternally inherited and in others apparently spontaneous, young people may have recurrent stroke-like episodes that do not correspond to blood vessel distribution or blockage of blood ves-
Fragile x syndrome

The strange disorder called fragile x syndrome was recognized, about ten years ago, to be the most common form of inherited mental retardation, probably accounting for about four percent of all mental retardation. Imprinting, it turns out, may play a role in its transmission.

The term fragile x refers to a gap or actual break in the X chromosome at a location called Xq27. It is seen only under cell culture conditions in which certain precursors of DNA are not available, and it is rarely visible in more than 50 percent of cells.

Not all people who carry the mutation show the fragile x site. Most affected individuals do, but many unaffected carriers show little or no cytogenetic sign of the fragile x, and not all females showing the fragile x site are retarded.

The syndrome is almost twice as common in males as in females. The extent of retardation varies sufficiently that people with the mild form, especially females, are not easy to identify. Most adult males with the syndrome have enlarged testes; some also have behavioral problems or coarse facial features with large ears.

Inheritance of the disorder is bizarre. Geneticist Robert Nussbaum of the University of Pennsylvania points out that daughters of a woman who carries the fragile x mutation but who is mentally normal and does not show the fragile x site have a 16 percent chance of showing the disease (an average 32 percent chance multiplied by the 50 percent risk of inheriting the fragile x chromosome). In contrast, daughters of a so-called transmitting (unaffected, or non-penetrant) male have a zero percent chance of showing the disease. However, male and female children of those daughters—in other words, grandchildren of the nonpenetrant males—are found to be at risk for the syndrome.

"Whether a female has the disease depends on the gender of the parent she inherits it from. Is this imprinting? We don't know," Nussbaum says. Of all the models that have been proposed, it's the most seductive in explaining the inheritance pattern," he adds.

Charles Laird proposed in a 1987 Genetics paper that a mutation at the fragile site leads to a block in expression of a nearby gene or genes on the mutant x chromosome, perhaps by effecting methylation of DNA. This all comes about, he believes, through failure of a normal process in a female. Early in embryonic life, one of the two x chromosomes in a female is inactivated in every body cell, in part through methylation. In germ line cells, this x is reactivated with the consequence that whichever x chromosome enters the zygote will be active.

Laird hypothesizes that imprinting in the fragile x syndrome is manifested as a failure of this reactivation process; that is, a small part of the fragile x chromosome is imprinted by remaining inactive. Transmitting males, their daughters, and some of their grandchildren would, in this scenario, avoid retardation because their fragile x chromosomes were not imprinted in a previous generation.

A male inheriting the imprinted fragile x chromosome could erase the imprint in his sperm, Laird proposes. But daughters of these males can also re-imprint and have affected children. Laird and colleagues also postulate that germ line cell mosaicism in the ovary resulting from random inactivation of the x chromosomes can explain the varying ratios of affected offspring.

An exciting current aspect of research on fragile x syndrome is the identification of the probable gene. Called the fragile x mental retardation-1 (FMR1) gene, it is expressed in brain tissue, as one might expect for a syndrome involving mental retardation, and is nearly as unusual as the inheritance pattern.

Writing in the May 31, 1991 issue of Cell, a large group of European and American investigators reported that the FMR1 gene is in virtually the same region on the x chromosome as the fragile x site itself. It had previously been shown that this fragment of the chromosome also contains, adjacent to FMR1, a stretch that is hypermethylated in fragile x patients and in females, on the normal x chromosome, when it becomes activated, but not in normal males. (Hypermethylation of genes usually renders them inactive, or unable to express.) Furthermore, in fragile x chromosomes there is a variable increase in the size of a restriction fragment, as compared with normal x chromosomes.
found depleted rather than deleted mtDNA. That is, mitochondria within the affected tissues were functional but contained virtually no mtDNA. The group has now found other children with this depletion disorder, and Eduardo Bonilla of the group has developed an antibody test for DNA in muscle sections that can aid in diagnosing the disease.

Studies of pedigrees of two families with the disease suggest autosomal recessive inheritance, says DiMauro, so a nuclear gene change may cause mtDNA depletion. Further study of this phenomenon may yield some insight into fundamental genetic mechanisms responsible for regulating mitochondrial copy number, says Schon.

Exposure to some chemicals can also deplete mtDNA. Patients taking AZT for AIDS can develop myopathy, ragged red fibers, and weakness. Mel Simpson of the State University of New York at Stonybrook found that the drug, a nucleotide analogue, can inhibit mtDNA replication by fooling the enzymatic machinery into using it instead of the normal nucleotide. Schon and co-workers have now demonstrated that this effect is reversible if the drug is stopped.

If it seems that mitochondrial diseases have overlapping phenotypes and

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Fragile X: Imprinting may play role in transmitting disease caused by a break in the X chromosome

somes, which suggests that something is inserted or amplified in quantity. Finally, the researchers discovered that within the methylated section and the FMR1 gene there is a "highly unusual" trinucleotide repeat sequence made up of cytosine and two guanines (CGG). It is likely that by amplifying itself, the CGG repeat sequence may form the fragile X mutation.

The function of FMR1 is as yet unknown, although it is likely to be quite significant, as the gene is highly conserved in evolution. Also unknown is the way in which all components operate on a molecular basis. For example, is the mutant FMR1 gene inactive in affected males as predicted by the imprinting model, or does it make an altered gene product as indicated by other models?

It is now possible, however, to use these findings to diagnose the disease prenatally. The involved fragment or area of a fragile X chromosome usually will show methylation in affected individuals and be quite large as compared to normal X chromosomes.

These new findings were initially facilitated by the work of Stephen T. Warren (one of the authors of the Cell study) of Emory University who, in 1987, devised an experimental strategy that laid the groundwork for isolating, on the X chromosome, the area containing the fragile site. Since then, his laboratory and a number of others, such as those of Grant Sutherland of Adelaide Children's Hospital in South Australia, Kay Davies in Oxford, England, and Jean-Louis Mandel of INSERM in Strasbourg, France, have cloned large fragments of the fragile site and have placed them into yeast artificial chromosomes (a method of growing up large quantities of large chromosomal fragments). Smaller pieces of DNA have been isolated from these for study, and some differences, most of which involve methylation, have been detected between affected, transmitting, and normal males. Most affected males show complete or partial hypermethylation, but nontransmitting males and normals do not. In addition, there is the correspondence between fragile X methylation patterns in affected males and inactive X chromosome methylation patterns in normal females, making diagnosis of females by methylation pattern more difficult.

It may be that the methylation is secondary to events taking place in the fragile site, Warren says, and does not actually influence expression of the disease. But secondary methylation events would not explain the apparent persistence of the same methylation pattern imposed by a female during X inactivation.

Referring to the CGG repeat sequence that was recently discovered, Warren believes that a model proposed five years ago may be correct. In that model, as Ledbetter and Nussbaum summarized in a 1987 Science article, "the fragile X site is a reiterated DNA sequence of variable length, the longest length being found in fully penetrant males and the shortest in phenotypically normal individuals who would carry a common fragile site at Xq27...." A small insertion or expansion of the CGG repeat may be the mutation that causes failure of X-chromosome reactivation and leads to imprinting, Laird says. But how does the additional increase in size occur in affected individuals, and how does it relate to the proposed imprinting process? Further molecular work will be required to answer these questions. • Gail McBride & Joann Rodgers
Inheritance of two different maternal chromosome 15s

Gametes

MII NDJ

Zygote

Somatic Tissue

Heterodisomy

Selectively loss of paternal chromosome

Isodisomy

Inheritance of the same maternal chromosome 15s

Uniparental disomy. In some cases of Prader-Willi syndrome, child inherits two copies of chromosome 15 from mother (isodisomy) or two different chromosomes from mother (heterodisomy). In both bases, paternal contribution is blocked.

that even people within pedigrees have variable phenotypes, it is true. "With mitochondria," says Wallace, "the concept of one gene: one enzyme: one phenotype gets thrown out. You start with thousands of copies of the same gene. Then each protein in the enzyme complex works in an aggregate with multiple different proteins to accomplish one function. Finally, the phenotype is a quantitative one related to the extent of the enzyme's defect and the tissues in which it occurs, so you have different phenotypes for the same mutation.

Variation and overlap

"Because of replicative segregation and heteroplasmy which can vary in different tissues of an individual, you get varying phenotypes for the same disease—phenotypic overlap. In our large MERRF pedigree, only two people have frank myoclonic epilepsy, but many are deaf or have muscle disease and one has migraine," he says, adding that classification of these diseases must become genetic. Disease classification should help with treatment, too. Currently available treatment is based on the assumption that everything is due to a paucity of ATP, and that one or more respiratory complexes is not operating well. Hence, mitochondrial ATP production needs boosting.

To accomplish this, electron shuttling substances, such as ubiquinone (coenzyme Q), have been given, and/or succinate, riboflavin, vitamin C, or a vitamin K compound. All act as surrogate electron transport molecules in specific areas of the respiratory chain. Coenzyme also stabilizes the inner mitochondrial membrane and helps eliminate free radicals that are generated as a result of membrane damage. Patients with KS/CPEO syndrome, MERRF, and MELAS have been reported to show improvement when treated with various of these agents, but as yet there have been no controlled trials.

Many investigators interested in mtDNA and oxidative phosphorylation diseases are now tackling new areas: degenerative diseases affecting the heart, brain, kidneys, peripheral nervous system—organ systems that rely heavily on mitochondrial energy but degenerate with age. "We see all these diseases as a continuum—as a complex association of genetic predisposition to ATP reduction, environmental stress that can also inhibit mitochondrial function, and aging that also results in mitochondrial decline," says Wallace.

Recently, in fact, Shoffner, Wallace, and co-workers have found deficiencies in respiratory enzyme complexes (used for oxidative phosphorylation) in skeletal muscle samples of five or six patients with Parkinson's disease. The differences in enzyme activities between the patients and 16 control subjects were statistically significant. Two of the patients had relatives who suffered with Parkinson's disease.
No mutations in mtDNA were apparent. But brain changes similar to those in patients with Parkinson's disease are found in a number of mtDNA diseases.

Shoffner and colleagues believe that the defects in oxidative phosphorylation inhibit electron transport through the enzyme complexes, thereby causing a decrease in cellular ATP production, and increase free radical production. The latter would be especially toxic to the brain (nigrostriatal dopaminergic pathway) that shows damage in the disease. All the effects increase with age, they believe, and could be enhanced by genetic predisposition and exposure to environmental factors. For example, symptoms very similar to Parkinson's disease can result from exposure to a chemical, MPTP, and to carbon monoxide and cyanide, all of which inhibit these respiratory enzyme complexes.

The lack of good model systems has impeded the study of mtDNA. Though spontaneous animal models do occur, from rats to racehorses, few have been recognized and studied.

A transgenic mouse with a mtDNA mutant would be helpful because it could reveal how mutant mtDNAs assort in different tissues and whether they have a replicative advantage in certain tissues or at certain stages of development. But genes cannot be inserted into mitochondria as they can into the nucleus (after placement into the cytoplasm) because it is difficult to get anything through the mitochondrial double membrane. Moreover, mitochondrial enzymes do not allow recombination with exogenous DNA as do nuclear enzymes. Many investigators are trying to use a gun containing tiny tungsten pellets coated with DNA to transform mitochondria, although the fact that the DNA can instead be taken up by the nucleus is a drawback.

The next best thing may be a line of human skin cells that have mitochondria but not mtDNA, similar to deletion mutants. These are called rho-O (respiratory deficient) cells, and they result when a drug called ethidium bromide is added to cells. It sits on the mtDNA and prevents its replication; eventually the mtDNA dies out. At this point, mitochondria containing mtDNA from patients are added to the system and studied without any contaminating background mtDNA from the host cell.

Mosaicism is a little different from the other mechanisms described in that it does not necessarily remain non-Mendelian in subsequent generations. When germline cells are affected by an event occurring early in development, for instance, the change can then be inherited in Mendelian fashion.

Mosaicism

Judith Hall tells of a family in which the parents appeared normal, with normal chromosomes, but their children, a boy and girl, had pseudoachondroplasia (a disease involving short stature, limitation of elbow movement, and other anomalies). "Pseudoachondroplasia usually appears as a new dominant mutation, and it's almost unheard of for it to occur twice in one family," she says. "We decided this family must have an unusual recessive form, and although in such cases each child has only a 25 percent chance of getting the genes from both parents, it happened."

The brother grew up and married an average-sized woman. "Since we believed he had a recessive form of the disease, and that it was highly unlikely his average-sized wife would be a carrier of the same gene for short stature that he had, we thought any children they had would at most be carriers of the disease," Hall says. "Well, we were wrong! The couple had a daughter with pseudoachondroplasia."

"Then and there I began to think more about mosaicism."

The changes involved in mosaicism, in which some cells differ from the majority of other body cells by a single gene or even by a whole chromosome, probably can occur anytime after conception, during embryonic or fetal life, or after birth. Depending on when the changes occur, cells harboring these genes or chromosomes may die out, coexist with other cells, or entirely take over in one area, exerting certain limited effects on the body, such as overgrowth of certain parts or cancer.

Mosaicism often goes undetected, however, because chromosomes are commonly studied in only one type of body cell on the assumption that all others are the same.

Hall and other investigators, such as R. Happle of the University of Nijmegen in The Netherlands, believe that some poorly understood, sporadically occurring disorders, such as McCune-Albright syndrome (characterized by sexual precocity as well as café-au-lait spots and bone problems in girls), may be the result of mosaicism. If all the cells had the mutant gene present, the effect might be lethal.

Of course, half the population is functionally mosaic with respect to the X chromosome. Thirty years ago, Mary Lyon of the MRC Radiobiology Unit in Harwell, England, showed that normal women have some cells in which most of the paternal derived X chromosome is active and the maternally derived one is not, while in other cells, much of the maternally derived X is active and the paternally derived one is not.

When mosaicism affects germline cells, the outcome can be totally unexpected. In the family with pseudoachondroplasia, for instance, Hall says the paternal grandfather may be "a mosaic." Although most of his cells do not have the gene for pseudoachondroplasia, some appear to, including some or all of his germline cells. This could account for transmission of the gene to his daughter and to his son, who in turn transmitted the gene to his child. Later close examination of the father revealed that despite his normal appearance, he had some limitation of elbow movement as do people with full-blown pseudoachondroplasia.

Peter Byers and co-workers at the University of Washington have reported several cases in which two children in a family have been born with a new, dominant—and deadly—form of osteogenesis imperfecta, a disorder affecting type I collagen in the body. By examining tissue obtained after death of the newborns, Byers found the siblings had an identical new mutation in one of the genes coding for type I collagen. Yet skin cells of the parents showed only normal collagen.

In one of the cases, a man had two such children by different wives. Molecular geneticist Daniel Cohn of the City of Hope Medical Center in Duarte, California, found that the father carried the same mutation as his dead children, but in fewer cells. These cells included lymphocytes and, unfortunately for the children, his sperm.

All of this means, Hall points out, that occasionally, the recurrence risk of a condition thought to be a new dominant mutation may be higher, sometimes much higher, than previously thought. Thus, in inherited diseases, as in other areas of life, appearances can be deceiving. Classification of inherited diseases by specific genetic defect rather than by phenotype is leading the way.