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AN ANALYSIS OF MITOCHONDRIAL DNA IN

RETT SYNDROME AND OTHER NEURODEGENERATIVE DISORDERS

by

Catherine Erickson Burgess
B.S. June 1987
Old Dominion University

A Dissertation submitted to the Faculty of
Eastern Virginia Medical School
and
Old Dominion University
in Partial Fulfillment of the Requirement for the Degree of

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EASTERN VIRGINIA MEDICAL SCHOOL
and
OLD DOMINION UNIVERSITY
December, 1994

Approved By:

Frank J. Castora, Ph.D., Director

Christopher J. Osgood, Ph.D.

Mark S. Elliott. Ph.D.

Bruce W. Tedeschi, Ph.D.

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ABSTRACT

AN ANALYSIS OF MITOCHONDRIAL DNA IN
RETT SYNDROME AND OTHER NEURODEGENERATIVE DISORDERS

Catherine Erickson Burgess

Eastern Virginia Medical School
and
Old Dominion University
1994

Director: Frank J. Castora

Mitochondrial dysfunction resulting from mutations on mitochondrial DNA (mtDNA) is being recognized in a growing spectrum of diseases. These diseases, resulting from single base mutations, large deletions, or insertions, have been largely neuromuscular in origin. However, as an understanding of the effects of mtDNA mutations progresses, attention is now focusing on neurodegenerative diseases. Rett Syndrome (RS), a progressive neurodegenerative disease with predominantly female cases, demonstrates morphologic mitochondrial changes, mitochondrial enzyme deficiencies and maternal inheritance (characteristic of mtDNA diseases). No investigation of mtDNA involvement has been previously conducted and, to date, no biological marker exists for this disorder.

Our preliminary studies in mitochondrial myopathies indicated that mtDNA could be detected from limited amounts of blood, amniotic fluid, and single human lymphoblasts. Multiplex PCR, restriction digestion and single cell sequencing were used

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to identify the LHON (Leber's Hereditary Optic Neuropathy) mutation in control and LHON single lymphoblasts. We then examined the state of mtDNA heteroplasmy in the heteroplasmic disease MELAS (mitochondrial encephalomyopathy, lactic acidosis, strokelike symptoms) in single human lymphoblasts and identified an intercellular distribution of mutant and normal mtDNA. This differs from analyses performed in LHON and MERRF (myoclonic epilepsy, ragged red fibers) pedigrees which demonstrate a predominantly intracellular distribution. This may implicate MELAS as a homoplasmic-lethal mutation.

We then defined a procedure to identify single base mutations within the mitochondrial genome. Mitochondrial myopathy patient samples were used. The procedure employs DGGE (denaturing gradient gel electrophoresis) to define an area of interest within the genome. PCR amplification and SSCP (single-stranded conformational polymorphism) analysis were used to localize the mutation to a 200bp region which was then sequenced to identify the lesion.

Rett Syndrome demonstrates a multicomplex effect of the respiratory chain enzymes indicating a possible translational dysfunction. The above approach was employed in an analysis of the 22 mitochondrial tRNAs for mutations relating to RS. SSCP analysis identified a number of conformational changes which, following DNA sequencing, proved to be non-specific for the disease. Sequencing of additional regions of the mtDNA confirmed the SSCP results indicating that a mutation in the mitochondrial tRNAs is not associated with Rett Syndrome.

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DEDICATION

This work is dedicated in no small way to the contributions of three people. First and foremost I would like to dedicate this dissertation to Dr. Frank Castora. Dr. Castora, you taught me to believe in myself when I wasn't even sure who I was. In all honesty, who I am and what I achieve in the future is due to the guidance, enormous patience and determination you provided during these graduate years.

Secondly, I would like to dedicate this work to my husband, David. Your love support and encouragement has kept me sane through all this. For me, you are a scientist, a cohort, a companion and my friend. I look forward to sharing the rest of my life with you.

And finally to Angela Seenes, a Rett Syndrome girl I had the privelege of meeting through the assistance of Dr. Don Lewis. I carry her smile with me everyday and the real understanding that the disease is more than just the science.

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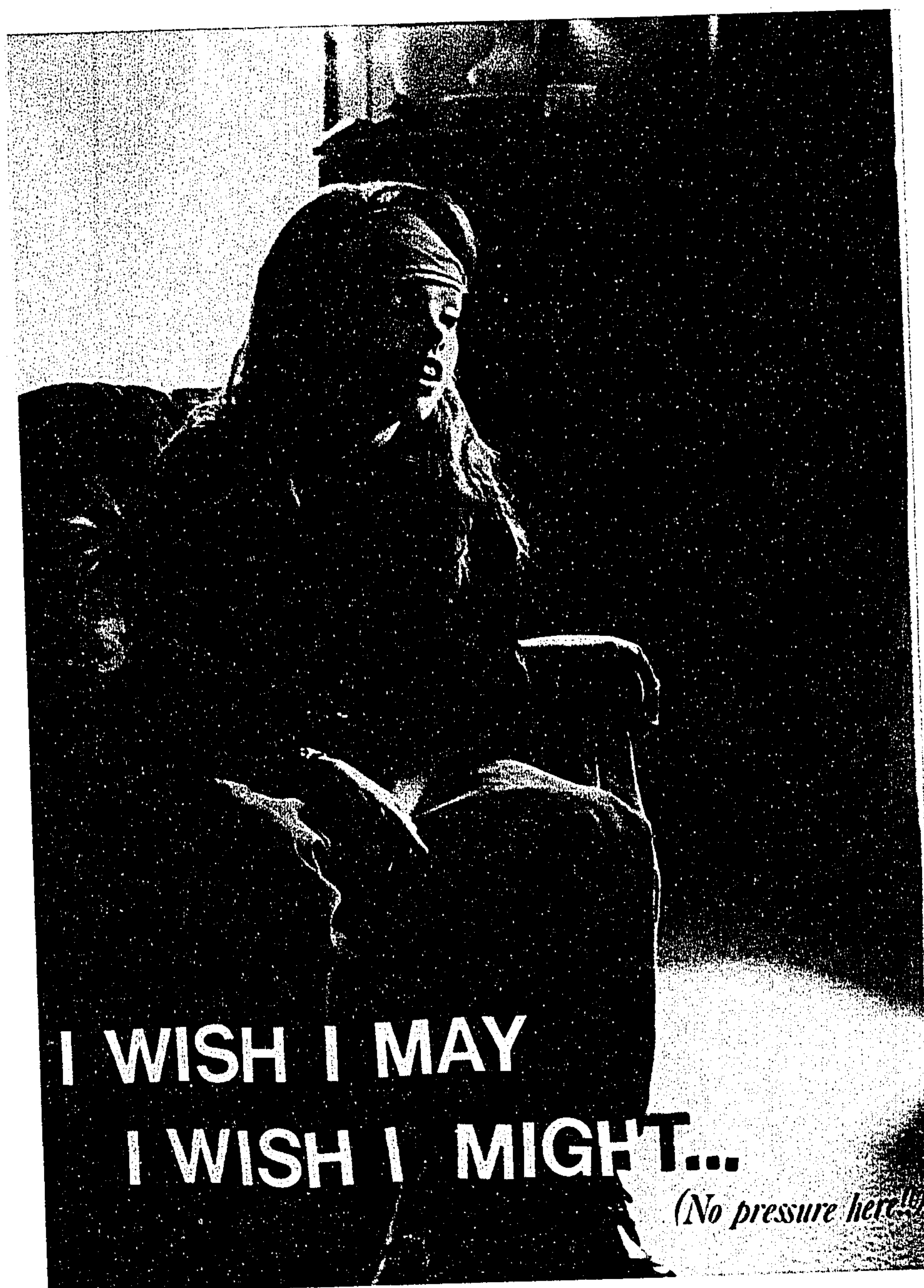

Angela Seenes

(A Gift From Her Parents)

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So many people have contributed to my research in both direct and indirect ways it is hard to acknowledge all their efforts. I would have to say, however, that Natasha Hamblet tops the list. Her patience seems beyond exhaustion - and that is no small feat with me in the lab. Her random and willing discussions on all aspects of my research (and often times my life) provided me with more valuable insight than I can truly appreciate. I wish all graduate students had such an accomplice with whom to share their successes and failures. Thank you.

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CHAPTER I

A. Background and Significance

Mitochondrial dysfunction resulting from mutations on mitochondrial DNA (mtDNA) is now being recognized in a growing spectrum of diseases from muscular dystrophy (1,2,3) to diabetes mellitus (4). These mutations, resulting from a variety of factors including oxidative damage and error-prone replication, become permanently incorporated into the mtDNA due to the lack of DNA repair mechanisms (5). Because cells contain many mitochondria and each mitochondrion contains two to ten mtDNA, cells can harbor mixtures of mutant and normal mtDNAs (heteroplasmy). Each time such a cell divides, the mutant and normal mtDNAs randomly segregate into the daughter cells (5). Over multiple divisions, the population of molecules can drift toward either predominantly mutant or normal mtDNAs. The severity of the clinical manifestations is a product of the nature of the mtDNA mutation and the proportion of mutant mtDNAs within the cell (6). Thus, such genetic heteroplasmy can result in a diversity of clinical manifestations, even between genetically-linked individuals.

Mitochondria are cytoplasmic, DNA-containing organelles that occupy a pivotal position in cellular energy metabolism. They are the main site of ATP synthesis in aerobic cells using the free energy created by the oxidation of metabolic fuels. Human

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mtDNA is a 16,569 bp, double-stranded, closed circular molecule encoding a large and small rRNA, 22 tRNAs and 13 proteins involved in respiratory complex formation by complementation with nuclear gene products (7,8). The 13 stable mRNAs are translated on mitochondria-specific ribosomes into 13 proteins forming subunits of the oxidative phosphorylation system. These include seven of 25 subunits for NADH-ubiquinone reductase (Complex I), one (cytochrome b) of ten subunits for ubiquinone-cytochrome c reductase (Complex III), three of 13 subunits for cytochrome c oxidase (Complex IV), and two of 14 subunits of the ATP synthetase. The remaining subunits are imported into the mitochondria after synthesis on cytoplasmic ribosomes from genes encoded by the nuclear DNA (9). MtDNA is not complexed with histones and mtDNA replication is not limited to a particular phase of the cell cycle. Several investigators have hypothesized that these factors make mtDNA more susceptible to mutagenesis (10,11,12,13,14).

Each mitochondrion contains two to ten mtDNAs and each cell can contain up to thousands of mitochondria. The genetics of mitochondria has several unique characteristics including maternal inheritance and replicative segregation. Following a mutational event (i.e. insertion, deletion, or substitution), heteroplasmy of the multiple mtDNAs occurs and, during cell division, the mutant and normal genomes are randomly segregated into the daughter cells resulting in a mosaic of mtDNAs. The severity of an OXPHOS defect resulting from such a mosaic is a product of the nature of the mtDNA mutation and the proportion of the mutant mtDNAs within the cell. The phenotype of a patient is therefore related to the severity of the defect and the energetic thresholds of the various organs and tissues involved (the higher the mutant percentage and the more

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energy-dependent the tissue, the more severe the symptoms). It is also noted that mutations in a variety of genes can cause the same phenotype once a threshold of mutated mitochondria is reached (15,16,17). For example, Leber's hereditary optic neuropathy (LHON) is a mitochondrial disease characterized by sudden onset vision loss. Four mtDNA point mutations occurring in different mitochondrial genes (both tRNA and protein-coding genes) have been identified as causative LHON mutations (15). In Kearns-Sayre syndrome (KSS), a variety of mtDNA deletions ranging from 2.0-7.0 Kb (depending on the individual) have also correlated with the clinical presentation of KSS when the mutant mtDNA population is greater than 45% of the total mtDNA (17).

Characterizing alterations of mitochondria, both morphologically and biochemically, remains the most useful line of investigation in suspected mitochondrial disease (18). Morphologically, these disorders are characterized by various structural changes usually found in muscle mitochondria. These include large aggregates of abnormal mitochondria, usually under the subsarcolemma (19), abnormal cristae, mitochondria with inclusions such as crystalline structures or globular bodies (20) and ragged red fibers (RRF) resulting from subsarcolemmal proliferation of mitochondria and detectable by modified Gomori Trichrome stain (14). Mitochondrial abnormalities, however, are not restricted to diseases resulting from primary errors of mitochondrial metabolism. Furthermore, the described morphologic abnormalities are not an obligate finding in association with a mitochondrial dysfunction (18,21).

Biochemical investigation of mitochondrial metabolism allows classification of enzyme activities into five groups (22): 1) Disorders of substrate transport involving

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carnitine and carnitine palmitoyltransferase (CPT) deficiencies; 2) Defects of substrate utilization involving pyruvate carboxylase or pyruvate dehydrogenase deficiencies or defects of beta-oxidation; 3) Defects of the Krebs cycle with fumarase or alpha-ketoglutarate deficiency; 4) Defects of oxidation-phosphorylation coupling; 5) Defects of the respiratory chain affecting one or more of the five complexes. Of these categories, only group five involves mitochondrial DNA. And of the five respiratory complexes comprising the OXPHOS system, biochemical analysis can distinguish monoenzymopathies involving single complexes from multienzymopathies involving multiple complexes. Although the precise relationship between clinical and biochemical phenotypes and mutations remains to be defined, defects of respiratory chain complexes illustrated in many diseases are raising the question of mtDNA involvement in a variety of neuromuscular and neurodegenerative disorders. For example, a reduction of complex I activity has been found in autopsy specimens of the substantia nigra and in platelet mitochondria from Parkinsonian patients (23,24,25). Additionally, there is evidence of a complex I deficiency in Huntington's disease (26) and a multicomplex involvement in Alzheimer's disease (27).

Mitochondrial DNA has been found to mutate at a much higher rate than nuclear DNA, accumulating as much as sixteen times more oxidative damage (12,28) and evolving five to ten times faster than single-copy nuclear genes (29). Additionally, repair of mtDNA damage is much less efficient than in the nucleus (30) since mitochondria appear to lack effective recombinational or excision repair systems (12,13). Mutations can take the form of deletions, insertions, and point mutations with a high

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transition/transversion ratio and a strong bias toward C->T transitions in the light strand (31). These mutations have been associated with impaired mitochondrial respiration and decreased bioenergetic capacity and protein synthesis (14,32). This vulnerability of mitochondrial DNA to genetic damage is now being demonstrated in many human diseases. For example,conditions associated with deleted mtDNA include Kearns-Sayre syndrome (33), Pearson's marrow/pancreas syndrome (34), Diabetes Mellitus (4), hypoxemia (35), cirrhotic liver surrounding hepatic tumors (36), cardiomyopathy (37), and even in normal ageing tissue (38). Many of these deletions map to the heavy mtDNA strand which is single-stranded during the first part of the mitochondrial DNA replication cycle (39). It has been suggested that this single strand may be the target of mitochondrial DNA deletions (1). This may occur if the single strand twists onto itself prior to the passage of the gamma polymerase, the mitochondrial replicative polymerase (12), resulting in a looping-out of a portion of the heavy strand. Point mutations are believed to be the result of oxidative damage (10,11,12,13,14,32) and spontaneous mutation arising in part from the 1/7000 insertion error rate of the gamma DNA polymerase. Point mutation diseases resulting from alterations of conserved amino acids on protein-coding sequences, detected as monoenzymopathies, include Leber's Hereditary Optic Neuropathy (LHON) (40), Neurogenic Muscle Weakness, Ataxia, and Retinitis Pigmentosa (NARP) (41) and Leigh's disease (42). Point mutations altering conserved nucleotide pairs in tRNAs, and detected as multienzymopathies due to their affect on mitochondrial protein synthesis, include Myoclonic Epilepsy with Ragged Red Fibers (MERRF) (2), Mitochondrial Encephalomyopathy with lactic acidosis and strokelike

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episodes (MELAS) (3), and Fatal Infantile Cardiomyopathy (FIC) (43). Additionally, free radical damage of mitochondrial DNA is now being linked to multiple disorders including carcinogenesis and ageing (6).

As an understanding of the effects of mtDNA mutations progresses, attention has focused on neurodegenerative as well as neuromuscular disorders. Rett Syndrome, a progressive neurodegenerative disease with predominantly female cases (44), demonstrates morphologic mitochondrial changes (45,46,47) as well as mitochondrial enzyme deficiencies (48,49). To date, however, no mitochondrial DNA analysis has been performed to investigate these occurrences.

Rett Syndrome

Rett Syndrome (RS) is a phenotypically distinct progressive neurologic disorder characterized by notable neurologic impairment including autistic behavior, dementia, seizures, acquired microencephaly, spasticity, hyperreflexia, breathing disorders and peculiar, stereotypic hand movements. It has been reported almost exclusively in females with an estimated occurrence of one in 10,000-12,000 live births (50). RS is approximately twice as common as phenylketonuria and may be responsible for one-fourth to one-third of the progressive disabilities among girls. The disease was first described in 1966 by Andreas Rett (51) but, due to its limited exposure only in German medical literature, it was not recognized as a nosologic entity until 1983 when Hagberg, et al (52) published their account of 35 girls demonstrating Rett Syndrome in the journal

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Annals of Neurology. Hagberg and Witt-Engerstrom (53) proceeded to characterize the disease into four clinical stages defined by the sequentially appearing characteristic features (Table 1). Stage one, early onset stagnation, occurs after 6-18 months of apparently normal development although some subtle earlier signs such as congenital hypotonia (54) and dyskinetic disturbance of hand movement (55) have been reported. Onset is characterized by a general delay of developmental progress, most specifically, the motor development. Stage 2, regression, involves a rapid deterioration of acquired skills as well as almost autistic withdrawals occurring at 1-2 years. Stage 3, a pseudostationary stage, begins a partial recovery with diminishing autistic symptamology (56) and increased social interaction. Neuromotor functions, however, continue to steadily decline until the patient becomes non-ambulatory and enters stage 4. Stage 4 involves late motor deterioration and is characterized by progressive muscle wasting, scoliosis, and growth retardation. Diagnosis of Rett's is based solely on evaluation of clinical presentation. To date, no biological marker has been identified and, therefore, a definitive diagnosis cannot be made until the patient is two to three years of age (stage 3) (57).

Morphologic Evidence for Mitochondrial Involvement

Major emphasis has been devoted to identifying both morphologic and biochemical abnormalities in Rett Syndrome patients. Morphologic examination of the nervous system demonstrates, macroscopically, a reduction in the size and weight of the

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TABLE 1: The four clinical stages of classic Rett Syndrome as defined at the 1985 Workshop on Rett Syndrome in Baltimore.

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TABLE 1
The four clinical stages of Classic Rett Syndrome

Stage I: Early Onset Stagnation

Onset age: 6 months to 1.5 years
Developmental progress delayed
Dissociated development
Early postural delay
"Bottom Shufflers"
Developmental pattern still not significantly abnormal
Duration: weeks to months

Stage II: Rapid Developmental Regression

Onset age: 1-4 years
Loss of acquired skills/communication
    fine finger; babble/words; active playing
Mental deficiency appears
Eye contact preserved
Seizures in 15%
Modest breathing problems
Duration: weeks to months, possibly one year

Stage III: Pseudostationary Stage

Onset: after passing stage II
Some communacative restitution
Prominent hand apraxia/dyspraxia
Apparently preserved ambulatory ability
Inapparent, slow neuromotor regression
Duration: years to decades

Stage IV: Late Motor Deterioration

Onset: when stage III ambulation ceases
Complete wheelchair dependency
Severe disability: wasting and distal distortions
Duration: decades

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brain (hypoplasia) of the RS patient. The weight reduction varies between 12 and 41 percent below age-matched controls (58,59,60,61,62,63). Quantitative neuroimaging studies of RS patients show a significantly reduced cerebral volume with evidence of a greater loss of grey matter (64) while Armstrong and Caviness noted decreased dendritic branching and synaptic density most prominently associated with the temporal lobe (65,66). Kitt, et al (67) also found a neuronal reduction in the basal forebrain with the cerebellar cortex more severely affected than the cerebral cortex. Accumulations of large and disintegrating mitochondria have been identified throughout the white matter and in the caudate nucleus. Investigations of muscle biopsies from other RS patients has revealed numerous accounts of swollen or dumbbell-shaped mitochondria (46,68,69,70,71,72) even within peripheral nerve biopsies (46). Eeg-Olofsson, et al, in 1989, found abnormal, swollen dumbbell-shaped mitochondria with "concentric laminated bodies" (69). No ragged red fibers (characteristic of mitochondrial encephalomyopathies, i.e. MERRF - myoclonic epilepsy with ragged red fibers) were detected. Completely normal mitochondria were found in muscle sections containing abnormal mitochondria. This characteristic may be indicative of mitochondrial heteroplasmy wherein a mixture of normal and mutant mitochondria coexist within the same tissue. Halestrap has proposed that the abnormality in mitochondrial size may be due to an ATP-decrease effect on the ATP-regulated potassium channel within the inner mitochondrial membrane (73). This channel may provide the locus for matrix volume regulation with high pyrophosphate (PPi) levels (due to increased ATP consumption) resulting in abnormal swelling of the mitochondria. Sustained high levels of PPi could be due to a dysfunction

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in the OXPHOS pathway resulting from a mtDNA mutation affecting the mitochondria's ability to replenish its ATP stores.

Enzymatic Evidence

The first year of life is a decisive time for human brain maturation. In the developing human brain, at approximately one year of age, there is a dramatic increase in RNA synthesis accompanied by a corresponding increase in ATP production (74). Brain development during this time is associated with regional changes in glucose cerebral metabolic rate (75) which is reflected by changes in regional cerebral blood flow (rCBF) (76,77). Between the ages of six and eighteen months, rCBF levels increase dramatically in several areas of the brain including the temporal pole (associated with language comprehension and sounding out of words), the prefrontal associative cortex (associated with intellect, complex learning abilities, personality and language comprehension), Broca's area (premotor and motor speech area), the thalamus (associated with sorting out, information editing, sensation and motor activity, cortical arousal and memory), and the vermis and cerebellar hemispheres (associated with processing inputs from the cerebral motor cortex, also provides timing for smooth and coordinated movements - damage in this area results in a loss of muscle tone and clumsy, disorganized movements). A deficiency in ATP-production due to an OXPHOS defect could result in little or no development of these brain regions during this brain 'growth spurt'. Analysis of neuroanatomical changes that might correlate with the devastating

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effects of Rett Syndrome has proceeded on many fronts. Investigations using magnetic resonance imaging (MRI) and computed tomography (CT) shows a small RS brain with some cortical atrophy (78,79,80) while neuropathology has revealed diffuse brain growth arrest and atrophy (60,61,63,81,82). The application of rCBF measurement techniques on RS girls has indicated a preserved immature pattern of frontal lobe hypoperfusion (83). Further analysis using SPECT (single photon emission computed tomography) (84) supported previously identified frontal lobe hypoperfusion but found midbrain/brainstem and cerebellar hypoperfusion as well. SPECT scans of the regional cerebral blood flow, investigated in a two and one-half year old RS patient and an age-matched control, revealed a pattern of frontal lobe hypoperfusion distinctly similar to that of a seven week-old control with a midbrain/brainstem hypoperfusion unique to the RS patient. The midbrain/brainstem dysfunction, indicated in the RS patient by breathing irregularities, sleep disturbances and auditory evoked response measurements (85,86,87), could result from this severely reduced perfusion.

A study of cerebral blood flow and oxygen metabolism in 1992 suggested that RS was also associated with impaired oxidative metabolism (88). Yoshikawa found that the oxygen extraction fraction (OEF) was reduced in four of his six RS cases studied. (Case one, the youngest and Case three, an atypical case showing relatively mild symptoms, were near normal values.) The decreased OEF value is also known to correlate with mitochondrial encephalomyopathies. Yoshikawa found that there was a tendency for the OEF values to decline with advancing age with the older children showing a greater deficiency than the younger - a common occurrence in mitochondrial disorders.

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Additionally, he found that as oxygen metabolism disturbance increased, the relative developmental age of the patient decreased. He proposes that, due to the similarities between OEF and CBF ratios in RS and mitochondrial cytopathies, a mitochondrial dysfunction must be considered. Other carbohydrate metabolism investigations have revealed significantly elevated lactate and pyruvate levels (72,89), a possible result of inhibited respiration. Matsuishi suggests that NADH-ubiquinone oxidoreductase may be deficient (89). Investigation of mitochondrial respiratory chain enzymes was also conducted. Using light and electron microscopy on three RS patient samples, Coker and Melnyk (48) found no alterations in ultrastructural morphology. However, enzyme assays revealed low levels of cytochrome c oxidase and succinate cytochrome c reductase in all cases. The oldest patient, approximately ten years senior of the other patients, revealed marked abnormalities in all four enzymes tested, evidencing a possible age-related degenerative disorder. Additional studies of mitochondrial respiratory chain enzyme impairment were also conducted by Dotti, et al (49) on two four-year-old Rett Syndrome girls. Their analysis shows a clear indication of respiratory chain impairment in both of their mitochondrial preparations, a result consistent with that of Coker and Melnyk. In contrast, however, Haas, et al (90) recently reported in an abstract that oxidative metabolism in muscle mitochondria from four Rett patients was normal although they report a complex IV/citrate synthetase ratio that is 22% lower in RS samples than in their controls. Difficulty in interpreting this study exists in the analysis of respiratory efficiency - it is presented as a ratio of respiratory complex enzyme activity/citrate synthase (CS) activity, a mitochondrial matrix enzyme, with the RS CS activity reported

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with a standard deviation larger than the CS mean (1.62 umol/min/mg protein, SD=1.76). With such a large comparative standard deviation, these RS ratios require further verification.

Genetic Evidence

Although the majority of RS cases are sporadic, increasing evidence provides compelling support for a genetic mechanism. This includes:

1. All currently accepted cases of RS are female, indicating the involvement of the X-chromosome, possibly in concert with some other genetic factor in an undefined way (91).
2. Studies of RS twins indicates concordance among monozygotic twins and discordance among dizygotic twins (91).
3. A patient with classical RS gave birth to a daughter who later developed RS (92).
4. Data relating to families with more than one RS female support a genetic inheritance (93).
5. There is an increased consanguinity rate which contributes further support (2.4% vs. 0.5%) (93).

The etiology of RS is still yet to be defined. Based on the above observations, several different causative mechanisms for the genetic heterogeneity have been proposed. Much of the emphasis has been on the X-chromosome due to the female inheritance

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pattern. Theories include a dominant X-linked mutation with lethality in males and reproductive lethality in females (94,95). However, this does not account for the familial inheritance pattern, the birth of an RS girl from an RS mother, or the lack of distortion of the 1:1 sex ratio in RS sibships (96). Nonrandom X-inactivation has been proposed as an explanation for familial cases (92). In this model, the Rett-affected X is predominantly inactivated due to natural selection for the normal X. In gametogenesis, however, the Rett gene is reactivated resulting in an affected female or an aborted male. Again, the 1:1 sex ratio in RS sibships is unaccounted for. Other hypotheses include two mutations at the same locus on both X-chromosomes, gene disruption, lyonization, parental imprinting and, the most accepted hypothesis, metabolic interference of X-linked and autosomal alleles (93). In this model a mutation arises on one of the X alleles resulting in a gene product (X*) that interferes with the product of the other unaffected allele. This results in an inhibited enzyme activity. There is also a non-allelic second locus involved wherein the mutant gene product (A*) interacts with X* in a suppressive manner allowing the normal X gene product to function properly. Therefore, a male with A* and X* would have a lethal combination due to the lack of a (normal) second X gene for enzyme activity. A female, however, would be normal. A Rett female would arise in the presence of the X* without the A* suppressor allele, due to the interference of the X* gene product with the normal X gene product resulting in reduced enzymatic function. In this scenario one would expect, as explained by Buhler, familial cases related by their fathers who would produce exclusively RS daughters. This, in fact, does not reflect the genetics of RS at all. Although there are numerous apparently spontaneous

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cases of RS, those familial cases that do exist demonstrate exclusively maternal inheritance (97,98,99,100,101). These pedigrees show affected sisters, first generation; affected half-sisters related through a common mother; and affected RS girls and their maternal aunt(s). Such a strong line of maternal inheritance argues that an extranuclear mutation must also be taken into consideration as a possible genetic mechanism of inheritance. The cytoplasm and mitochondria of a human zygote arises from the oocyte. This inheritance would account for the maternal inheritance seen in Rett Syndrome. A mitochondrial DNA mutation steered by a further X-chromosome mutation (102) could account for the almost exclusive female penetrance of the disorder. The occurrence of both mutations in a single individual might appear sporadic, as in Rett Syndrome. Such a mechanism might also have graver affects on a male zygote resulting in death in utero whereas the female zygote would survive due to the normal allele of the second X-chromosome. This could also account for the increased incidence of abnormalities reminiscent of RS seen in the brothers of RS girls (93). Both X-chromosome single gene mutations or mtDNA point mutations are known to result in degenerative diseases. Inheritance of one or the other could result in the diversity of ailments exhibited in these lineages.

Certain mitochondrial disorders may exhibit complex genetics due to the involvement of a second genetic factor. One example of such a disorder is Leber's Hereditary Optic Neuropathy (LHON). LHON has been associated with a point mutation in the mitochondrial DNA at nucleotide position 11778 (15). This mutation alters a highly conserved amino acid in the NADH dehydrogenase subunit four protein complex.

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The clinical expression of this disease, which includes sudden onset bilateral vision loss, varies greatly within and between families. Within families, individuals with identical mtDNA composition (mutant percentage vs. normal percentage mtDNA) display a variety of phenotypes ranging from ophthalmoscopically normal fundi to complete optic atrophy. Genealogical data shows a particularly strong male penetrance with 50% of males and 20% of females in the LHON maternal lineage developing optic atrophy. This suggests that the liability to develop optic atrophy is determined by the presence of an altered X-linked gene. Evidence for an X-chromosome gene linked to DXS7 has been reported (103). Thus the complex genetics associated with the inheritance and penetrance of LHON may resemble that observed in Rett Syndrome.

Mitochondrial DNA mutations often occur in a heteroplasmic state wherein there is a mixture of mutant and normal mtDNAs within a single cell or tissue. Such diseases demonstrating this heteroplasmy include Kearns-Sayre Syndrome (KSS), and some forms of familial Type II Diabetes Mellitus (IDDM), both resulting from a heteroplasmic state of large deletions. Other disorders resulting from heteroplasmic point mutations include MERRF (myoclonic epilepsy with ragged red fibers), MELAS (mitochondrial encephalomyopathy with lactic acidosis and strokelike symptoms), and FIC (fatal infantile cardiomyopathy). Variations in the mtDNA levels (normal percentage vs. mutant) often results in a diversity of clinical symptamology including age of onset, progression, and severity of the disease. Thus, mtDNA heteroplasmy could account for much of the variability seen in RS girls. For example, although concordance among monozygotic (MZ) twins is the norm, the reports of discordance among MZ twins (104) could result

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from different amounts of mutant mtDNA in the twin zygotes following fission as mitochondria, being a cytosolic organelle, are known to segregate randomly according to cytoplasmic location during cytokinesis (105). A higher mutant concentration in one twin could result in the almost immediate regression after birth followed by the rapid progression to the later stages of RS seen in the discordant MZ twins. The second twin, possibly with a lower initial mutant level, develops symptoms at a later stage but ultimately progresses to the same stage as her RS twin. As in other neuromuscular disorders involving heteroplasmic mtDNA mutations, there would be a slow drift towards mutant homoplasmy - which may account for the gradual progression of muscular degeneration seen in late stage III and stage IV of RS. Similarly, the variability in other Rett phenotypes and the forme fruste cases of RS (106) could reflect the variations in the proportions of the mutant to normal mitochondrial DNA ratio.

Because the etiology of Rett Syndrome has not as yet been identified, it is essential that all rational possibilities be investigated. A great deal of evidence regarding the morphology, enzymatics and genetics of Rett Syndrome points to a possible mitochondrial DNA involvement. To date, there has been no published reports of mtDNA analysis in RS.

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B. Statement of Problem

Rett Syndrome is a progressive neurologic disorder of unknown etiology. To date, most of the genetic investigation of this disorder has been directed at the X-chromosome due to Rett's almost exclusive female penetrance. Much of the available literature, however, points to a possible mitochondrial DNA involvement. The mitochondria is the powerhouse of the aerobic cell generating the majority of ATP necessary to meet the cell's energy requirements. The quantity of mitochondria within each cell is determined by this energy demand. Any alteration in the function or synthesis of the oxidative phosphorylation enzymes directly affects the ability of the mitochondria to provide ATP and therefore the cell's ability to function. Since 1989, many instances of mtDNA mutations have been found to correlate with human disorders including MERRF, MELAS, KSS, LHON, Diabetes Mellitus, Leigh's Disease, and Fatal Infantile Cardiomyopathy. Rett Syndrome shares many of the characteristics common to these mitochondrial encephalomyopathies. Such characteristics include muscle wasting, EEG variations, distorted mitochondrial morphology, oxidative metabolism deficiencies and maternal inheritance. Studies of the mitochondria, its genome, and potential mutations associated with Rett Syndrome will increase our understanding of the mitochondrial genome, its relationship to mitochondrial function and its possible involvement in this neurologic disease.

Initial steps in this investigation will involve an optimization for mitochondrial DNA of the polymerase chain reaction (PCR), a technique essential to genetic analysis.

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PCR optimization will be initiated in blood samples to allow future patient evaluation. Hemoglobin, EDTA and/or heparin levels will be evaluated using both nuclear and mitochondrial primers. Next, a series of genetic tests will be devised to test for identified mitochondrial mutations using PCR, restriction digestion and electrophoretic analysis. Single cell analysis will then be used to determine the sensitivity of the PCR and to investigate the status of heteroplasmy of disease mutations within individual cells compared to the levels within the associated tissue.

Once the PCR analysis is optimized an approach will be designed to identify potentially disease-causing mtDNA mutations. Patient DNA samples will be acquired from the local population using a defined set of criteria designed to identify mtDNA-based diseases. The patient DNA will be screened for known mutations and the appropriate mtDNA genes will be analyzed for potential disease-related mutations. Specific gene regions will be chosen based on enzymatic biochemistry and/or clinical symptamology. Potential disease regions will be analyzed using a series of mutational detection techniques including restriction fragment length polymorphism (RFLP) analysis, denaturing gradient gel electrophoresis (DGGE), single-stranded conformational polymorphism (SSCP) analysis, and dideoxy DNA sequencing. Any candidate gene mutation will be further evaluated in family members and controls.

The etiology of Rett Syndrome is unknown although attention is now turning toward a possible mitochondrial involvement in its development. RS patients will be acquired from the local population, as a gift of Sakkubai Naidu at the Kennedy Krieger Institute in Johns Hopkins University, and from the Brain Tissue Resource Center at

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Harvard Medical School. Samples will be screened for known mitochondrial mutations and then designated gene regions will be evaluated based on enzymatic deficiencies. MtDNA analysis will make use of SSCP and dideoxy DNA sequencing analysis. Potential mutations will be further evaluated against other RS patients, family members and normal controls.

These studies will help researchers to further understand the involvement of heteroplasmy in disease progression. Additionally, this preliminary analysis of the mitochondrial genome may impact upon our understanding of the disease process of Rett Syndrome itself. Further, a defect in the mitochondrial genome can be easily screened for from a variety of sources including blood, amniotic fluid and chorionic villi. Even the absence of a mtDNA mutation in the analyzed genes will enhance the available knowledge regarding this disease and allow later investigators to narrow their own region of evaluation.

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CHAPTER II. MATERIALS AND METHODS

A. Materials

Human placenta was obtained through the Labor and Delivery division of Sentara Norfolk General Hospital. Ten milliliter blood samples were acquired through collaboration with Dr. Mathew Frank of Child Neurology Associates and Dr. Donald Lewis of the EVMS division of Child Neurology with the consent of the patient or his/her guardian. Muscle and skin biopsies were acquired in collaboration with Dr. Mathew Frank and Dr. Larry Leichtman of the EVMS Pediatrics (Genetics Division) and with the consent of the patient's guardian. Samples were recieved fresh from biopsies scheduled for standard testing. Cultured fibroblasts were recieved with patient consent from the EVMS Cytogenetics Laboratory and were primary cultures from muscle biopsies. Brain samples were the kind gift of Dr. Sakkubai Naidu of the Kennedy Krieger Institute at Johns Hopkins University and from the Brain Resource Center at Harvard Medical School. LHON and MELAS cultured cells were a kind gift of Dr. Douglas Wallace, Emory University School of Medicine. Taq DNA polymerase was purchased from either Perkin-Elmer/Cetus or Promega Corporation. SFA NI (cat #172S) was purchased from New England Biolabs. Mae III (cat #822230) and Apa I (cat #899208) was purchased from Boehringer Mannheim. All other restriction enzymes were purchased

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from Promega. Pyruvate, uridine, L-glutamine and neomycin were purchased from Sigma. Cellgro RPMI 1640 was from Mediatech. Fetal Bovine Serum was from Hyclone. The Nick Translation System (cat #8160SB) was from Gibco BRL. The Silver Stain Plus kit (cat #161-0449) and the Prep-a-gene DNA purification kit (cat #732-6015) was from Bio Rad Laboratories. MDE Gel (J.T. Baker #4739-00) was purchased through Baxter Corporation. The fmol DNA Sequencing system and Wizard Prep DNA purification kit (A7170) was from Promega as was the MMLV-RT, DNase I, and the oligo dT and Random Hexamers. Primer Synthesis Reagents and purification columns were purchased from Milligen, a Division of Millipore Corporation. All radioactive nucleotides, including [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]dATP and [α-^{32}P]dCTP (800 Ci/mmol), were purchased from New England Nuclear. Nylon Membrane, DNA grade agarose, acrylamide and bis-acrylamide were purchased from Fisher. All other chemicals were of analytical grade or higher.

B. Equipment

E-C 103 power supply by E-C Appartus Corporation was used for agarose and some acrylamide gel electrophoresis including DGGE (denaturing gradient gel electrophoresis). An MBP 3000D or Bio Rad Power Pac 3000 electrophoresis power supply and IBI STS45 DNA Sequencing System was used for SSCP (single stranded conformational polymorphism) and DNA sequencing analysis. Polymerase Chain Reaction was performed on a Barnsted Thermolyne Temptronic Thermalcycler.

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Ultracentrifugation was done using a variety of Beckman rotors and a Beckman L7-80 ultracentrifuge. Centrifugation was performed on a Beckman J-21C centrifuge. Stratagene pressure control station, posiblot appartus, and Stratagene UV Stratalinker 1800 were used for DNA transfer from agarose gels. For acrylamide gels, a Hoeffer TE Electroblot appartus and TE-51 Transphor power supply were used at 4°C. Agarose gel electrophoresis was performed using an IBI Model MPH unit. Acrylamide gel electrophoresis was performed using Hoeffer SE-400 free-standing units or an SE-600 aquqrium for DGGE with a Haake temperature controller and Buchler polystatic pump. UV transilluminator was from UVP, Inc. The camera system was a Polaroid MP-4 Land Camera system and stand. An LKB Ultrascan XL enhanced laser densitometer was used for scanning gels and data analysis. DNA samples were dried and concentrated in a Savant Speed-Vac concentrator connected to a Savant RT-490 refrigerated condensation trap and Welch 1400 vacuum pump. A Bio Rad model 483 slab dryer was used for drying gels. DNA primers were synthesized on a Cyclone DNA synthesizer by Milligen Biosearch, Inc.

C. Methods

PLACENTAL mtDNA ISOLATION:

Placental mtDNA is recovered from fresh placenta by the combined methods of Swierczynski (107) and Drouin (108). The complete protocol is listed in Appendix I.

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Basically, the placenta is drained and the membranes are removed. The tissue is washed five times, ground, homogenized and filtered. The supernatant is differentially centrifuged to recover the mitochondrial pellet which is then resuspended and differentially centrifuged to recover the mitochondria. The mitochondria are resuspended and lysed in 2% SDS at 37°C until the solution clears. Proteins are precipitated with CsCl (0°, ten minutes) and removed by centrifugation. The supernatant is added to a CsCl/ethidium bromide gradient and ultracentrifuged. The lower band is collected, ethidium removed and then ethanol precipitated, and resuspended in TE (10 mM Tris (pH 8.0), 1 mM EDTA).

PATIENT TOTAL DNA ISOLATION:

1.) From Cultured cells, muscle, or skin biopsy: Total cellular DNA is prepared from 0.1 to 0.7 grams(g) of sample (muscle or skin) or from a T-25 flask of cultured cells (pelleted). Solid tissues are minced and homogenized to create a cell suspension in TE and then centrifuged to pellet cells. Pellets are resuspended in 25 mM Tris (pH 7.5), 50 mM EDTA, 75 mM NaCl, 1% SDS, 40 mM DTT, and 1 mg/mL Proteinase K and then lysed overnight at 50° C. Suspensions are phenol extracted, phenol-chloroform-isoamyl alcohol extracted until no white (protein) appears at the interface, and then chloroform-isoamyl alcohol extracted. The DNA in the aqueous layer is precipitated in 0.3 M sodium acetate by addition of two volumes ethanol at -70° C, and recovered by centrifugation at 10,000-12,000 RPMs for 30 minutes. The supernatant is decanted and

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the pellet is air-dried and resuspended in 50 uL TE. The DNA concentration is determined by measuring the absorbance at 260 nM. A one milligram per milliliter solution of double-stranded DNA will have an absorbance at 260 nM of 20 O.D. units. Since nucleotides or low molecular weight species will also absorb at 260 nM, we always verified our calculated DNA concentration by agarose gel electrophoresis.

2.) From Blood - Method 1 (109): Mononuclear cells were isolated from the buffy coat following centrifugation on ficoll-hypaque gradients. Digestion was performed as described above with precipitated DNA resuspended to 300 uL in TE. Concentration was determined by absorbance at 260 nM.

3.) From Blood - Method 2 (110): One to ten milliliter blood samples were collected in either (Na)heparin (15 U/mL) or EDTA(K_3) (1.7 mM/mL) and stored at 4°C. A small volume of blood, approximately 30 uL, was added to 60 uL of sterile water. The mixture was mixed gently and then heat lysed at 100°C for 10 minutes. The sample was then centrifuged in a microcentrifuge for 5 minutes and the supernatant collected and stored at 4°C. Five microliters was used in each PCR amplification. Amplification was performed within 0-4 days after collection unless otherwise stated.

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SINGLE CELL ISOLATION:

Tissue cultured cells (HL60, LHON, Control, MERRF, MELAS - (all lymphoblasts); amniotic fluid cells) were collected following dilution from a suspension overlaid on a mineral oil base as previously described (111). Single cells were identified using a dissecting or inverted microscope and collected using either a capillary tube with a 0.1 mm diameter opening prepared by drawing out a flamed capillary tube or by using a sterile ultramicropipet. Single cell isolates were verified by ejecting volume into a seperate region of the oil base and scanning for cell number. Each single cell sample was delivered into a 0.5 ml microfuge tube containing either ten microliters of sterile water or ten microliters of 5x amplification buffer (83 mM ammonium sulfate, 335 mM Tris-HCl, pH 8.8, 33.5 mM $MgCl_2$, 50 mM beta-mercaptoethanol, 34 mM EDTA, and 850 micrograms per microliter Bovine Serum Albumin (BSA)) overlaid with thirty microliters of paraffin oil. The single cells were lysed at 95°C for ten minutes, microfuged, and stored at -20°C until needed.

Single mouse blastomeres were obtained by micromanipulation using the technique of Handyside, et al (112). The procedure was performed by Dr. Manal Morsy formerly of the Jones Institute Research Laboratories for Reproductive Medicine, Norfolk, Virginia. The individual blastomeres were delivered into ten microliters of autoclaved, deionized water, overlaid with paraffin oil and lysed by boiling as above. After microcentrifugation, samples were stored at -20°C.

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PCR AMPLIFICATION

1.) Standard Protocol (113): The 100 microliter reaction mixture centained 200 uM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.5 uM primers, 2.5 units Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCL, pH 8.0, 1.5 mM $MgCl_2$, and either 250 ng total DNA or 30 ng purified placental mtDNA. The amplification cycle was: ten minute denaturation (94°C) for one cycle; 30 seconds denaturation (94°C), 30 seconds annealing (60°C), and 60 seconds extension (72°C) with a two second increase in extension per cycle repeated for 30-35 cycles in a thermalcycler and followed by a seven minute final extension. Samples were then stored at 4°C until needed.

2.) Multiplex Protocol: This protocol is essentially that of Chamberlain, et al (114). Approximately 250 ng of total DNA (or 30 ng of purified placental mtDNA) was amplified in a 50 microliter reaction volume containing 0.5 mM of each dNTP, 20 picomoles of primers, ten percent dimethyl sulfoxide (DMSO), two units of Amplitaq DNA polymerase, 16.6 mM ammonium sulfate, 67 mM Tris-HCl, pH 8.8, 6.7 mM $MgCl_2$, 10 mM beta-mercaptoethanol, 170 micrograms per milliliter BSA, and 6.8 uM EDTA. Cycling parameters varied depending upon the primer sets and derived product length but were generally: seven minute denature (94°C), one cycle; 30 second dentaure (94°C), 30 second anneal (53°C), 4 minute 45 second extension (65°C) for 24 to 28 cycles with a final extension of seven minutes. For incorporation of radioactive radionuclide, the appropriate dNTP was reduced to half concentration and approximately

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one microliter of the radiolabeled dNTP was introduced into the reaction.

HEMOGLOBIN DETERMINATION

Hemoglobin determination was made using the Total Hemoglobin Kit 525A (Sigma Diagnostics). The procedure was performed according to the cyanmethemoglobin method for quantitation of hemoglobin in blood (148).

SCREENING PROTOCOLS

Screening for known mutations is essential to prevent time-consuming analysis of a previously identified mutation. Using published protocols and PCR primers, we screened for four of the most common point mutations - those found in LHON, NARP, MELAS and MERRF - and the deletion mutations associated with KSS and CPEO. These mutation detection assays were performed as follows:

1.) KSS and CPEO (deletion mutations): Five micrograms of total DNA were digested in three separate aliquots with *PvuII*, *EcoRI*, and *PstI* overnight at 37°C. The digested products were fractionated on an 0.8% agarose gel, transferred to nylon and probed with nick-translated placental mtDNA. Alterations in fragment migration distance relative to a normal mtDNA control sample indicate an insertion or deletion of mtDNA.

Alternatively, PCR across the common 4.9 Kb deletion (115) using primers to the

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ATPase 6 and cyt b gene regions would detect the majority of the known deletions. In this case, since the primers would be 5478 bp apart on the wild-type mtDNA, the occurrence of smaller bands would reflect the presence of deleted mtDNA in the sample. Likewise, a larger band would indicate the presence of a mtDNA insert between these sites.

2.) MERRF - Mismatched Primers For a Point Mutation: The MERRF mutation is detected using PCR amplification with mismatched primers followed by Nae I digestion (116). The mismatched three prime primer contains a two base pair alteration is two nucleotides internal to the three prime end of the sequence. During PCR amplification, a *NaeI* site is created in the presence of the MERRF mutation (Figure 1A). Following PCR, the fragments are digested with *NaeI* and a 90 bp amplified fragment will be digested into a 26 and 64 bp fragment if the MERRF mutation is present.

3.) MELAS, NARP and LHON - Point Mutations: All three of these point mutation diseases are detected by PCR amplification of the target region and Hae III (117), Ava I (118) or Mae III (119) digestion, respectively. Each of these digests results in a positive assay for the mutation by cleaving the normal fragment into two smaller fragments (Figure 1B).

The restriction fragments (or PCR products) are resolved by gel electrophoresis on either a 0.8%-1.0% agarose or ten percent acrylamide gel, stained in one microgram per milliliter ethidium bromide, and visualized and photographed through UV-induced

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FIGURE 1: A. Strategy for detection of the point mutation MERRF using mispairing primers for PCR to generate a restriction site at the three prime end in the presence of the MERRF mutation. The sequence of the mtDNA with the point mutation in the $tRNA^{Lys}$ gene is shown along with the sequence of the mismatched primer (MERRF Reverse) beginning at the site of the mutated MERRF nucleotide (np 8345). The MERRF Reverse primer includes two mismatches at positions 8350 and 8351 in order to generate the *NaeI* restriction site in the mutated but not normal mtDNA. The forward primer is homologous to nts. 8282 to 8305 of the heavy strand mtDNA. **B.** Acrylamide gel representing the analysis to be used for LHON, NARP, and MELAS mutations. LHON analysis uses a PCR-amplified 704 bp target region (nts. 11490-12194) which is restriction digested with *MaeIII* to detect the mutation. Normal fragments are 255,231,167, and 31 bps.; a mutated target is cut into the fragment sizes 231,167,128,127, and 31 bps. NARP analysis uses a 554 bp PCR-amplified fragment (nts. 8646-9200) restriction digested with *AvaI* to produce fragments of 347 and 207 bps. in the presence of the NARP mutation; the normal fragment is not cut. MELAS analysis uses a 372 bp PCR-amplified target (2980-3352) followed by digestion with the restriction enzyme *ApaI* to produce 263 and 109 bp. fragments in the presence of the MELAS mutation. The normal fragment is not cut.

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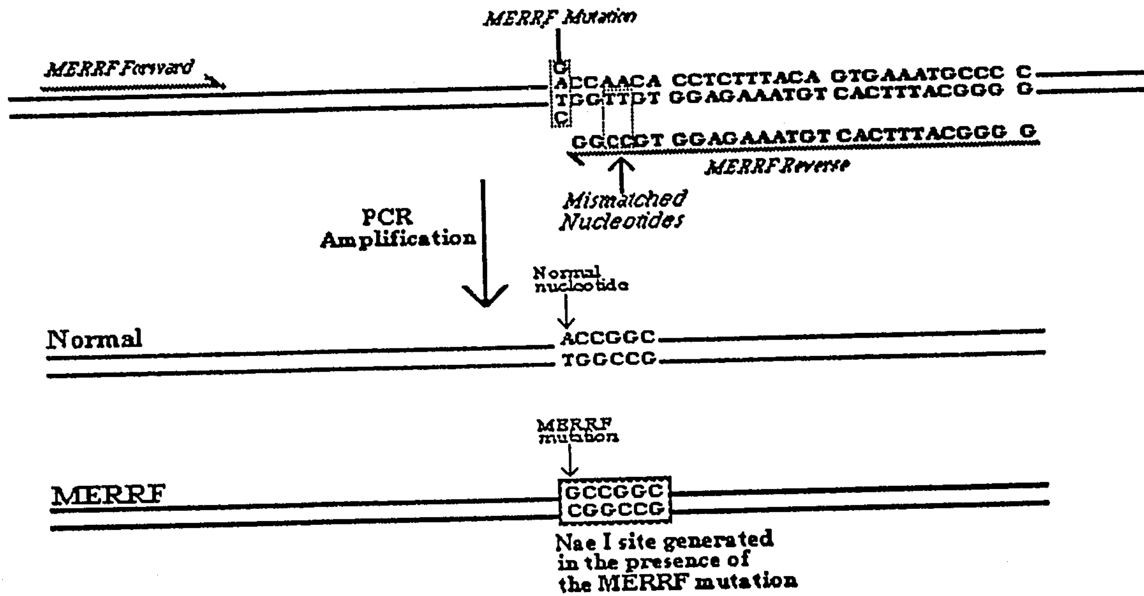
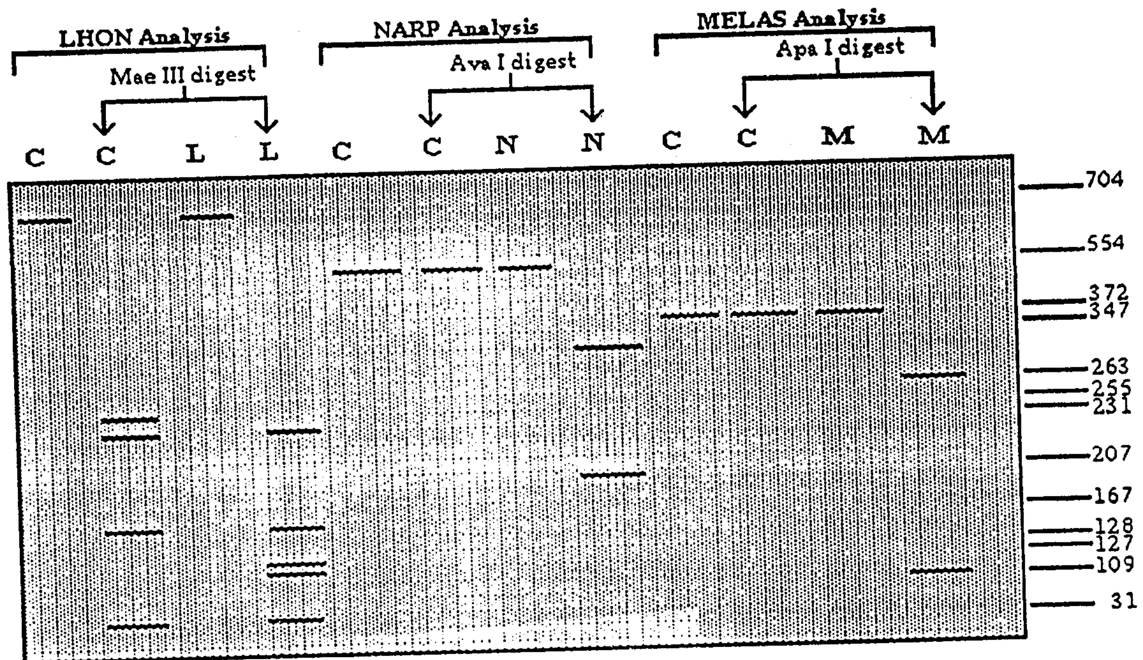
A.



B.



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fluorescence. If known mutations were detected, this was reported to the appropriate physician and the specimen removed from further analysis.

DNA LABELING

1.) End-Labeling: Twenty picomoles of a PCR primer was radiolabeled at the five prime end in a reaction mixture containing ten pmol [γ-^{32}P]ATP, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 100 μM spermidine, eight units T4 Polynucleotide Kinase, and sterile, deionized water to ten microliters final volume. The sample was incubated at 37°C for 10-30 minutes. Kinase was then inactivated at 90°C for two minutes and the labeled sample was stored at -20°C until needed.

2.) Nick Translation: Human mtDNA and/or PCR-amplified DNA was nick-translated using the Gibco BRL Nick Translation Kit. Radiolabeled DNA was recovered by ethanol precipitation. DNA was dissolved in TE and stored at -20°C until needed.

MUTATION DETECTION METHODS

1.) Single-Stranded Conformational Polymorphism (SSCP) (120,121,122): Mitochondrial DNA regions of interest were multiplex-PCR amplified with [α-^{32}P]dATP incorporation and unlabeled primers as described above. Fragments of 200-300 basepairs (bp) were analyzed directly while fragments greater than 300 bp were restriction enzyme

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digested to an appropriate size (200-300 bp) and then analyzed. Approximately three microliters of the labeled PCR product was added to nine microliters of SSCP dye (95% formamide, 10 mM NaOH, 0.05% bromophenol blue (BPB), 0.05% xylene cyanol(XC)) and heat denatured at 95°C for five minutes. Denatured DNA was transferred to an ice bath and cooled for five minutes. Five microliters of the denatured DNA was loaded onto a 0.5x MDE gel (JT Baker) of 35x43 cm in a buffer of 0.6x TBE (53 mM Tris-borate, 53 mM Boric acid). Samples were electrophoresed for 18 hours at 8 watts or until the XC was approximately 35 cm from the bottom of the wells. After electrophoresis, the glass plates were seperated, the gel was transferred to Whatman, covered with plastic wrap and autoradiographed for 24-96 hours.

2.) Denaturing Gradient Gel Electrophoresis (DGGE) (123): Approximately five to ten micrograms of patient total DNA was digested in four aliquots each with one of four restriction enzymes (Dde I, Hae III, Hph I, Mbo II) which generate fragments of 200-700 bp. Alternatively, PCR-amplified DNA was purified on Wizard Prep columns and digested, if necessary, to the appropriate length. The restriction fragments were electrophoresed in 6.5% acrylamide gels with a 20%-80% denaturant range (100% = 7 M urea/40% formamide) in a 60°C aquarium (Hoeffer SE-600). PCR-amplified DNA can be detected by ethidium staining and UV illumination or the gel can be electroblotted and probed. For electroblot, the gel was denatured in 0.5 M NaOH for five minutes, neutralized in 0.5 M Tris-HCl, pH 8.0, for five minutes and equilibrated in 2x TE for ten minutes. Fragments were electroblotted to a nylon membrane in a 4°C Hoeffer Model

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TE transphor aquarium with 2x TE buffer at one amp for two hours. Membranes were washed in 6x SSC (900 mM sodium chloride, 90 mM sodium citrate), air-dried and stratalinked on the "auto" setting (30 seconds, 120,000 μjoules/cm^2). Fragments were then localized by Southern Hybridization.

3.) Southern Hybridization (124): DNA samples run in an agarose gel matrix were transferred to a nylon membrane using a posiblot appartus (Stratagene) under 75 mm Hg for 45-60 minutes. DNA samples in an acrylamide gel matrix can be electroblotted to a nylon membrane as described under DGGE. The DNA was UV-crosslinked to the membrane using a Stratagene Stratalinker at 120,000 uJ/cm^2 for 30 seconds. The membrane was prehybridized in 5x SSPE (750 mM sodium chloride, 44 mM sodium monophosphate, 5 mM EDTA) at 42°C for one hour followed by an overnight hybridization at the same temperature in 0.5% SDS, 5x SSC, 5x Denhardt's Solution, 0.1 milligram per milliliter single-stranded salmon sperm DNA, 50% deionized formamide, and 25 to 50 nanograms per milliliter probe DNA. The membrane was washed twice in 2x SSPE/0.1% SDS for 30 minutes each, twice in 0.2x SSPE/0.1% SDS for 30 minutes each and briefly rinsed in 2x SSPE. The membrane was dried, wrapped in plastic wrap and exposed to X-ray film at -70°C with an intensifying screen. The length of exposure is dependent upon the counts present on the gel as detected by a hand-held Geiger-Muller counter. A workable rule of thumb is that 50-100 cps will require an overnight exposure.

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4.) PCR Sequencing (125,126,127): Sequence analysis was performed using Promega's fmol DNA sequencing kit. The reaction mixture included 40 femtemol of template DNA (a PCR-amplified DNA fragment purified using Wizard Prep PCR purification kits or Bio Rad Prep-a-gene DNA purification kits), 50 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 2 picomoles [gamma-^{32}P] labeled primers, 5 units sequencing grade amplitaq DNA polymerase, and sterile water to 17 microliters. Four microliters of this reaction mixture was added to two microliters of dNTP/ddNTP mixtures in four tubes labeled A,C,G,T. The samples were cycled as follows: 7 minute initial denaturation (94°C) - 1 cycle; 30 second denaturation (94°C), 30 seconds annealing (42°C), 1 minute extension (70°C) for 30 cycles. Six microliters of sequencing stop solution (10 mM NaOH, 95% formamide, 0.05% BPB, 0.05% XC) was added to each sample. Samples were then heated to 95°C for five minutes and three microliters per lane was loaded on the sequencinmg gel. The gel was electrophoresed at 65 W until the BPB dye reached the bottom of the gel. At the end of the electrophoretic run, the plates were separated and the gel transferred to Whatman paper and wrapped in plastic. The wrapped gel was exposed overnight without an intensifying screen at -70°C.

MUTATION QUANTITATION

A region containing a heteroplasmic DNA mutation as determined by DNA sequencing was PCR-amplified from total patient DNA using normal or mismatched primers. (Mismatched primers create a restriction site in the presence or absence of the

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mutation by altering one or two nucleotides upstream of the mutation site.) The amplified fragment was then treated with the restriction enzyme that detects the mutation through the alteration of a restriction site. Digested samples were run on acrylamide gels. For non-restriction recognized mutations, mutant and normal fragments were seperated using SSCP. The percentage of the mutant fragment was determined by quantifying normal and mutant bands using a LKB Laser Densitometer and determining the ratio between the mutant and normal fragments.

CELL CULTURE

LHON, MERRF, MELAS, and Control lymphoblast cell lines were cultured in RPMI 1640 with 100 micrograms per milliliter neomycin, 2 mM L-glutamine, 10% fetal bovine serum, 1 mM pyruvate, and 50 micrograms per milliliter uridine. Cell lines were fed every two days and split weekly. Pyruvate and uridine are used to compensate for oxidative phosphorylation deficiencies in the mitochondrial myopathy cell lines.

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CHAPTER III. RESULTS

This project began with the intent of investigating mitochondrial DNA involvement in the development of mitochondrial myopathies. During the course of this investigation, a variety of tangents were explored that contributed to the project as a whole. Therefore, this Results Chapter is presented in sections to reflect the three areas that were explored. Section A examines Single Cell Analysis. This section contains the preliminary work that preceeded the mitochondrial myopathy investigation. A study of single cell mtDNA emerged from this area as an attempt to uncover the sensitivity and applicability of the techniques used. The second section, an investigation of mtDNA and its contribution to mitochondrial myopathy disorders, made use of the techniques optimized in Section A as well as incorporating newer mutation detection techniques. Patient samples studied were acquired through local physicians, with parental consent, and from patients that met criteria that grouped their disorder as mtDNA-based. However, none of the patients demonstrated the same disorder. The final section, C, is a study of mitochondrial DNA involvement in Rett Syndrome, a disorder of unknown etiology but exhibiting a strong mitochondrial involvement. This section closely examines the tRNA regions of the mitochondrial genome and their possible contribution to this disorder.

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A. Single Cell Analysis

1. Blood DNA Analysis

The polymerase chain reaction (PCR) is a sensitive technique designed to amplify known DNA sequences from a variety of DNA sources. Identification of DNA sequences from blood previously required isolation of white cells and subsequent digestion and DNA precipitation prior to PCR (see Methods: DNA isolation from blood - Method 1). In our preliminary studies, a rapid protocol was developed to isolate DNA from human blood (110). This protocol, shown in Figure 2, could be accomplished in 15 minutes from as little as 30 microliters of blood and yielded enough DNA for multiple PCR analyses. The isolated DNA was tested for PCR amplification efficiency and then investigated to determine the effects of hemoglobin and the anti-coagulants EDTA(K_3) and (Na)Heparin on the PCR amplification. If the procedure could be used in the presence of these potential inhibitors, samples could be acquired from a variety of sources without the necessity of immediate analysis.

We prepared oligonucleotide primers flanking a part of the cytochrome b gene located at positions L14812 to L14838 and H15151 to H15177 of the mtDNA in order to assess whether the rapid isolation procedure yielded sufficient DNA to allow amplification of a specific mtDNA sequence. Figure 3 shows the results of such an amplification from EDTA- or heparin-treated blood samples following 30 cycles of PCR. As is seen in lanes D through G, the 365 bp cytochrome b fragment is produced as

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FIGURE 2: Flow diagram for rapid DNA isolation from anticoagulated and non-anticoagulated blood for PCR amplification. This procedure requires approximately 15 minutes and the supernatant has been found to be stable at 4°C.

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FLOW DIAGRAM OF DNA PREPARATION FOR PCR AMPLIFICATION

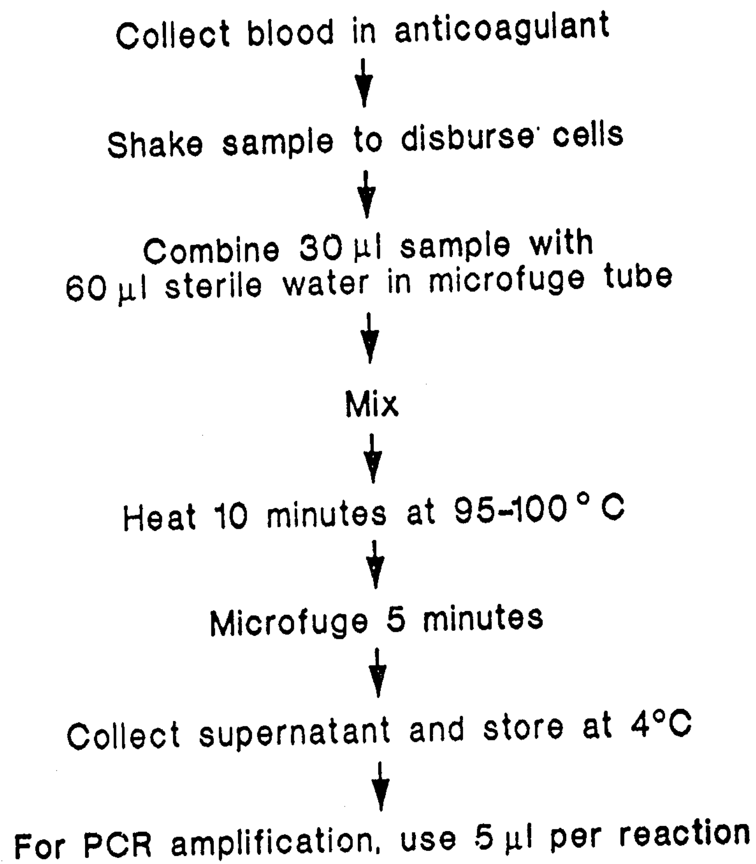

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FIGURE 3: PCR amplification of the mtDNA cytochrome b region (nts. 14812-15177) using the procedure described in Figure 2. Lane A, pGEM DNA molecular weight marker; lane B, negative control; lane C, positive control, HL60 mtDNA; lane D and E, EDTA anticoagulated blood with 1.0 and 0.5 μM primers, respectively; lanes F and G, same as lanes D and E except using heparin anticoagulated blood; lanes H and I, whole blood, no anticoagulant, using 1.0 and 0.5 μM primers, respectively.

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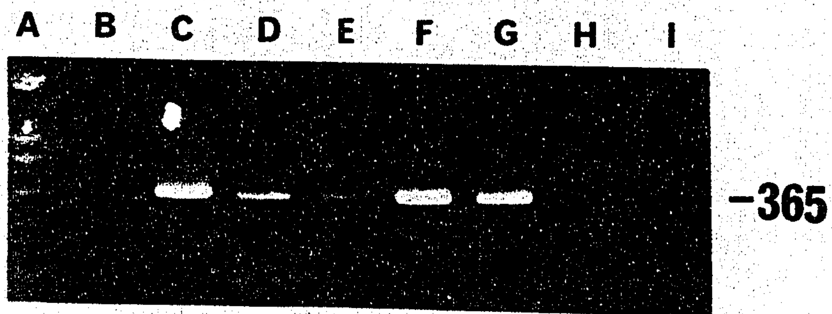

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expected. Using the same amount of template DNA, it appears that there is an almost two-fold stronger amplification using blood collected in heparin rather than in EDTA (compare lanes F and G to lanes D and E). Under identical conditions, no amplification was detected when non-anti-coagulated whole blood was used (lanes H and I).

Hemoglobin has been reported to interfere with Taq polymerase activity necessitating lengthy DNA isolation procedures in order to eliminate hemoglobin contamination (128). In addition, blood samples used for laboratory analysis are collected in heparin or EDTA to prevent coagulation before testing can be completed. These agents have also been reported to inhibit the PCR reaction (129). To determine the levels at which these agents inhibit Taq activity, an investigation of amplification efficiency was conducted.

Cesium chloride purified HL-60 cell DNA was used for PCR amplification in the presence of varying amounts of (Na)heparin, EDTA or hemoglobin. Equal amounts of the amplification reaction were loaded on a gel, electrophoresed, stained, photographed and then densitometrically analyzed for amplification efficiency in comparison to a control with no added reagents. Analysis of the raw data was performed with the aid of a computer program that quantified the amounts of amplified template for each condition. Results were plotted on the basis of amplification efficiency versus reagent concentration. Graph 2 shows the results of this analysis.

In agreement with previous studies, hemoglobin between 50-1000 micrograms caused a concentration-dependent inhibition of DNA amplification with 500 micrograms completely inhibiting amplification. Whole blood hemoglobin concentration was

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GRAPH 2: Inhibitory effect of hemoglobin, heparin, and EDTA on PCR amplification. One hundred percent amplification corresponds to PCR in the absence of any inhibitors.

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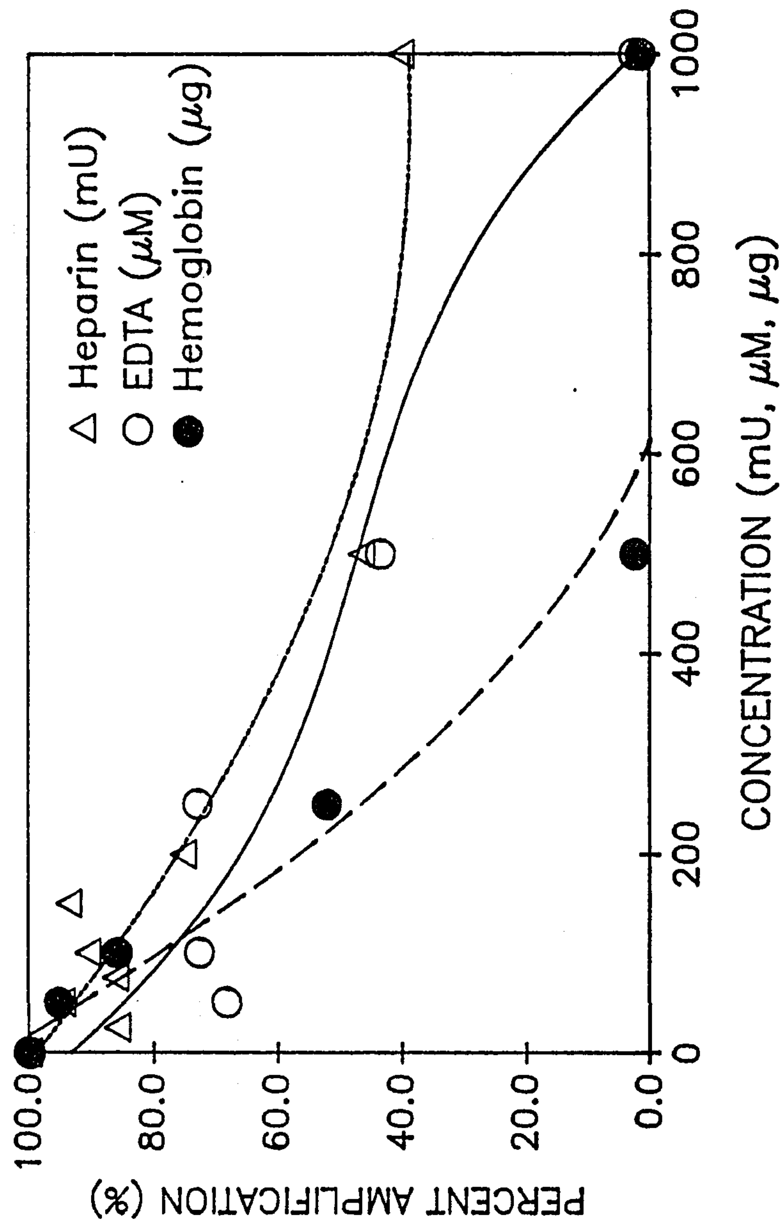

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determined to be 1.2 g/mL (130). After processing the whole blood as shown in Figure 2, the concentration of hemoglobin in the amplification reaction was approximately 100 micrograms (1.0 mg/mL). As seen in Graph 2, this level of hemoglobin would have minimal to no effect on the PCR reaction. Additionally, heparin and EDTA are shown to inhibit DNA amplification in a concentration-dependent manner also, with 100 mU of heparin resulting in as much as a 60% inhibition of amplification and one millimolar EDTA, 100% inhibition. The concentration of heparin using this protocol is reduced from the collected blood sample to 25 mU while EDTA is reduced from the collected sample to 27 uM. The concentration of either anti-coagulant (following processing by this method) in conjunction with the hemoglobin remaining in the sample should result in less than a 15% inhibition of Taq activity making this a rapid and reliable protocol for DNA analysis.

The stability of the DNA in the anti-coagulated whole blood sample was also examined. Graph 3 shows that heparinized blood can be processed and amplified at 100% of the maximum amplification level (determined on the day of collection) for up to 25 days following sample collection if the samples are stored at 4°C. In contrast, EDTA-anti-coagulated blood deteriorates in a time-dependent manner. Blood collected and stored in EDTA could be processed and amplified five days after collection, but significant inhibition was observed 14 days post-collection and little or no DNA amplification was detectable 18 days post-collection. Hence, although there is no significant difference in the amplification efficiency between the two anti-coagulants, the advantage of longterm storage warrants the use of heparin over EDTA.

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GRAPH 3: Effect of storage of blood sample on DNA amplification. Blood collected in the presence of anticoagulants was stored for various times at 4°C before workup and amplification. One hundred percent amplification corresponds to amplification on the day of sample collection.

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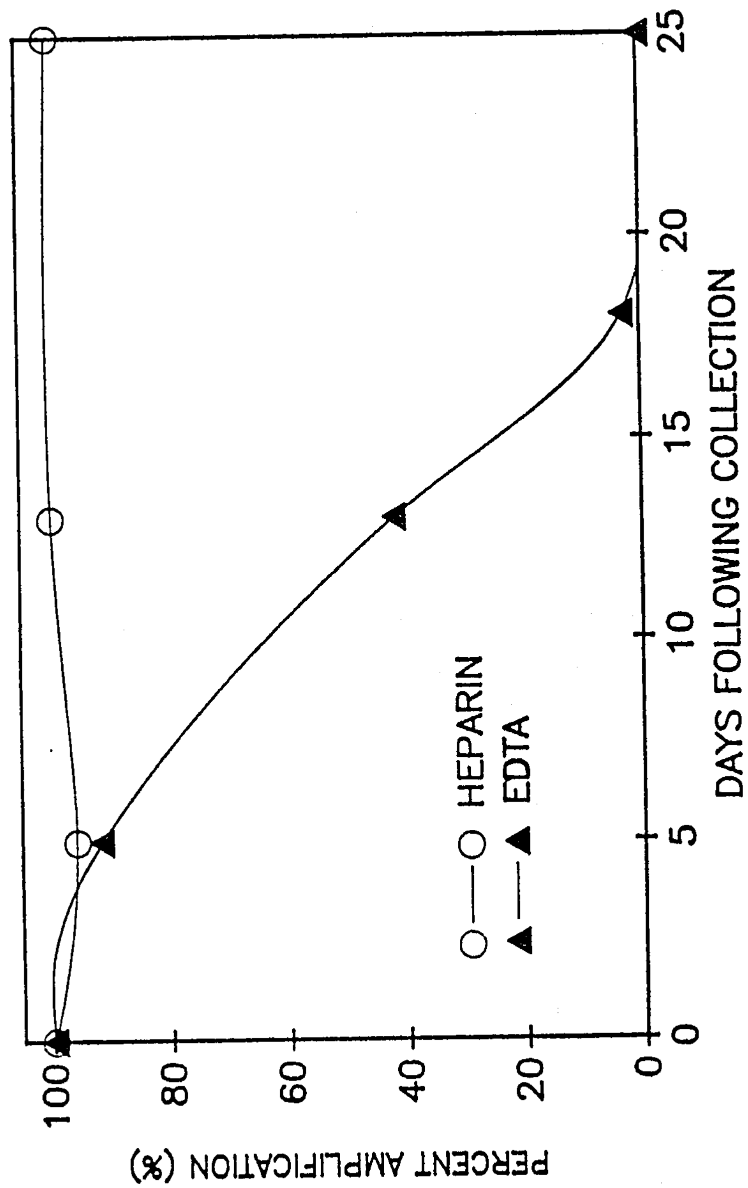

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2. Single Cell Analysis

The ability to detect specific mitochondrial DNA target sequences from the equivalent of one drop of blood spurred us to investigate the possibility of amplifying mtDNA from even smaller samples, e.g. a single cell. Individual HL-60 cells were collected from a growing culture, following dilution, under a dissecting microscope using a flamed capillary tube as described in Methods. Single cells were collected into ten microliters of sterile deionized water. Figure 4 shows an acrylamide gel electrophoresis of the mtDNA fragment from microscopically selected HL-60 cells amplified through 35 cycles of PCR using the cytochrome b primers. Lanes F, G and H clearly indicate the production of the expected mtDNA fragment from two, five and ten HL-60 cells, respectively. Although a very faint band of the appropriately sized DNA was present in the one cell sample (lane E), we attempted to improve the level of amplification by altering the PCR conditions, i.e. temperature, buffer composition, cycle time, and number of cycles. In addition, we prepared a second set of primers to a region of the mitochondrial ATPase six gene in order to attempt a 'duplex' amplification from a single cell. These primers mapped to the mitochondrial gene regions L8646 to L8666 and H9200 to H9180 generating a PCR fragment of 554 bp. Previous use of the multiplex amplification protocol (see Methods) had proved to increase the efficiency of normal amplifications in comparison with the standard protocol. Because we were dealing with very small amounts of DNA from the single cell, the multiplex approach was attempted. Figure 5 shows the results of this experiment. As can be seen, 28 cycles under these

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FIGURE 4: Acrylamide gel electrophoretic separation of PCR amplified mtDNA. Lanes B-H represent PCR amplified DNA using primers flanking a portion of the cytochrome b gene (nts. 14812-15177). Lane A, pGEM molecular weight markers; lane B, negative control; lane C, positive control, 10 ng purified HL60 mtDNA; lane D positive control, 10 ng total DNA; lanes E, F, G, and H, HL60 total DNA from 1, 2, 5, and 10 HL60 cells, respectively.

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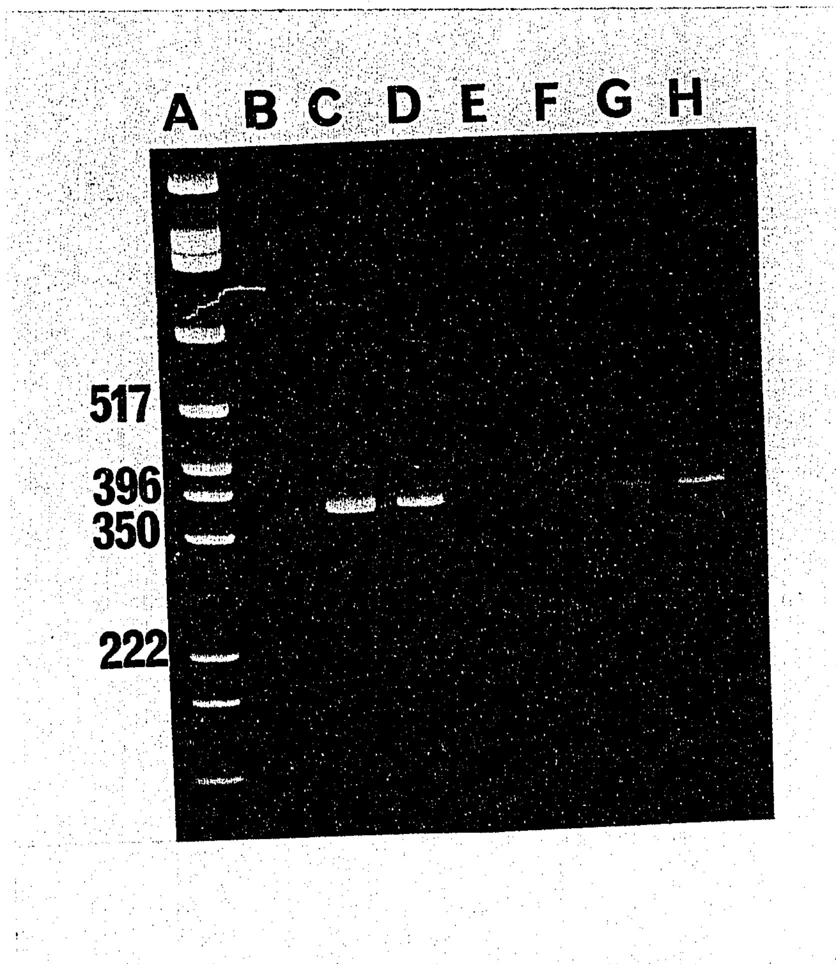

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FIGURE 5: Mitochondrial cytochrome b and ATPase 6 gene (nts. 8646-9200) amplification by PCR of individual HL60 cells. Lane A, pGEM molecular weight markers; lane B, negative control; lane C and D, positive controls for ATPase 6 and cytochrome b, respectively, using 10 ng purified HL60 mtDNA; lane E, duplex amplification of control mtDNA using cytochrome b and ATPase 6 primers; single HL60 cells were used for the amplification in lanes F-H; lane F, cytochrome b; lane G, ATPase 6; lane H, duplex amplification using cytochrome b and ATPase 6 primers.

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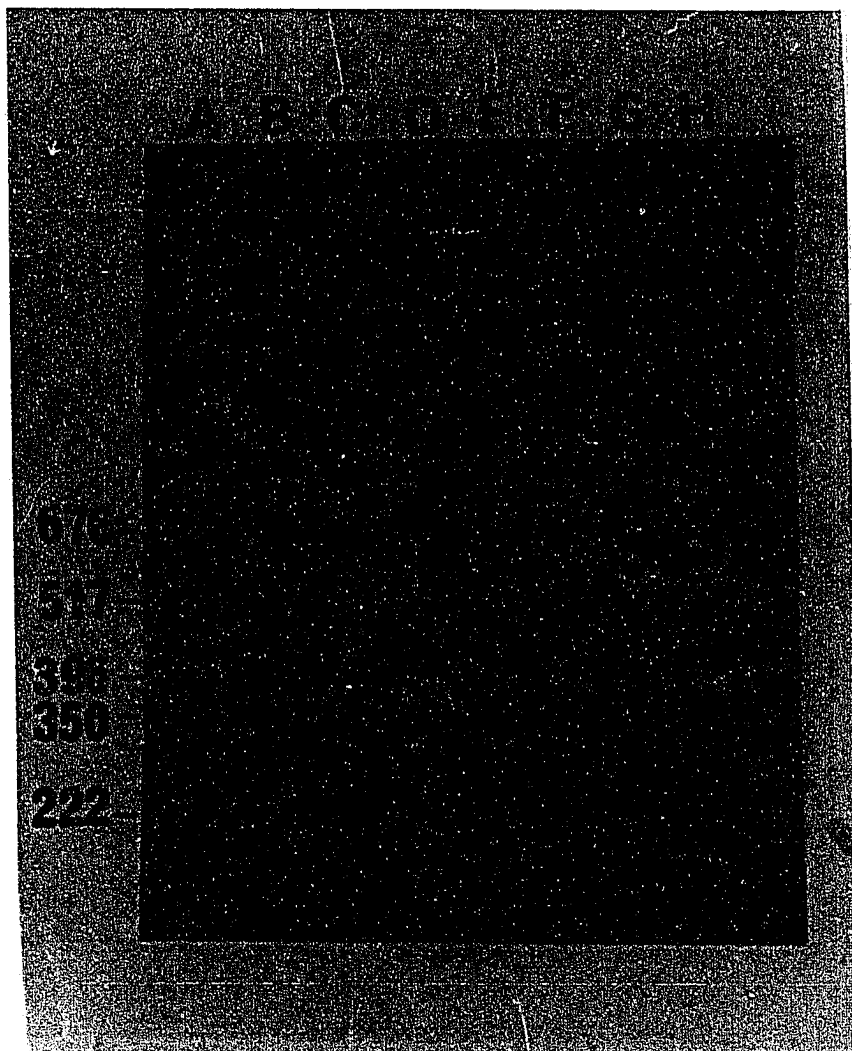

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'duplexing' amplification conditions were sufficient to produce readily detectable DNA from a single HL-60 cell (lanes F and G). Moreover, as seen in lane H, the presence of both the cytochrome b and ATPase six primers led to efficient and equivalent 'duplex' amplification products being generated. In fact, the multiplex amplification reaction was much improved over the standard protocol (compare figure 4 and 5) and was incorporated in all further single cell experiments.

Having shown that mtDNA target sequences could be amplified in readily detectable amounts from single cells, we were interested in the potential to detect mtDNA lesions in small samples in order to assess the potential to perform prenatal diagnosis of mitochondrial myopathies. To this end, we performed a 'duplex' cytochrome b and ATPase six amplification on mtDNA in five and ten microliters of amniotic fluid. These results are shown in Figure 6. As seen in lanes F and G, the amniotic fluid produced sufficient material for detection by PCR of both the cytochrome b and ATPase six targets. Figure 6 also shows the results of PCR amplification of the cytochrome b target from a single cell isolated by micromanipulation from an eight-cell stage mouse blastomere (lane E). Although analogous to the experiments in Figure 5, the results in lane E of Figure 6 indicate the potential for performing analysis of mtDNA lesions on preimplantation embryos from single cells extracted from the blastocyst.

We next obtained leukocytes cultured from a normal patient and from one suffering from Leber's Hereditary Optic Neuropathy (LHON). LHON is a neurodegenerative disease characterized by sudden bilateral blindness in the second or third decade due to optic nerve degeneration. Certain forms of LHON have been shown

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FIGURE 6: Amplification of mouse blastomere and human amniotic fluid using cytochrome b and ATPase 6 primer sets. Lane A, pGEM molecular weight marker; lane B, negative control; lane C and D, positive controls using ATPase 6 and purified human placental mtDNA; lane E, cytochrome b amplification of a single mouse blastomere cell; lanes F and G, duplex amplification using cytochrome b and ATPase 6 primers on five and 10 microliters of human amniotic fluid.

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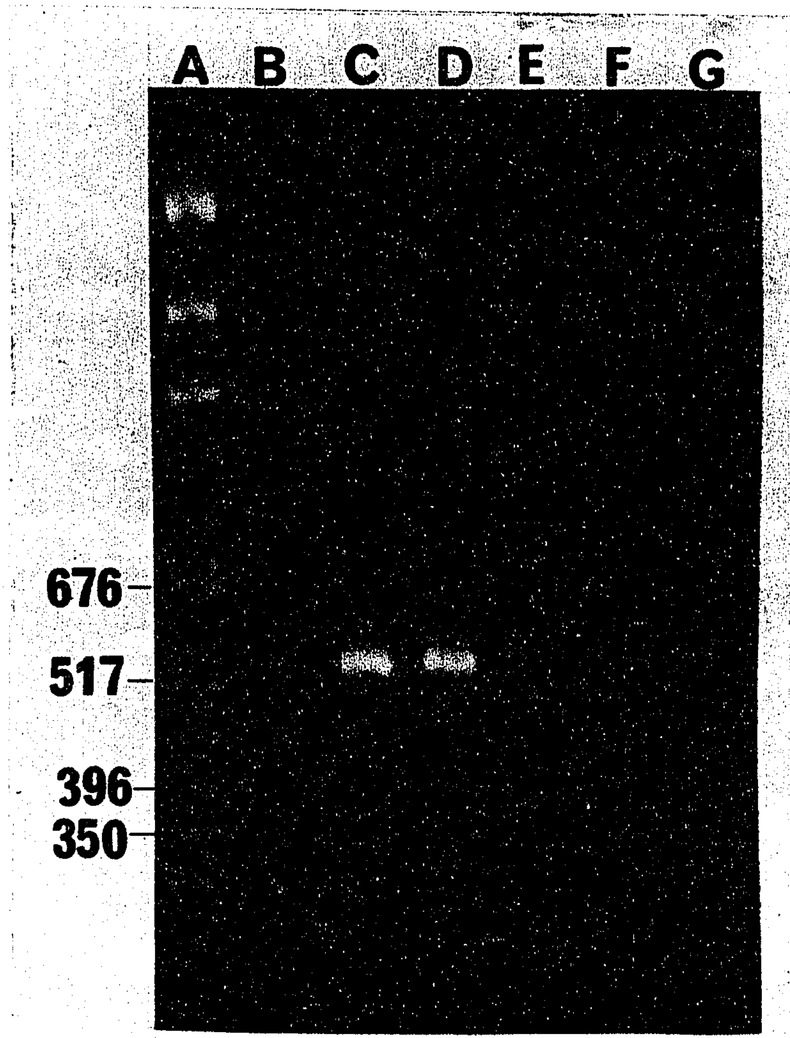

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to be associated with a G->A transition in mtDNA at residue 11,778 which converts a highly conserved arginine to a histidine at amino acid 340 of the NADH-dehydrogenase subunit four encoded on the mtDNA (40). After selecting single cells by micromanipulation, a region of the mtDNA containing the 11,778 nt LHON mutation was amplified by PCR. These primers localized to L11490 to L11511 and H12194 to H12172 and generated a 704 bp fragment. The nucleotide change associated with LHON leads to the loss of an *SfaNI* restriction endonuclease site, so lack of digestion of the PCR-generated fragment by *SfaNI* has often been used as a diagnostic test for LHON. However, other silent mutations in the adjacent nucleotides could also lead to the loss of the *SfaNI* site without being diagnostic for the LHON mutation. The G->A transition, however, also generates a *MaeIII* site which can give a positive test for the presence of the mutation (131). Figure 7 shows the results of PCR amplifying from a single control and single LHON cell and the digestion of this PCR-amplified material with *MaeIII*. The product amplified from normal mtDNA contains three *MaeIII* sites which produces fragments of 255, 231, 161, and 57 bp. The G->A LHON mutation results in a fourth *MaeIII* site within the 255 bp band to yield fragments of 127 and 128 bp. Lanes E and F show the expected pattern of *MaeIII* fragments from normal (bands at 255,231,161,57) and LHON (band at 255 is missing, new bands are seen at 127 and 128 bp) mtDNA.

The ability to amplify and identify lesions of mtDNA from single cells suggests that, technically at least, it might be feasible to perform prenatal diagnosis of certain mtDNA defects from small sample volumes including chorionic villi, amniotic fluid, and single blastomeres from the pre-implanted blastocyst. Due to a restricted sample supply,

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FIGURE 7: Detection of the nt. 11778 mutation in mtDNA from a single cell of a patient with Leber's Hereditary Optic Neuropathy (LHON). Samples were amplified by PCR using NAD4 primers (nts. 11490-12194), treated with the restriction enzyme *MaeIII* and the digestion products were separated by acrylamide gel electrophoresis. Lane A, negative control; lane B, positive control, 10 ng purified placental mtDNA; lanes C-F, single cells amplified with NAD4 primers; lane C, normal patient; lane D, LHON patient; lane E, same as lane C treated with *MaeIII*; lane F, same as lane D treated with *MaeIII*; lane G, 100 bp ladder.

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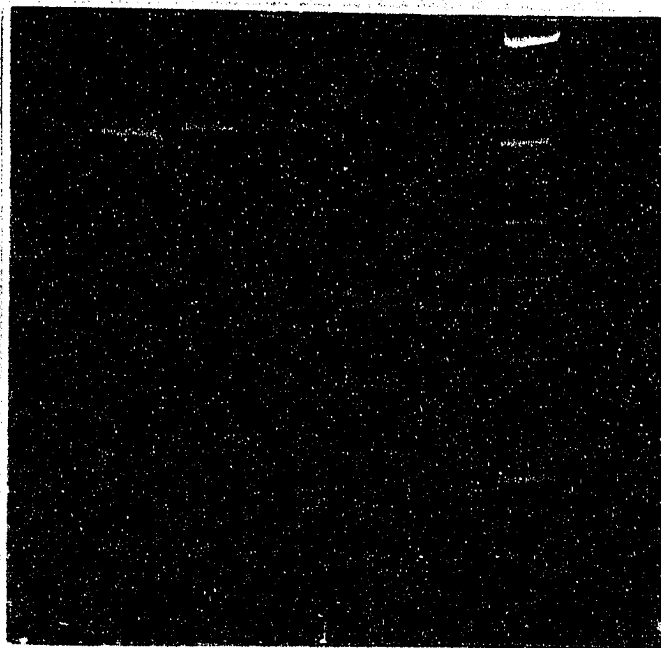

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it would be advantageous if multiple regions could be analyzed from a single sample. Therefore, we investigated the application of multiplex analysis and subsequent fragment-specific sequencing as a correlate with other available mutation identification technologies. Because sequencing is a highly sensitive technique, it is exquisitely responsive to contaminations that might otherwise affect normal detection procedures.

We synthesized two other primer sets of approximately equal size to the LHON set but with enough size difference to distinguish the fragments during electrophoresis. The other two fragments were designed to detect the MELAS (mitochondrial encephalomyopathy, lactic acidosis, and strokelike symptoms) and MERRF (myoclonic epilepsy, ragged red fibers) mutations by RFLP analysis. Their positions on the mtDNA and sizes are shown in Figure 8. Also, because primer sets can often interact with each other due to common sequences resulting in primer dimers and aberrant band production, we tested each primer set alone and in various combinations. This experiment is shown in Figure 8B. As can be seen, each primer set amplifies independently and no extraneous banding is apparent. This is important to both the sequencing and other detection techniques because aberrant band formation could influence the determination of the presence or absence of the mutant.

Next, we repeated the multiplex experiment using the LHON and control single cells isolated from culture. Figure 9 is the multiplex amplification of the single cells (lanes F-control, and G-LHON) and of each individual primer set with placental mtDNA (lanes C-E). Lane C is the MELAS primer set producing a 925 bp band; lane D is the LHON primer set of 704 bp; and lane E is the MERRF primer set generating a 572 bp

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FIGURE 8: **A.** Mitochondrial genomic map showing the positions of the three primer sets and their sizes synthesized for multiplex analysis. Primer sets are: MELAS, nts. 2427-3352; MERRF, nts. 7800-8372; LHON, nts. 11490-12194. **B.** Acrylamide gel electrophoresis of the amplification using combinations of the described primers with 10 ng human placental mtDNA. First lane is pGEM molecular weight markers; for remaining lanes, M = MELAS primers; L = LHON primers; F = MERRF primers.

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A.

O_H

P_H F D·Loop T

V P

12s P_L

E ND6

MELAS 925 bp

16s

L ND5

ND1

L S H

I Q

M LHON 704 bp

ND4

ND2

A N O_L C Y

W ND4L

ND3 R

G

S

A6

A8

D K

MERRF 572 bp

B.

M + L + F | M + L | L + F | M + F | L | F | M

676-

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FIGURE 9: Multiplex amplification of mtDNA from a single cell using the described primers. Samples were amplified using all three primer sets together or each primer set individually. Lane A: negative control; lane B: pGEM DNA markers with band sizes 2645, 1605, 1198, 676 and 517, respectively; lane C-E: positive control using 50 ng of cesium-purified placental mtDNA with: lane C: 925 bp MELAS fragment; lane D: 704 bp LHON fragment; lane E: 572 bp MERRF fragment; lane F: multiplex PCR from a single control cell using all three primer sets; lane G: multiplex PCR from a single LHON cell using all three primer sets.

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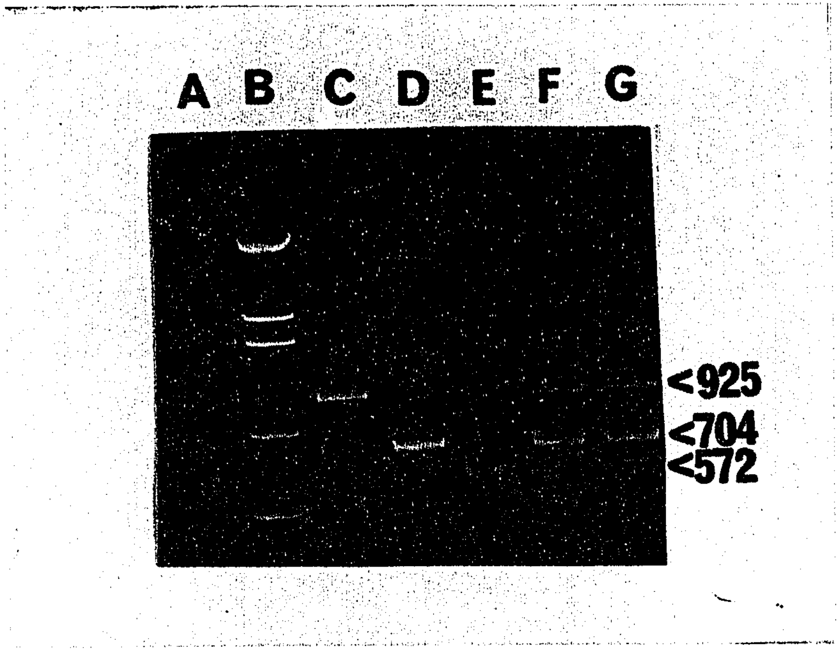

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fragment. No aberrant band formation is seen in the single cell lanes. The remaining template was purified using Wizard PCR purification columns and concentrated to one-half the elution volume. Four microliters of the purified PCR product (MERRF, MELAS and LHON fragments) was used for the sequencing reaction. Primers for the reaction were designed to amplify a 704 bp product from which the G->A 11778 nt LHON mutation could be detected using restriction enzyme digest with *MaeIII*. This mutation is clearly detected in the sequencing gel in Figure 10. The left sequence is the control set with the normal G nucleotide. The right set is the LHON mutant with the altered A nucleotide. The arrow identifies the 11778 position.

This approach demonstrates amplification from a single cell and subsequent target-specific sequencing analysis of the LHON mutation from the pool of mixed fragments. In an analogous fashion, appropriate primers could have been used to allow sequencing of either the MERRF or MELAS regions. Using this technique, multiple areas of the mtDNA can be examined for sequence variations even when the sample size is severely limited.

3. Heteroplasmy Analysis

This work is the first to show that individual cells can be used as a source for amplifying one or more specific mtDNA sequences. The analysis of DNA from a single cell has been reported previously but in almost all cases these studies have examined gene loci on diploid or haploid chromosomal DNA (132,133,134). The only previous

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FIGURE 10: Sequence analysis of the 704 bp LHON fragment showing the LHON 11778 mutation (6) from the multiplex PCR amplification of a single cell. Lanes are A,C,G. Samples are: left: the normal control patient with the G nucleotide at position 11778 (arrow). Right: LHON patient showing the G -> A mutation associated with the disease (arrow).

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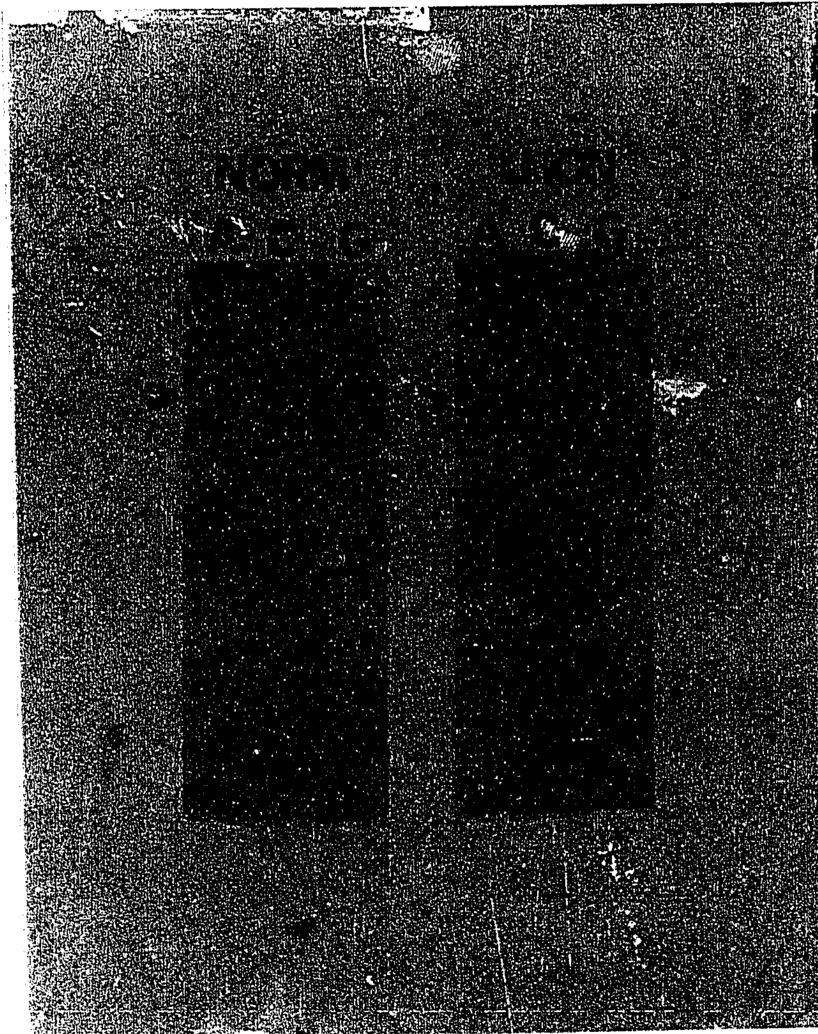

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analysis of mtDNA from single cells has been the study of mutant mtDNA in skeletal muscle fibers from patients with CPEO (135) and MELAS (136). These studies found a heteroplasmic state of the mtDNA within the muscles that, in some cases, correlated with the severity of the clinical presentation of the disease. Chomyn et al (137) argued that this heteroplasmy was actually a variety of homoplasmic cells that occur within the same tissue. Studies by Moraes et al (136), however, found that all single muscle fibers observed from a MELAS patient maintained heteroplasmy that never exceeded 99%. These myofibers are multinucleate single cells resulting from the fusion of multiple precursor myoblasts. Hence, the possibility exists that the mitochondrial contingent may be heteroplasmic because the organelle derive from different myoblasts which contained different mitochondrial genomes. Therefore, we sought to investigate the proportion of heteroplasmy in a non-fused uni-nucleate cell line.

Our lymphoblast cell line was derived from a patient with a heteroplasmic MELAS presentation. White blood cells were recovered from whole blood, Epstein-Barr virus (EBV) transformed and mixed B-lymphocyte cultures grown. Because these cells were not clonally-derived, they should depict an accurate representation of the heteroplasmic state of the tissue. Due to their OXPHOS defect, these cell lines were cultured in the presence of uridine and pyruvate. Uridine is a component of pyrimidine catabolism and is converted to an intermediate (malonyl CoA) which is used for fatty acid oxidation. Synthesized fatty acids can be beta-oxidized to propionyl CoA, converted to succinyl CoA, and used in the citic acid cycle for ATP synthesis. Pyruvate is converted to oxaloacetate and ultimately to glucose via gluconeogenesis. Once the cell

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culture was established, an aliquot was removed for single cell isolations and total DNA recovery. Isolates were PCR-amplified using the primers L2980 to L3001 and H3352 to H3330 generating a 372 bp mtDNA fragment. When digested with *ApaI* this fragment is cut into a 263 bp and 109 bp band if the MELAS mutation is present. The normal fragment remains uncut. Following digestion, the restriction digest was run on an acrylamide gel and the gel was silver-stained. In one case, the PCR was performed in the presence of a radioactive precursor and the gel autoradiographed following silver-stain to permit a comparison of the two techniques (Figure 11A). The visualized bands were photographed and scanned (silver-stain and autorad) by laser densitometry.

The heteroplasmic analysis conducted on MELAS B-lymphoblasts is shown in Figure 11A and B. Figure 11A shows the silver-stain and autoradiographic analysis previously described. As can be seen, in this experiment the silver-stain technique and autoradiography have approximately equal sensitivity. In fact, the scanning analysis was essentially equal (data not shown). All future analyses were conducted using the silver-stain technique.

Our initial investigation into the state of single cells in heteroplasmic mitochondrial diseases found that mutant and normal populations of a diseased tissue were not separated into homoplasmic cell populations. Instead, each cell contained a heteroplasmic grouping. Following scanning of our individual cells, we also found that the proportion of heteroplasmy ranged from 14.4% mutant to 58.4% (Figure 11A) while our control DNA (total DNA isolated from 1.5 milliliters of cultured cells) had an average mutant mtDNA level of 28.3%. When we averaged all mutant proportions in our

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FIGURE 11: Heteroplasmy analysis showing an acrylamide gel separation of *ApaI*-digested, PCR amplified total DNA from single MELAS cells using primers at positions 2980-3352. **A.** PCR amplification was performed with [α-^{32}P]dATP. Upper panel: Silver-stained acrylamide gel. Digestion creates a 263 and 109 bp fragment in the presence of the MELAS mutation. The normal fragment is uncut (372 bp). Lower panel: autoradiography of the same gel. **B.** Same analysis as described above but single cells were isolated following one month in culture prior to PCR amplification. Percentage numbers in both **A.** and **B.** indicate the ratio of mutant and normal molecules from the single cells following scanning analysis by laser densitometry. Control lane indicates the ratio of the cell population.

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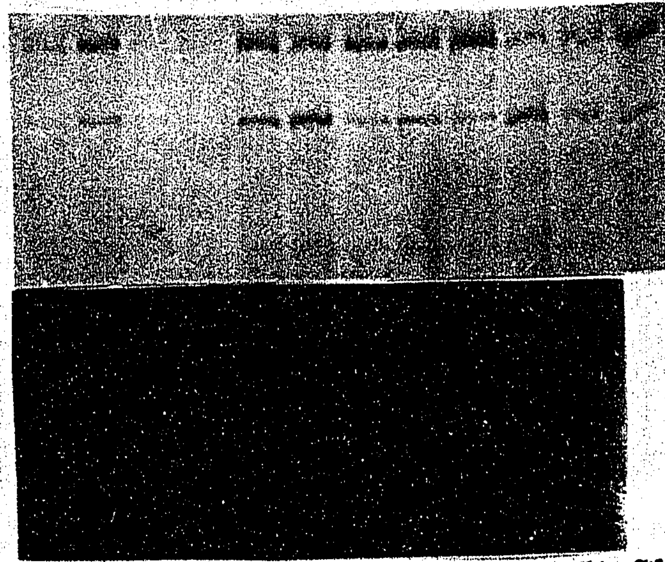
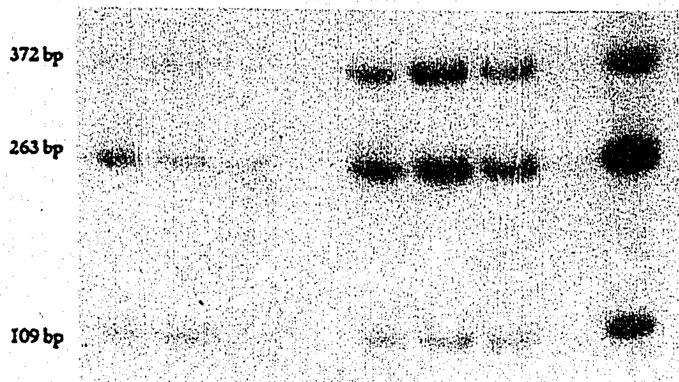
A.



B.



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single cells, we found that the whole culture mutant percentage of 28.3% reflected the average of our single cell values. This provides further evidence that the heteroplasmic state of the tissue is the result of the heteroplasmic state of the individual cells.

Following approximately one month in culture, we reassessed our single cells for heteroplasmy (Figure 11B). We found a significant increase in our mutant to normal mtDNA ratio (54.8% to 80% mutant) with the average of the analyzed cells still reflecting the total population ratio (63.2% mutant) (data not shown). What caused this significant increase in the mutant population? Previous research by Yoneda et al (138) into the segregation of mutant and wild-type mtDNA indicated that an intracellular replicative advantage of the mutant mtDNA was mainly responsible for the dramatic shift toward the mutant genotype. This was not attributable to a faster growing mutant or due to unequal segregation during division. Yoneda suggests a possible feedback mechanism that induces the selective replication of mtDNA in organelles functionally compromised due to the mutant mtDNA. The observation that, phenotypically, the MELAS mutation may be complemented by as little as six percent wildtype genomes (138,139) may indicate that MELAS homoplasmy is detrimental to cell survival. This is somewhat substantiated by the fact that in neither our study nor that of Moraes et al (136) were 100% MELAS homoplasmic cells identified.

B. Mitochondrial Myopathy Patient Analysis

Mitochondrial DNA diseases had not been recognized as true diseases until 1988

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when Holt et al (33) correlated large mitochondrial deletions with Kearns-Sayre Syndrome (KSS) and Wallace et al (40) identified a point mutation in association with LHON. Since these initial discoveries, a number of mtDNA mutations have been isolated in association with a variety of diseases. A map of the mitochondrial genome with some of the more common disease-related mutations and their positions is shown in Figure 12. The point mutation diseases are specified by nucleotide position. Deletion mutations encompass a broad range of sizes but are categorized as either major or minor deletions. The limits that define these subtypes are shown. Due to the common origin of these diseases on the mtDNA, they also share a variety of characteristics (40). These include (140):

1. **Maternal Inheritance.** Mitochondrial DNA demonstrates maternal inheritance and can be traced through a maternal lineage (141,142). The ovum harbors several thousand mtDNAs while the sperm has only a few hundred (142) and those few that enter the egg (less than one percent) have little effect on the genotype (143).

2. **Symptoms indicate a defect associated with the Oxidative Phosphorylation System.** All of the mtDNA genes encode subunits of the OXPHOS enzymes or tRNAs or rRNAs that function in the synthesis of these proteins (144). A mtDNA defect that alters the function of a protein or its synthesis will be reflected as a defect in the activity of the OXPHOS system. These alterations can be identified more definitively through analysis of the respiratory chain complexes.

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FIGURE 12: Human mitochondrial genome map showing the location of all genes and mutations. Gene definitions are presented in the associated legend; mutations are: MELAS: Mitochondrial Encephalomyopathy, Lactic Acidosis, Strokelike episodes; MMC: Mitochondrial Myopathy and Cardiomyopathy; LHON: Leber's Hereditary Optic Neuropathy; MERRF: Myoclonic Epilepsy, Ragged-Red Fibers; and NARP: Neurogenic Ataxia, Retinitis Pigmentosa. Deletion limits indicate the largest regions involved in the major and minor deletions.

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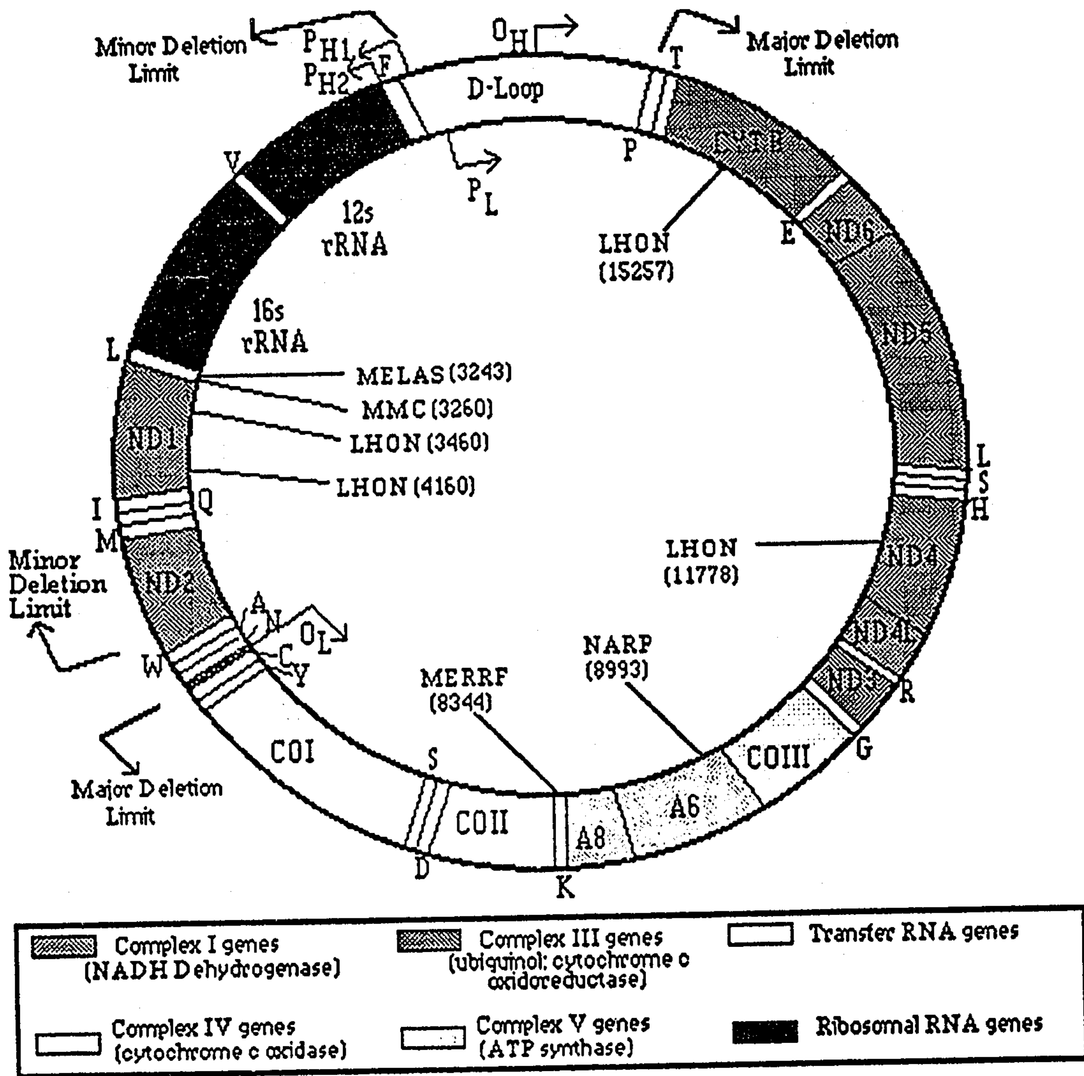

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3. The extent of the OXPHOS deficiency increases with each generation along the maternal lineage. Because a mtDNA-based disease is maternally inherited, the symptoms of the disease should increase in severity with each generation as the proportion of mutant mtDNA increases. However, the mtDNA diseases also present a 'threshold effect' wherein the phenotypic expression of a mutation depends on the relative proportion of the mutant and wildtype mtDNAs within the cell (22). Therefore, the variation in clinical symptoms is not always obvious and is used as a guide rather than the norm.

4. Different tissues are affected sequentially as the respiratory capacity along the maternal lineage declines. Different organ systems rely on mitochondrial energy to different extents. This differential reliance on the OXPHOS capacity is reflected in the type of tissues affected by mtDNA mutations (140,145) starting with the most highly oxidative tissue (i.e. brain, CNS) and progressing through the less and less OXPHOS-dependent tissues (i.e. Type I oxidative skeletal muscle, heart, kidney, liver) (40). The most notable effect, however, is in the skeletal muscle often exhibited as muscular atrophy.

Using these common characteristics as a framework by which to search, we began to investigate mtDNA-based diseases (or mitochondrial myopathies). To prepare for an extensive search of the mtDNA, we designed primers that encompassed the entire mitochondrial genome. A slight overlap in the primer sets was incorporated to ensure that

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no point mutations were missed. Additionally, primer sets were synthesized to permit detection of the known mutations to allow our screening procedure to identify only new mutations. These synthesized primers are shown in Table 4. Each primer is listed according to position, left or right primer, and a common name. This setup allowed us to match any left primers with any right in a rapid fashion as well as to determine appropriate sequencing primers.

Next, we contacted local area neurologists indicating our desire to investiagte patients meeting the above criteria. The IRB-approved patient form we developed is attached as Appendix 2. As samples became available, we acquired as much background information as possible and began screening for the known mutations while waiting for respiratory complex evaluations. Patients were initially screened for the mutations associated with KSS, MERRF, MELAS, LHON, and NARP. An example of this analysis is shown in Figure 13. In 13A, patients three, four and six are screened against the control population for large deletions. This procedure involves three different restriction digests of the total DNA followed by Southern analysis to detect the mtDNA fragments. An alteration in band migration from that of the control would indicate the presence of a deletion (or insertion) mutation. Patients were also analyzed for deletion mutations by PCR using primers for the 4977 kb common deletion (data not shown). In 13B, patients two, three, four and six are being assessed for the MERRF mutation. Mismatch primers, which generate a *NaeI* restriction site in the presence of the mutant are used in a radioactive PCR. Following restriction digest, the reactions are run on an acrylamide gel, dried and autoradiographed. An alteration in band migration from the control would

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TABLE 4: Primers synthesized for mutational analysis of the mitochondrial genome. Left primer positions indicate the five prime-most heavy strand binding site; right primer positions indicate the three prime-most light-strand binding sites. Names were arbitrarily determined to easily distinguish the primers.

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TABLE 4

LEFT PRIMERS			RIGHT PRIMERS	
NAME	NT POSITION		NAME	NT POSITION
A_1	130		A_2	1259
B_1	1257		Bi_2	2444
Bi_1	2427		B_2	3352
$Term_L$	2980		C_2	4506
C_1	3352		D_2	4644
D_1	4504		E_2	4977
E_1	4642		F_2	5720
F_1	4976		G_2	6286
G_1	5717		H_2	6473
COI_1	5900		COI_2	7600
H_1	6281		I_2	8189
I_1	6471		$MERRF_2$	8372
4_1	6644		J_2	9073
5_1	6818		$A6_2$	9200
6_1	7009		$COII_2$	9205
7_1	7186		$COIII_2$	10145
$COII_1$	7600		K_2	11492
J_1	8188		L_2	12194
$MERRF_1$	8282		M_2	13168
$A6_1$	8646		K_M	13666
K_1	9070		cyt b_2	15177
$COIII_1$	9186		N_2	15346
1_1	9342		O_2	133
2_1	9523			
3_1	9731			
L_1	11490			
M_1	12195			
N_1	13167			
cyt b_1	14812			
O_1	15345			

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FIGURE 13: Screening analysis for known mutations in mitochondrial myopathy patients. **A.** Southern blot analysis of patient 3, 4, and 6 with a human mtDNA control. First of three lanes is a *BamHI* digest (cut site 14,258 np.) producing a 16,569 bp fragment; second lane, *PvuII* digest (cut site 2650 np.) producing a 16,569 bp fragment; *PstI* digest producing 14,459 bp and 2110 bp fragments. **B.** MERRF analysis of patients 2, 3, 4, and 6 with a human mtDNA control. DNA is PCR amplified using mismatched primers (see Materials/Methods) and digested with *NaeI*. Normal fragments remain uncut while the MERRF mutation results in a *NaeI* cut site producing 27 and 64 bp fragments. First lane is negative control; 'u' = undigested and 'd' = digested. **C.** MELAS analysis of patients 7 and 8 with a human mtDNA control. DNA is PCR amplified with [α-^{32}P]dATP to produce a 372 bp target fragment which is digested with *HaeIII*. Normal fragments produce 169, 166 and 37 bp fragments while the presence of the MELAS mutation results in 166, 97, 72, and 37 bp fragments. First lane is a negative control; lanes 2, 4, and 6 are undigested fragments; lanes 3, 5, and 7 are digested with *HaeIII*. **D.** LHON analysis of patients 2, 3, 4, 6, 7, and 8 and human mtDNA control. *SfaNI* digestion of normal PCR amplified DNA results in 416 and 208 bp fragments while the LHON mutant remains uncut (704 bp). Lanes 1, 3, 5, 7, 9, 11, and 13 represent undigested. Lanes 2, 4, 6, 8, 10, 12, and 14 are digested with *SfaNI*.

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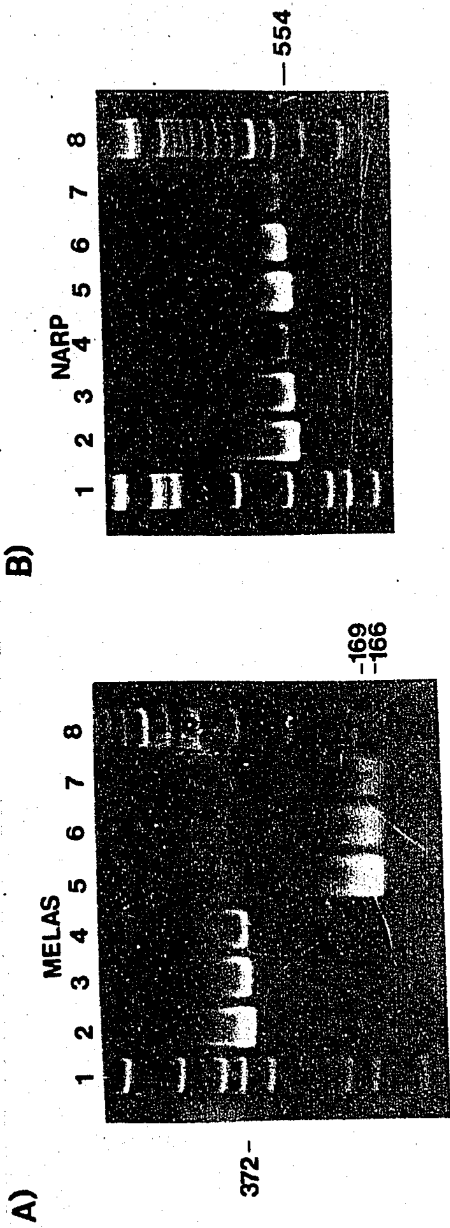

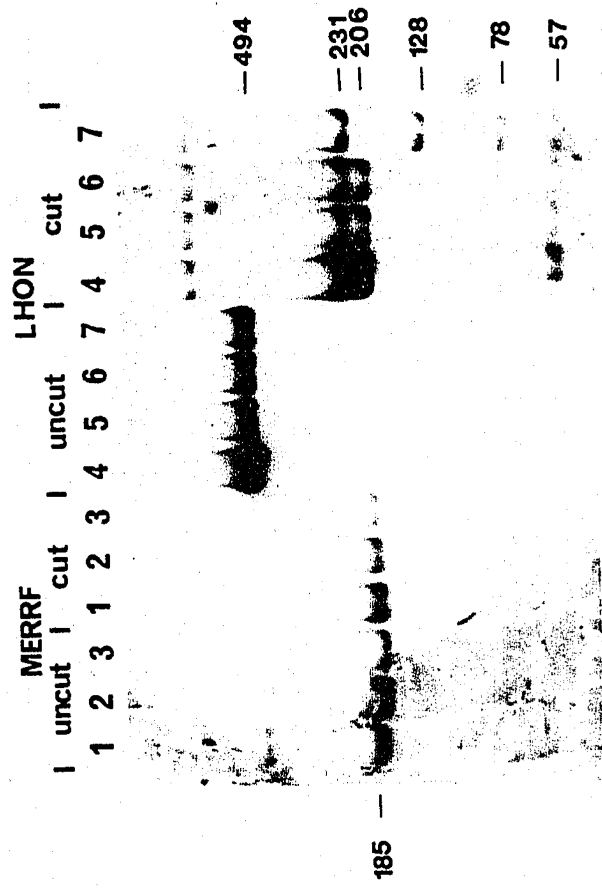

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indicate the presence of the mutation. As can be seen, no MERRF mutants are present. Figures 13C and 13D are also screening tests for the MELAS and LHON mutations, respectively. These tests were performed using radioactive PCR followed by restriction digest and gel analysis. No mutants were found.

A compilation of the patient samples received, their clinical symptoms and biochemistry are shown in Table 5. Any subsequent diagnosis during our analysis is shown under the biochemistry column. Additionally, results from our known mutational analysis are also shown. Of the patient samples received for analysis, only three showed positive maternal inheritance - patients four, seven and nine. It was found during the course of our initial screening, however, that both patient nine and the patients' mother had been exposed to high doses of carbon monoxide which can sometimes mimic the symptoms of a mitochondrial disease. This patient was dropped from further investigation. Although some preliminary analysis was also performed on the other patient samples, emphasis was focused on patients four and seven.

1. Patient Four Investigation

Fibroblasts from the mother of patient four were acquired from Dr. Mathew Frank in December, 1992. The patient was a four-year-old male diagnosed with severe mental and physical developmental delay. Symptoms included ptosis, hypospadias, and hypotonia. There was a positive family inheritance with the mother demonstrating a history of less severe hypotonia and bilateral congenital ptosis. A brother and sister of

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TABLE 5: Mitochondrial myopathy patients. Information regarding symptoms and biochemistry were obtained from the attending physician with patient or guardian permission. Results from known mutation screening are shown under the associated disease name.

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Pt.	AGE	STATE	SYMPTOMS	BIOCHEMISTRY	NARP	MERRF	MELAS	LHON	KSS
1	15 yrs	deceased	Huntington's Disease; negative family history	tests nondiagnostic					
2	16 mo	deceased	hypotonic, horizontal nystagmus, intermittent ptosis, developmental delay, negative family history; Leigh's disease?	icreased lactate and beta-hydroxybutyrate, high lactate/pyruvate ratio, normal complex II,III,IV. Possible complex I deficiency	-	-	-	-	-
3	3 yrs	alive	hypotonia, myoclonus, developmental delay, slight optic aptrophy, esotropia, chronic encephalopathy, congenital lactic acidosis, negative family history	abnormal lactate production, increased lysine and cysteine, normal skeletal muscle, normal complex II,III,IV; Complex I deficiency	-	-	-	-	-
4	4 yrs	alive	severe mental and physical developmental delay, ptosis, hypospadias, diffusely weak and hypotonic; positive family history	cytochrome c oxidase deficiency Mother Affected:	-	-	-	-	-
5	4 yrs	alive		defect of fatty acid oxidation, ketotic dicarboxylic aciduria					
6	16 yrs	alive	eposodic ataxia, migraine headaches, retinopathy, seizures, negative family history	variable lactate levels, OXPHOS biochemistry under investigation	-	-	-	-	-
7	5 mo	alive	hypotonia, dysmorphic features, deafness, ataxia, positive family history	organic aciduria, succinyl choline sensitivity; OXPHOS biochemistry under investigation	-	-	-	-	-
8	6 yrs	alive	hypotonia, ataxia, choreoathetoid movements, developmental delay, short stature	mitochondrial abnormality	-	-	-	-	-
9	5 yrs	alive	positive family history	mitochondrial abnormality	-	?	-	-	?

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the patient were unaffected (Figure 14A). A muscle biopsy was acquired and forwarded to Dr. Brian Robinson at the Hospital for Sick Children, Toronto, Canada for enzymatic analysis. The results of this analysis are shown in Figure 14B as reported by Dr. Robinson's Clinical Genetics lab. The depressed cytochrome c oxidase level (2.99 versus 6.71 in controls) and the lactic acidosis (37.7 versus 27.0 in controls) indicate a possible defect of cytochrome c oxidase expression. With a positive family history, there is a possibility of a mtDNA defect as mentioned in the 'comments section' of Figure 14B. This information prompted our investigation of the three cytochrome c oxidase gene regions on the mtDNA. Although we only had access to the mother's DNA (through the fibroblasts), we decided to proceed with our own analysis for two reasons - i) the mother displayed symptoms similar to her son's but much milder; and ii) mtDNA diseases with a positive family history in the maternal lineages often demonstrate a common mutation with increasing heterozygosity through each generation (22).

The cytochrome c oxidase (CO) subunits encoded on the heavy-strand mtDNA are: COI (5904-7444); COII (7586-8262); and COIII (9207-9990) - spanning approximately 3000 nucleotides (nts) of the genome. They are separated from the genome and each other by five tRNAs and the ATPase six and eight genes. These cytochrome c oxidase genes encode three of the 13 polypeptides that constitute complex IV of the respiratory chain (the other subunits are nuclear-encoded). Cytochrome c oxidase is a complex metalloprotein that provides a critical function in cellular respiration by catalyzing the reaction between hydrogen ions, electrons and oxygen to form water (146) with cytochrome c being the physiological electron donor. This exergonic reaction results

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FIGURE 14: A. Pedigree of patient four, the six-month-old proband, showing the affected mother and unaffected father and siblings. **B.** Biochemical analysis of patient four by Dr. Brian Robinson at the Division of Clinical Genetics, showing decreased cytochrome c oxidase levels and lactic acidosis.

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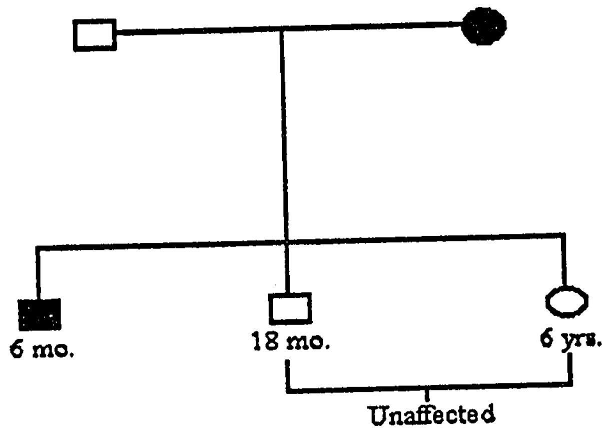
A.



B.

The following are results on patient 4

	Patient	Controls
	nmoles/min/mg protein	
Pyruvate Dehydrogenase (native)	0.98 (1)	1.04 ± 0.26 (7)
Pyruvate Dehydrogenase (dichloroacetate activated)	1.22 (1)	1.35 ± 0.14 (5)
Pyruvate Carboxylase	—	—
Phosphoenolpyruvate Carboxykinase	—	—
Lipoamide Dehydrogenase (E_3)	—	—
Dihydrolipoyl Transacetylase (E_2)	—	—
Cytochrome Oxidase	2.95 ± 1.37 (5)	6.71 ± 0.75 (6)
	nmoles/hr mg protein	
Pyruvate Decarboxylase E_1	—	—
Cellular Lactate/Pyruvate ratio	37.7 ± 6.4 (4)	27.0 ± 2.9 (7)

Comments: This seems to be a partial defect of cytochrome oxidase expressed in fibroblasts. With mother history of ptosis it would possibly be a mt DNA defect. We will try and pursue this further.

Yours Sincerely,

Brian H. Robinson, Ph.D.,

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in the conservation of the pH gradient and the membrane potential across the mitochondrial inner membrane necessary for Oxidative Phosphorylation. Cytochrome c oxidase also appears to be the highly regulated control site for the overall process of Oxidative Phosphorylation. This enzyme catalyzes the only irreversible step of the chain and, therefore, functions as the 'bottleneck' of the system. The mitochondrial-encoded subunits (COI, II, III) form the catalytic core of the eukaryotic enzyme. Subunits I and II form the redox (or metal) centers of the enzyme (147,148) with subunit II also containing the high affinity binding site for cytochrome c. Subunit III appears to function in proton-pumping (149) although its precise role is unclear. Any alterations of these subunits (i.e. mutation) that affect the capacity to function therefore can severely hamper the ability of the respiratory chain to provide ATP.

We decided to directly sequence these three gene regions due to their relatively small sizes (COI is approximately 1500 nts. while COII and COIII are both less than 800 nts.). Our preliminary analysis began with the mother's COIII region where direct sequencing identified four nucleotide changes as compared to the published sequence (150) of which three actually altered amino acids (Figure 15A). Determination of a significant alteration is essential for disease-associated mutations, especially those involved in altering the amino acid sequence. Wallace et al (40), in identifying the LHON mutation, defined a set of criteria to identify disease-associated mutations in protein-coding regions. Of primary importance is the necessity for the mutation to alter an amino acid (i.e. not be 'silent'). This criteria now excludes the mutation at 9809 bp. The A->C mutation at this site occurs in the wobble position of the codon and does not

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FIGURE 15: Identified mutations in patient four cytochrome c oxidase genes. Sequence of the mtDNA in both the control (upper sequence) and patient four (lower sequence) is shown with any associated amino acid changes. Mutation is boxed. **A.** COIII gene. **B.** COI gene. No mutations were found in the COII gene region.

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PATIENT FOUR
CYTOCHROME C OXIDASE MUTATIONS

A. COIII

```
                                  9477
                        S    E    [V]   F
CONTROL  ...............TCA  GAA  [G]TT TTT.................
PT. FOUR ...............TCA  GAA  [A]TT TTT.................
                        S    E    [I]   F

                             9559
                        P   [R]       T
CONTROL  ...............CCC [C]G A  ACA.................
PT. FOUR ...............CCC [C]C A  ACA.................
                        P   [P]       T

                             9667
                        E    N      N
CONTROL  ...............GAA  A[A]C  AAC...............
PT. FOUR ...............GAA  A[G]C  AAC...............
                        E    S      N

                                9809
                        A    T      G
CONTROL  ...............GCC  AC[A]  GGC.................
PT. FOUR ...............GCC  AC[C]  GGC.................
                        A    T      G
```

B. COI

```
                                6050
                        L    G      N
CONTROL  ...............CTA  GG[T]  AAC...............
PT. FOUR ...............CTA  GG[G]  AAC...............
                        L    G      N

                                6956
                        V    G      G
CONTROL  ...............GTA  GG[T]  GGC.................
PT. FOUR ...............GTA  GG[C]  GGC.................
                        V    G      G

                                7028
                        V    A      H
CONTROL  ...............GTA  GC[C]  CAC.................
PT. FOUR ...............GTA  GC[T]  CAC.................
                        V    A      H
```


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alter the normally encoded threonine amino acid. The other three changes, however, do change the protein-coding sequence. The second criteria is that the mutation alter an amino acid conserved across species lines. The conservation of an amino acid in many different species indicates that its position is involved in a fundamental function of the protein it helps encode. Alteration of this amino acid may result in the inhibition (or the enhancement) of the catalytic activity of the OXPHOS subunit or it may alter membrane binding or substrate binding. Of the three mutations that alter the amino acid sequence, only one is conserved. The changes at 9559 bp and 9667 bp are both found in the bovine sequence indicating that these alterations are not functionally detrimental to the proton-pumping role of the COIII subunit. The last mutation at 9477 bp was conserved in bovine, however it violated the third criteria used for determining significance - it is a normal nucleotide variant as defined by Marzuki et al (151). This is a published database of 128 polymorphic nucleotide positions in the coding regions of the mitochondrial genome from ten unrelated individuals. The final two criteria used to identify a mutation should it pass the first three are that the mutant be disease-specific (found in multiple (but not necessarily all) patients demonstrating symptoms of the same disease) and that it not be observed in a population of control individuals. If a variant violates any of these five criteria, it is considered a natural polymorphism as are all the identified mutations in COIII.

The COII gene product contains the redox center of the cytochrome c oxidase subunit as well as the high affinity binding site for cytochrome c. The COII gene is less than 700 bp in length and appears to be more highly conserved than the other two

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domains. In sequencing this region we found no mutations in our mother fibroblast DNA. Our observation is also supported by Marzuki's (151) who identified only three COII mutations in ten patients. The other gene regions, COI (>1500 bp) and COIII (<800 bp) had fourteen and seven alterations, respectively (151).

Prior to sequencing the COI gene region, we received the fibroblast DNA of patient four from Dr. Robinson to use in comparison with the analysis of the mother. The two DNA sources are therefore referred to as 4m for the mother and 4s for the son to distinguish the results. We then decided to sequence the proband for the COI gene region. Dr. Robinson's postdoctoral assistant had already sequenced the COII and COIII regions and found no changes of significance. We agreed to compare our final results as well.

In the COI gene region we identified three changes to the son's mtDNA (Figure 15B). All three proved to be silent mutations (6050 bp, 6956 bp, 7028 bp) that did not affect the amino acid sequence. Contacting Dr. Robinson with these observations he also confirmed that, to date, his postdoctoral assistant had found nothing of significance.

Although the family history and respiratory chain activities together indicated a possible mtDNA defect of the cytochrome c oxidase regions, we found no significant disease-specific mutations. One could postulate that other mutations in mtDNA gene regions (i.e. tRNA or rRNA) involved in mitochondrial transcription affected the enzymes activity, but such a mutation should be reflected by a multicomplex involvement which we do not see in this patient. Therefore, the mutation probably resides on a nuclear gene element from the cytochrome c oxidase subunit. Although family history

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hints at mtDNA involvement, a recessive gene on the X-chromosome or a recessive autosomal mutation should also be considered. A broader family history involving the mother's siblings and ancestors would do much to clarify the inheritance pattern. Because no mutation on the mtDNA cytochrome c oxidase gene regions was found and we are not prepared to pursue nuclear gene mutations, we shifted our investigations to a second patient.

2. Patient Seven Investigation

Tissue from a muscle biopsy was received from patient seven in October, 1992 from Dr. Lawrence Leichtman. The patient was a five-month-old female diagnosed with failure to thrive, poor feeding and interval growth, swallowing difficulties, and marked hypotonia. A family history consisted of a mother with generalized muscle weakness, succinyl choline sensitivity, vertigo, and deafness, but no other maternally-affected relations (Figure 16). A muscle biopsy showed no abnormal ultrastructural morphology and normal NADH and cytochrome c oxidase distribution (Figure 17A and B). Analysis conducted by Emory Molecular Diagnostics Laboratory found no deletion or duplication of the mtDNA by Southern analysis and no point mutations at positions 3250 or 3260 of the $tRNA^{Leu}$ gene - two mutations that demonstrate generalized muscle weakness and heart defects in the disease state (Figure 18A and B). A biochemical analysis was scheduled.

Due to the apparent maternal inheritance and skeletal muscle effects, we decided

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FIGURE 16: Patient seven pedigree. Three generations on both maternal and paternal lines are represented. 'Proband' is patient seven. Note the affected mother and unaffected father.

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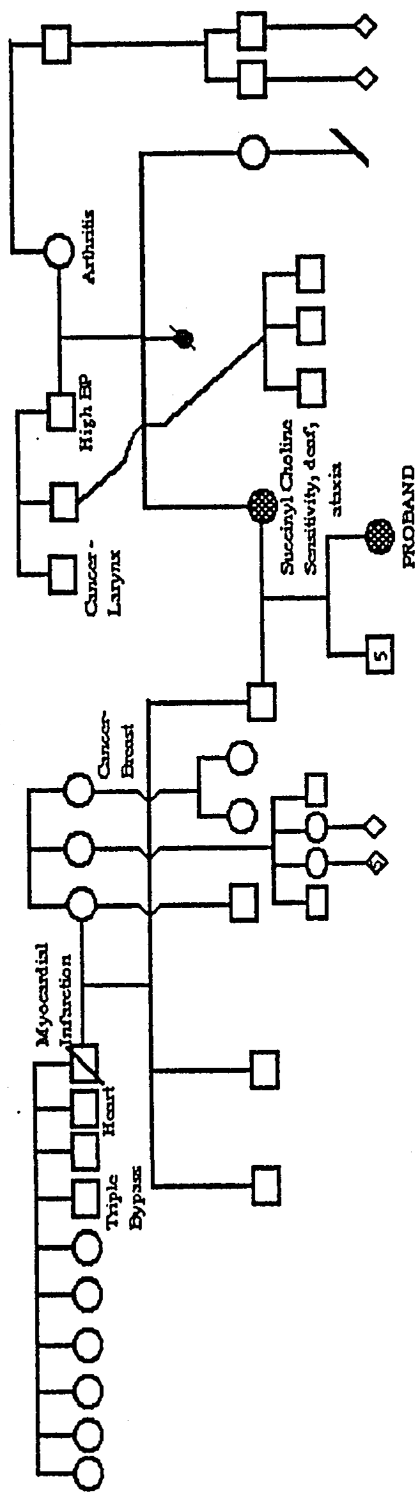

Patient 7 Pedigree

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FIGURE 17: Results of electron microscopy evaluation of patient seven muscle biopsy.

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ELECTRON MICROSCOPY

MICROSCOPIC DESCRIPTION: Multiple grids containing transversely and longitudinally oriented ultra thin sections of skeletal muscle were stained with lead citrate and uranyl acetate and examined under the electron microscope. In longitudinally oriented sections myofibrillar architecture and organization is normal in all fibers examined. In transversely oriented sections. Muscle fiber diameters are in the range of normal for age. There are no necrotic, degenerating, or regenerating fibers seen. Plasma membranes and basement membranes are normal. Myonuclei are normal. Muscle fiber glycogen and lipid droplet content is normal. Numerous mitochondria are seen in both transversely and longitudinally oriented sections and are normal in their architecture, number and distribution. Components of the transverse tubular system and endoplasmic reticulum including triadic formations are normal. In the endomysial space, capillaries and other connective tissue elements are normal.

IMPRESSION: Normal skeletal muscle by ultrastructural criteria.

ADDENDUM: The muscle specimen on this patient examined by light microscopy and histochemistry was normal. In addition to routine histochemistry, additional frozen sections were reacted for cytochrome C oxidase and showed a normal content and distribution of this enzyme.

MUSCLE BIOPSY

GROSS DESCRIPTION: A single specimen of skeletal muscle frozen in liquid nitrogen and mounted in trigacanth was received from the Childrens Hospital of the Kings Daughters. A specimen fixed in glutaraldehyde was also received.

MICROSCOPIC DESCRIPTION: Duplicate transverse frozen sections of the specimen were stained with Hematoxylin and Eosin and modified Gomori's trichrome. Histochemical reactions were carried out for NADH, ATPase following alkaline and acid preincubation, acid phosphastase, lipid and glycogen.

Muscle fibers range from 5 to 18.5 microns in diameter. The majority of fibers are in the 12 to 15 micron diameter range. There are no necrotic, degenerating or regenerating fibers. In the trichrome stained sections the intermyofibrillar membranous network in individual fibers is normal. There is one small area of collagenous scarring in the muscle. Otherwise no abnormalities are seen in the connective tissue or vascular elements. There are no inflammatory infiltrates. In the NADH reacted sections the distribution of oxidative enzyme activity is normal. In the ATPase reacted section there is a normal distribution of histochemical fiber types with no evidence of pathological grouping or other type specific changes. No abnormalities are seen in the acid phosphatase reacted sections. Muscle fiber lipid and glycogen content appears to be normal.

IMPRESSION: Normal skeletal muscle for an infant of this age.

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FIGURE 18: Results of southern blot and point mutation analysis performed by Emory Molecular Diagnostics Laboratory on patient seven muscle biopsy.

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Southern Blot Analysis:

Southern Blot Tissue: Skeletal Muscle

Southern Blot Results:

Normal BamHI digest with a single band.
Normal EcoRV digest with bands at 6.9, 6.1, and 3.6 kb.

Nuclear DNA/Mitochondrial DNA Ratio:	Densitometry (%Mutant; %Normal Mitochondrial DNA):
Not Tested	Not Tested

Southern Blot Interpretation

Normal Southern blot analysis. Mitochondrial DNA (mtDNA) analysis in skeletal muscle using BamHI and EcoRV restriction endonucleases was normal, excluding mtDNA deletions and duplications as the cause for this patient's clinical manifestations.

Clinical Classification: No information available for review.

Recommendations:
(1) No deletion or duplication of the mtDNA was identified in skeletal muscle.

(2) If possible, referral to see me at Emory University is optimal for proceeding with the evaluation. This will permit me to review the clinical presentation, perform a muscle biopsy, isolate fresh mitochondria for oxidative phosphorylation biochemistry, make appropriate cell lines, and perform more comprehensive testing as dictated by the results of routine testing. Please let me know if this is a viable option for your patient. If so, we will assist the patient in making appropriate arrangements for their visit to Emory.

(3) Thank you for the opportunity to work with you on your patient's problem. We will keep you appraised of any developments that are relevant to the care of your patient.

Point Mutation Interpretation:

Normal point mutation analysis. Mitochondrial DNA point mutation analysis in skeletal muscle for mutations at positions 3250 and 3260 of the tRNA-Leucine gene was normal, excluding these mutations as the cause of your patient's manifestations.

Clinical Classification: No clinical information available for review.

Recommendations:
(1) Pathogenic mitochondrial DNA point mutations were not identified in skeletal muscle.
(2) The analysis of the mitochondrial DNA for deletion or duplication mutations is underway and will be reported shortly.
(3) I would be happy to review a clinical summary and give recommendations on this patient if you would like.

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to investigate this patient for a disease-specific mitochondrial mutation. We screened the mtDNA for known mutations prior to a broader analysis. No alterations were identified. We then proceeded toward an analysis of the mitochondrial genome. Because direct sequencing of the entire genome would be impractical, we employed a variety of screening procedures designed to detect single base pair changes so as to scan the mtDNA in segments. The two most successful applications were denaturing gradient gel electrophoresis (DGGE) and single-stranded conformational polymorphism (SSCP).

The principle of the DGGE technique is electrophoretic separation of small DNA fragments based on the T_m of that fragment in a denaturing gradient gel at increased temperature. The sensitivity of the system allows distinction of T_m in fragments differing by only one base pair. The advantage of DGGE is its ability to detect mutations that are present at as little as one percent of the total sample (123). In this method, five to ten microgram aliquots of patient DNA are digested with one of four restriction enzymes, *DdeI*, *HaeIII*, *HphI*, *MboII*, which cut the mtDNA into fragments of 200-700 bp, the optimal size for DGGE analysis. The restriction fragments are electrophoresed in 6.5% acrylamide gels with 20%-80% denaturant range (100% denaturant = 7 M urea/ 40% formamide) in a 60°C aquarium. Migration distance of each mtDNA fragment in the gel depends on the melting behavior which reflects the base composition. Fragments are electroblotted to a nylon membrane and located by Southern blotting with regionalized labeled-mtDNA probes. Alteration of fragment mobility as compared to a control sample indicates a change in base composition reflected as an alteration in melting behavior (Figure 19A). A fragment with a band pattern not seen in control mtDNA can be

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sequenced directly from PCR amplified DNA. The technique is accurate and sensitive and an electroblotted membrane can be stripped and reprobed up to 14 times. The technique, from DNA to autorad, takes approximately three days to complete.

Single-stranded conformational polymorphism (SSCP) is based on electrophoretic detection of conformational changes in single-stranded DNA molecules resulting from point mutations or other small nucleotide changes (121). Single-stranded fragments form hydrogen bonds between the bases within the strand that result in a unique conformation for each DNA sequence. If a nucleotide change occurs within a region involved in the secondary conformation it may alter the hydrogen bonding pattern and, ultimately the molecules' conformation. Conversely, a nucleotide change in a non-structural region may result in a new bond formation that also affects the secondary structure. For larger DNA fragments (over 350 bp), a variety of restriction enzyme digests can be used to test multiple conformations of the strand thereby increasing the probability of placing the nucleotide change in a structurally important region. Conformational alterations are identified by electrophoresing labeled single-strands on a high-resolution non-denaturing gel at room temperature. Gel mobility is a reflection of strand conformation and changes in migration of like strands indicate base pair changes within the sequence that can be identified by direct sequencing (Figure 19B). Although the technique can only detect those nucleotides altering conformation (80% detection rate) the use of multiple restriction digests creating two or more fragments each with their own conformation, increases the probability of detection.

For patient seven we used DGGE to investigate the complex I (NADH

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FIGURE 19: Figures depicting the principals behind the DGGE (**A.**) and SSCP (**B.**) approach. **A.** DGGE analysis of control and patient DNA showing the changes in migration due to alterations in sequence. These base changes alter the 'melting' characteristics of the fragment causing a change in the migration of the fragment. **B.** SSCP approach showing how alterations in sequence can change the secondary structure of the fragment. Note in digest A that the A -> G mutation results in a new binding region that increases the migration rate of the fragment. In digest B, the T -> G mutation removes a binding site and slows fragment migration.

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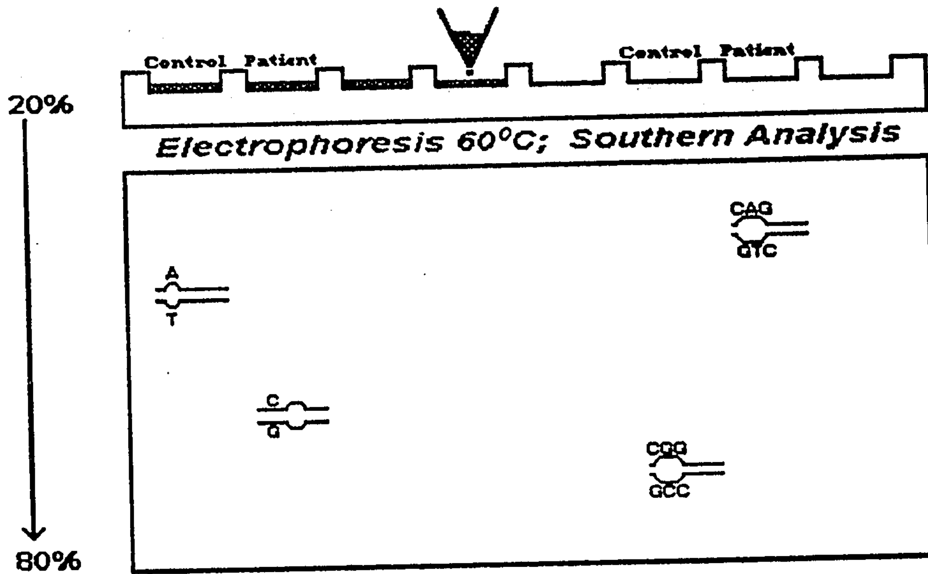
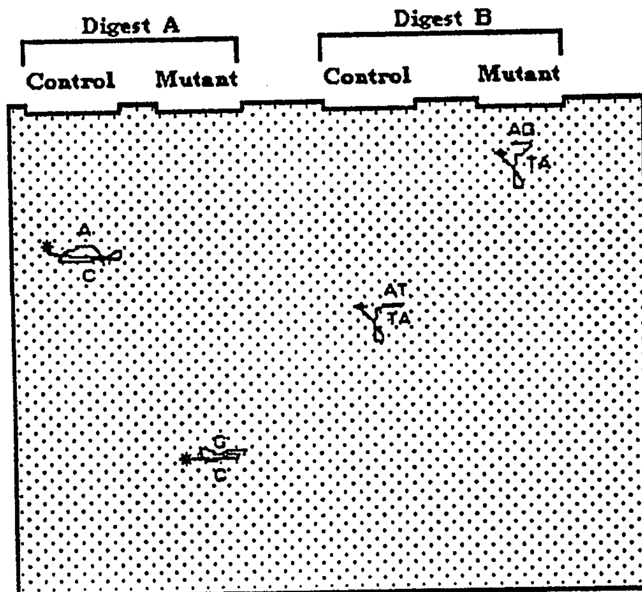
A. DGGE ANALYSIS



B. SSCP ANALYSIS



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dehydrogenase) regions. Because DGGE only maps the mutation to a large region (probe size plus distance to nearest restriction sites), we localized our analysis using SSCP followed by direct sequencing of the identified fragment. A DGGE analysis conducted on patient seven DNA is shown in Figure 20. The probe location (925 bp) and restriction map is indicated in 20A while the DGGE analysis is shown in 20B. Note the altered migration of the band in the *HaeIII* digest for patient seven. A change in the banding pattern is also seen in the *DdeI* lane although not as clearly as the *HaeIII* lane. These pattern alterations indicate a nucleotide change but not a specific location. Using the restriction map, the mutation can be localized to an area of approximately 1000 nucleotides in the 16s rRNA region indicated in Figure 20 by the starred (*) sites.

The probed area of the control and patient DNA was then PCR-amplified with [α-^{32}P]dATP and analyzed by SSCP following restriction digestion (Figure 21). Note the change in migration between the patient and control fragments. Although the variation appears small, it was repeated twice to verify the results. SSCP analysis narrowed the area to be sequenced to 191 bp within the 16s rRNA region. Direct sequencing was then used to identify a mutation at nucleotide position 2706 (Figure 22) which was later classified as a Marzuki normal variant.

The advantage of this approach - DGGE, SSCP, sequencing - is indicated by our ability to identify a single mutation within an approximately 1500 bp region. Allowing for experimental overlap, this screening process took about one week to identify the mutation. The only technique which may present some difficulty is DGGE which requires four five to ten microgram aliquots of the sample DNA for analysis. For analyses where

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FIGURE 20: **A.** DGGE analysis of patient seven total DNA and human mtDNA using a probe to the region 2427-3352 bp. Restriction enzymes used are indicated for each lane. Note the appearance of a second band in the patient seven *HaeIII* lane. **B.** Regional map of the human mitochondrial DNA showing the probe position and positions of the restriction enzyme sites.

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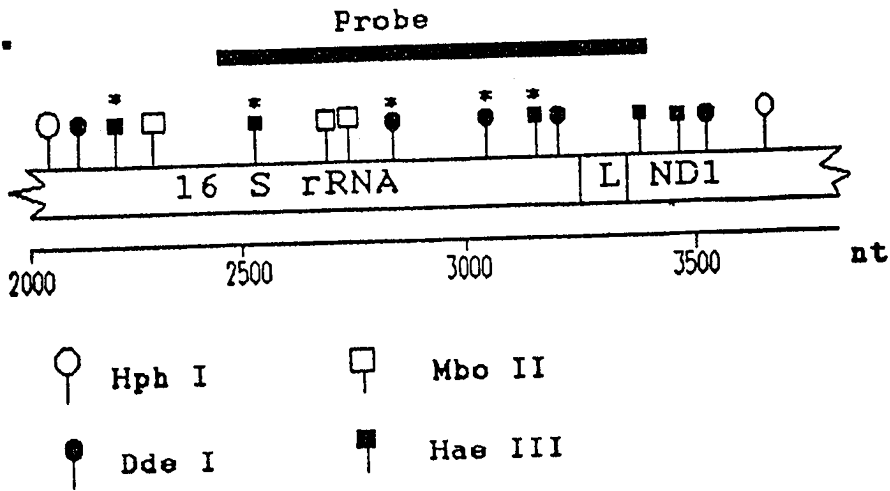
A.



B.

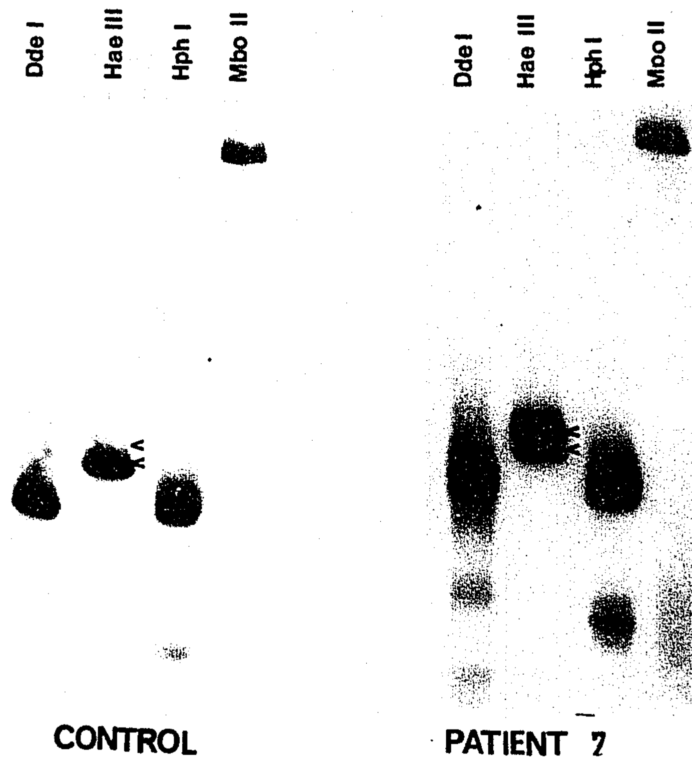


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FIGURE 21: SSCP analysis of the region probed in the DGGE (Figure 20). Bands are the 191 bp fragment generated from the 925 bp PCR amplified DNA and restriction digested with *HhaI* producing bands of 514, 220 and 191 bps.

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FIGURE 22: DNA sequencing gel showing the A -> G mutation at position 2706 of the 16s rRNA gene. The region was sequenced using the primer Bi_1 (2427-2445). The mutation is unconserved in bovine and considered a Marzuki et al (150) normal variant.

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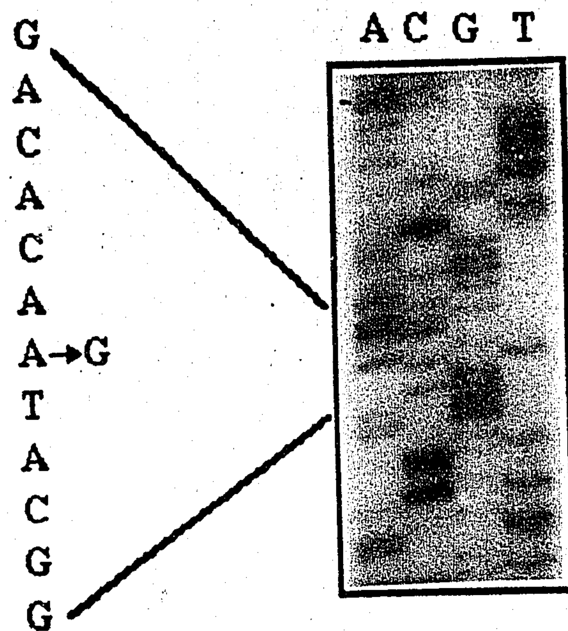
Patient Seven 2706 np Mutation



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sample size is restricted, this could prove to be problematic.

We then began to screen other regions of complex I in patient seven using the same approach. Additional investigations by the EVMS genetic clinic, however, revealed a normal muscle electromyograph and a formal diagnosis of Stickler Syndrome and gastroesophageal reflux disease. Stickler syndrome is an autosomal-dominant disorder of connective tissue characterized by skeletal, orofacial and ocular abnormalities and results from a mutation in the type II procollagen (COL2A1) gene. We, therefore, abandoned our mtDNA analysis of this patient.

Table 6 is a compilation of all mutations identified in our nine patients. The position, patient, nucleotide alteration, and status (normal variant, conserved, unconserved, silent) of the mutation are indicated. No mutation identified has been disease-specific. Because most of our patients had ill-defined biochemistry and no demonstratable maternal inheritance, we began to try to focus our interests into specific diseases with strong mitochondrial criteria as previously described.

3. Rett Syndrome Analysis

Rett Syndrome (RS) is a phenotypically distinct progressive neurologic disorder characterized by notable neurologic impairment. It has been reported almost exclusively in females with an estimated occurrence of one in 10,000-12,000 live births. Morphologic characterization of the nervous system includes hypoplasia (reduced brain size and weight) with a reduced cerebral volume and loss of grey matter. Often,

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TABLE 6: All mutations identified during the analysis of mitochondrial myopathy patients. Patient, position and type of change, effect on amino acid sequence and relevance of the mutation are indicated (i.e. conserved, unconserved, normal variant, silent).

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EFFECT	PT.	NP.	ALTERS	GENE	OLD->NEW NT	OLD->NEW AA
Silent/ N.V.	3	4769	A->G	ND2	ATA->ATG	Met->Met
Silent	3	5670	A->C	$tRNA^{Asn}$		
Silent	3	11593	A->G	ND4	CGA->CGG	Arg->Arg
Unconserved	3	12012	C->G	ND4	TCA->TGA	Ser->Trp
Conserved/N.V.	4_m	9477	G->A	COIII	GTT->ATT	Val->Ile
Normal Variant	4_m	9559	G->C	COIII	CGA->CCA	Arg->Pro
Unconserved	4_m	9667	A->G	COIII	AAC->AGC	Asn->Ser
Silent	4_m	9809	A->C	COIII	ACA->ACC	Thr->Thr
Silent	4_s	6050	T->G	COI	GGT->GGG	Gly->Gly
Silent	4_s	6956	T->C	COI	GGT->GGC	Gly->Gly
Silent/N.V.	4_s	7028	C->T	COI	GCC->GCT	Ala->Ala
Unconserved	6	4596	G->C	ND2	GTT->CTT	Val->Leu
Silent	6	4769	A->G	ND2	ATA->ATG	Met->Met
Unconserved	6	4869	G->C	ND2	CAA->GAA	Gln->Glu
Silent	6	7831	C->G	COII	CGC->CGG	Arg->Arg
Silent	6	8473	T->C	ATPase8	CCT->CCC	Pro->Pro
Silent	6	8649	A->G	ATPase6	CGA->CGG	Arg->Arg
Normal Variant	6	8860	A->G	ATPase6	ACA->GCA	Thr->Ala
Normal Variant	7	2706	A->G	16s rRNA		
Silent	8	8269	C->T	COII	ATC->ATT	Terminator

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accumulations of large and disintegrating mitochondria have been found in association with the disease. Enzymatically, there is reduced regional cerebral blood flow patterns characteristic of decreased glucose metabolism and an immature brain state. Two investigators found decreased levels of respiratory chain complexes possibly following an age-related decline. Genetically, the disease mechanism has eluded researchers but strong maternal inheritance in familial cases is indicated. Additionally, variation in clinical expression in monozygotic twins (104) and increasing clinical severity in a mother-daughter Rett pair (91) hint at possible mitochondrial heteroplasmy. Finally, the progression of Rett Syndrome stages from developmental delay (stage I) through mental deficiency and seizures (stage II), neuromotor regression (stage III) and muscle wasting with loss of ambulation (stage IV) indicate a decline that follows the respiratory capacity of the tissues. Using this information, Rett Syndrome appears to meet all the criteria previously defined for mitochondrial myopathies.

We therefore began to examine the literature relating to enzymatic analysis of respiratory chain complexes to try to determine an area on which to focus our interests. Studies by Coker and Melnyk (48) and Dotti et al (40) showed a decrease in all five complexes of the Rett patients studied. This multicomplex effect usually results from a mutation of a gene directly involved in polypeptide production (transcription or translation) of which only the tRNAs and rRNAs are coded for on the mitochondrial genome. Mitochondrial myopathies stemming from such a multicomplex effect usually result from the mutation of a tRNA gene (2,3,15,43,152,153) while only one disorder has been identified as resulting from a rRNA defect (154). Based on this evidence, we

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decided to investigate the mitochondrial tRNA genes for a disease-specific mutation.

Transcription of the mitochondrial genome results in two polycistronic messages - one from the light strand (L-strand) and one from the heavy strand (H-strand). Because the mtDNA is very compact, no introns separate the genes. As an adaptation, the tRNA genes punctuate the H-strand and L-strand transcripts allowing each message to be separated following tRNA excision. The tRNAs are removed by RNase P-like activity that recognizes the tRNA secondary structure (155,156) at which point each freed message is polyadenylated and translated within the inner mitochondrial matrix by the synthesized tRNAs. There are only 22 tRNAs coded on the mtDNA (as compared to 32 nuclear tRNAs) and these are all that is required to read the mitochondrial genetic code due to a simplified codon-anticodon pairing within the mitochondrial genome. In this system, a tRNA can recognize an entire four-member codon family (i.e. ACA, ACC, ACG, ACU) rather than the two tRNAs per codon family found in the nuclear genetic code. This is accomplished by the inclusion of an unmodified uridine in the wobble position of the mitochondrial tRNA (157). The remaining codons are read by a tRNA with a modified uridine which restricts recognition to a U-G wobble (158). Genetically, there is a cross-species structural variation with the most conserved regions being the loop and distal portions of the stem in the anticodon arm, the stem of the 'DHU' arm, and the tip of the amino-acyl stem (159). Considerable variation is apparently allowable in the rest of the structure. Except in the $tRNA^{Leu(UUR)}$. This tRNA has a secondary role in the regulation of transcription of the mitochondrial genome. Within its sequence is a terminator region that functions to restrict transcription of the total mitochondrial genome

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allowing a high level of rRNA without an associated high level of protein-coding message. An alteration of the terminator region may be associated with the MELAS presentation by altering the ratio of rRNA to protein in the mitochondrial inner matrix (160). With the mitochondrial tRNAs functioning not only in translational processing but also as message 'splicers' and in functional regulation, an alteration of sequence in these regions could severely alter the functional capacity of the tRNA and, subsequently, Oxidative Phosphorylation.

Although the tRNAs are each only about 100 nucleotides in length, they are located in some twelve different areas of the mitochondrial genome (see Figure 12). Additionally, our available primers were not always within the range of tRNA gene to allow sequencing (Table 7). We therefore devised an SSCP approach that would screen each region for mutations with areas of interest being sequenced either with available primers or newly synthesized. This approach involved PCR amplification of the tRNA region followed by an array of restriction digestions (Table 8) to produce fragments that could be analyzed by SSCP. At least two patterns of digestion were analyzed by SSCP for each region. The advantage of this approach over DNA sequence analysis was that multiple samples and regions could be evaluated in a single gel and only primers for regions of interest showing variability in multiple samples need be synthesized.

Our first samples were received from Dr. Donald Lewis as ten milliliter blood samples from two Rett females. The white blood cells were isolated on Ficoll-Hypaque gradients, collected and then digested overnight with proteinase K (as described in Materials Methods Blood DNA Isolation - Method 1). Precipitated DNA was resuspended

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TABLE 7: Primer sets used to analyze the 12 tRNA regions of the mitochondrial genome. Region (zone), tRNA, position and primers used are indicated. Primers available to sequence the region are also indicated.

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ZONE	tRNA	POSITION	PRIMERS USED	SEQUENCE?
1	Phe	577-677	A_1(130)-A_2(1259)	N
2	Val	1602-1670	1488_1-Bi_2(2444)	Y (1488_1)
3	Leu(UUR)	3230-3304	T_L(2980)-B_2(3352)	Y (B_2)
4	Iso	4263-4331	C_1(3352)-C_2(4506)	Y (C_2)
	Glu	4329-4400	"	Y (C_2)
	Met(fMet)	4402-4469	"	Y (C_2)
5	Trp	5512-5576	F_1(4976)-G_2(6286)	Y (F_2)
	Ala	5587-5655	"	Y F_2
	Asp	5657-5729	"	Y (F_2,G_1)
	Cys	5761-5826	"	Y (F_2,G_1)
	Tyr	5826-5891	"	Y (G_1)
6	Ser	7445-7516	7_1(7186)-COI_2(7600)	Y (COI_2)
	Asp	7518-7585	"	Y (COI_2)
7	Lys	8295-8364	J_1(8188)-8438_2	Y (8438_2)
8	Gly	9991-10058	3_1(9731)-$COIII_2$(10145)	Y ($COIII_2$)
9	Arg	10405-10469	3_1(9731)-K_2(11492)	N
10	His	12138-12206	L_1(11700)-M_2(13168)	Y (L_2)
	Ser(AGY)	12207-12265	"	Y (M_1)
	Leu(CUN)	12266-12336	"	Y (M_1)
11	Glu(L-str)	14674-14742	N_1(13167)-Cyt b_2(15177)	N
12	Thr	15888-15953	O_1(15345)-O_2(133)	N
	Pro(L-str)	15955-16023	"	N

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TABLE 8: SSCP approach designed to screen the 12 tRNA regions for point mutations and small deletions. Regions, position of the tRNA, restriction enzymes used, and resulting band sizes are indicated.

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tRNA DIGESTION ANALYSIS

577-677 Phe A_1(130) - A_2(1259) = 1129

MaeIII sites: 429,902 bands: 299, 473, 357

HphI sites: 422, 626, 713, 725, 945, 972, 1227

bands: 292, 204, 87, 12, 220, 27, 255, 32

1602-1670 Val 1488_1 - Bi_2(2444) = 956

DdeI sites: 1637, 1667, 1715, 1895, 1923, 2036

bands: 149, 30, 48, 180, 28, 113, 408

HhaI sites: 1768 bands: 280, 676

3230-3304 Leu(UUR) T_L(2980) - B_2(3352) = 372

Uncut

HhaI sites: 3161 bands: 181, 191

HaeIII sites: 3146, 3315 bands: 166, 169, 37

4263-4331 Iso \

4329-4400 Glu C_1(3352) - C_2(4506) = 1154

4402-4469 Met(fMet) /

EcoRI sites: 4121 bands: 769, 385

MaeIII sites: 3736, 4084, 4284 bands: 384, 348, 200, 222

5512-5576 Trp \

5587-5655 Ala \

5657-5729 Asp F_1(4976)-G_2(6286) = 1310

5761-5826 Cys /

5826-5891 Tyr /

HincII sites: 5691, 5917 bands: 715, 226, 369

HphI sites: 5315, 5806, 5884 bands: 339, 491, 78, 402

7445-7516 Ser

7518-7585 Asp 7_1(7186) - COI_2(7600) = 414

uncut

MboII sites: 7371, 7431 bands: 185, 60, 169

MaeIII sites: 7219, 7382 bands: 33, 163, 218

8295-8364 Lys J_1(8188) - 8438_2 = 250

uncut

HphI sites: 8425 bands: 237, 13

9991-10058 Gly 3_1(9731) - $COIII_2$(10145) = 414

uncut

SfaNI sites: 9778 bands: 47, 367

10405-10469 Arg 3_1(9731) - K_2(11492) = 1761

DdeI sites: 9740, 10226, 10356, 10631, 11326

bands: 9, 486, 130, 275, 695, 166

HaeIII sites: 10365, 10690, 10726 bands: 634, 325, 36, 766

12138-12206 His \

12207-12265 Ser(AGY) $LCRT2_1$(11779) - M_2(13168) = 1389

12266-12336 Leu(CUN) /

MboII+HinfI sites: 11949, 12170, 12471, 12678, 12696, 13031, 13098, 13103

bands: 221, 301, 207, 18, 335, 67, 5, 65

AluI sites: 11824, 12212, 12282, 12560, 1257; bands: 45, 388, 70, 278, 11, 597

14674-14742 Glu(L-strand) N_1(13167) - Cyt b_2(15177) = 2010

DdeI+HphI sites13173,13176,13446,13554,14055,14347,14395,14434,14608,14872,14904,15166

bands: 6, 3, 270, 108, 501, 292, 48, 39, 174, 264, 32, 262, 11

MboII sites: 13329, 13609, 14100, 14103, 14624, 15019

bands: 162, 280, 491, 3, 521, 395, 158

1588-15953 Thr

15955-16023 Pro O_1(15345) - O_2(133) = 1357

DdeI sites: 15704, 15996, 16380 bands: 359, 292, 384, 322

HinfI sites: 15376,15724,15757,16001,16066 bands: 31, 348, 33, 244, 65, 636

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in TE and aliquots were PCR amplified using LHON, MERRF, MELAS, and NARP primer sets. LHON and MERRF amplifications were performed in the presence of [α-^{32}P]dATP. Fragments were digested in the appropriate restriction enzyme to detect the known mutations and then electrophoresed on an acrylamide gel. Radioactive gel samples were dried and exposed overnight while nonradioactive gel samples were stained in ethidium bromide and photographed under UV-illumination. The MELAS and NARP analysis (Figure 23A and B) show no alterations of Rett Syndrome fragments as compared to control fragment mobilities. The MERRF and LHON analysis (Figure 23C) also showed no changes (a positive control was included in lane 7cut of the LHON analysis). Testing for the KSS mutations found no variations from age-related controls (data not shown). This data indicates that Rett Syndrome does not result from a known mtDNA mutation.

Prior to extensive SSCP analysis being conducted on the Rett Syndrome WBC DNA, brain samples were received from Dr. Sakkubai Naidu of the Kennedy Krieger Institute. The letter detailing the ages of the received samples and the cause of death is shown in Figure 24. The samples were processed as described in Materials/Methods. SSCP analysis was then initiated using the brain samples in conjunction with the WBC DNA. The inclusion of brain DNA was important to our analyses for two reasons:

(1) Blood DNA is a highly replicative tissue and mutations in high concentration (90%) in the muscle or brain tissue may be in very low concentration in the blood (2-3%) (161). Additionally, due to the high replicative rate, normal variant mutations are more often incorporated into

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FIGURE 23: Known mutation analysis in Rett Syndrome. **A.** Analysis of RS mtDNA for MELAS. Lane 1, pGEM molecular weight markers; lane 2-4, 372 bp fragment from placental control, RS#1 and RS#2, respectively; lanes 5-7, same as lanes 2-4 except for treatment with *HaeIII*. **B.** Analysis of RS mtDNA for NARP. Lane 1, pGEM molecular weight markers; lane 2-4, 554 bp PCR fragment from placental control, RS#1, RS#2, respectively; lanes 5-7, same as lanes 2-4 except for treatment with *AvaI*; lane 8, 100 bp ladder. **C.** Analysis of RS mtDNA for MERRF and LHON. Lanes 1 and 4, placental mtDNA; lanes 2 and 5, RS#1; lanes 3 and 6, RS#2; lane 7, LHON sample. Uncut = not treated with restriction enzyme; cut = treated with either *NaeI* (MERRF) or *MaeIII* (LHON).

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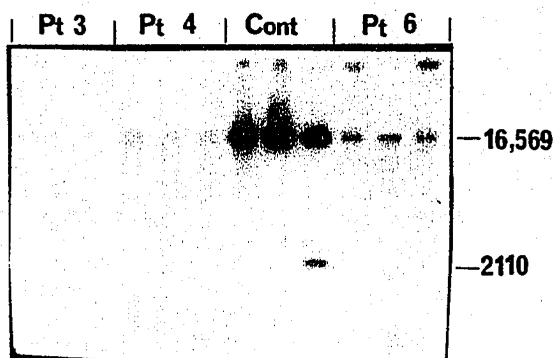
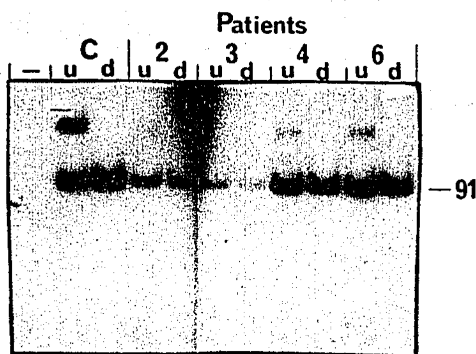
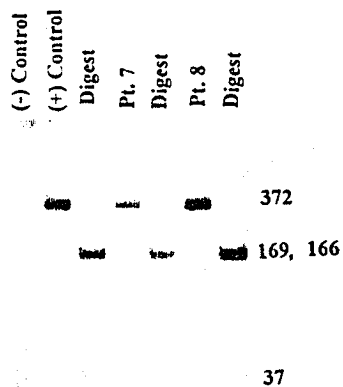
A. Southern Analysis



B. MERRF (Nae I) Digest



C. MELAS (Hae III) Digest



D. LHON (Sfa NI) Digest

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FIGURE 24: Letter from Dr. Sakkubai Naidu of the Kennedy Krieger Institute detailing the Rett Syndrome and Control brain samples recieved for analysis.

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Kennedy Krieger Institute

Sakkubai Naidu, M.D.
Director
Neurogenetics Unit

A comprehensive resource for children with disabilities

April 19, 1994

Frank J. Castora, Ph.D.
Associate Professor of Biochemistry
Eastern Virginia Medical School
Department of Biochemistry
700 Olney Road
Norfolk, Virginia 23507-1898

Dear Dr. Castora,

In response to your letter and our telephone conversation regarding a collaborative study of mitochondria in Rett Syndrome, I am enclosing the following brain tissues:

<u>Control</u>

#21	4-year-old male postmortem interval of 18 hours, accidental death
#24	10-year-old female postmortem interval of 17 hours, accidental death
#55	19-year-old female postmortem interval of 17 hours, accidental death

<u>Rett</u>

#93-244	4-year-old who died of asphyxiation
#621	12-year-old postmortem interval of 5 hours, who died of drowning
#664	21-year-old postmortem interval of 5 hours, who died of pneumonia

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the blood DNA than in more stable tissues resulting in many 'false positive' SSCPs.

(2) Brain is a highly oxidative tissue (with a low replication rate) and is the primary tissue affected in Rett Syndrome. Therefore, mutations identified in more than one brain sample have a greater possibility of being disease-specific.

The devised SSCP approach was therefore repeated for all regions on the brain tissue samples. The DNA was first PCR-amplified in the presence of [α-^{32}P]dATP using the designated primer sets. Eight microliters of amplified DNA was incubated with one of the indicated restriction enzymes and digested for a minimum of three hours at the appropriate temperature. Three microliters of digestion product was mixed with nine microliters of SSCP dye, heat-denatured at 95°C for ten minutes and cooled in an ice-water bath for ten minutes prior to loading. Five microliters was loaded on a 1x MDE non-denaturing gel and electrophoresed at eight watts until the xylene cyanol band was at least 30 centimeters from the wells. The gel was then transferred to Whatman paper and exposed with an intensifying screen. Mobility shifts were identified by comparing the control and RS patient lanes.

Many more variations in SSCP fragment mobility were seen in the blood than in the brain samples. In the event that only one RS sample was altered, this region was not investigated. Alterations seen in RS samples as well as control samples were disregarded because the mutation would not be disease-specific. Figure 25 shows examples of SSCP analyses that were not pursued further. This includes a lack of mobility changes (Figure

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25A and B), mobility changes mimicked in the control (Figure 25C), and mobility changes seen in only one RS patient (Figure 25D). None of these areas appeared to have a mutation with a disease-specific involvement in Rett Syndrome.

Two tRNA areas showed variations in fragment mobility that were seen in two separate patients. These changes occurred in patients one (blood sample) and R664 (brain sample) in the $tRNA^{Ser}$ and $tRNA^{Asp}$ regions (Figure 26A and B) and in patients one (blood sample) and R664 (brain sample) in the $tRNA^{Gly}$ region (Figure 26C and D). Both of these alterations show an increased band mobility (note arrow position in relation to control lanes).

Although only two patients show a band alteration, the mutation may still be characteristic of RS. In many mitochondrial myopathies, several different mtDNA mutations may result in clinical expression of the same disease (for a review of pathogenic mitochondrial mutations, see 162). One example of such a myopathy is LHON which can result from six different point mutations occurring in either the cytochrome b gene, NADH dehydrogenase four or one genes, the cytochrome c oxidase I gene, plus a variety of synergistic mutations that, in various combinations, may also result in LHON. MERRF and MELAS have also been correlated with multiple point mutations (two and three, respectively). Therefore, precedence indicates that these alterations may be associated with one form of RS expression.

The 7_1 (nts. 8188-8208) - COI_2 (nts. 7600-7580) region encompassing the $tRNA^{Ser}$ and $tRNA^{Asp}$ regions (nts. 7445-7585) was sequenced from PCR-amplified DNA purified on Wizard PCR purification columns (Promega) using the fmol DNA sequencing system

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FIGURE 25: SSCP analysis showing various changes between control and patient samples. **A.** and **B.** Samples showing no alteration in fragment migration as compared to controls. **C.** Samples showing an migration alteration in the Rett sample that also appears in the control. **D.** Samples showing an Rett Syndrome specific change in migration.

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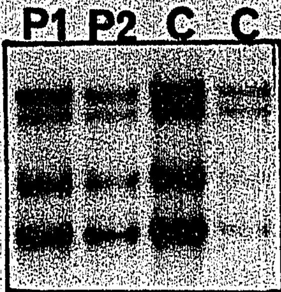
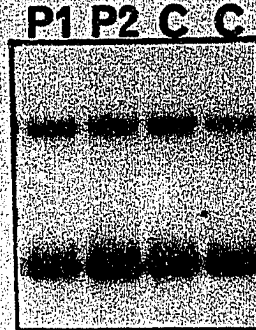
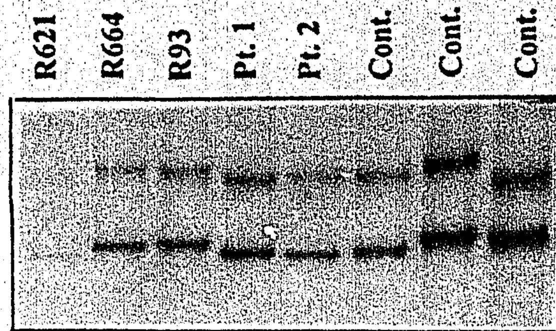
A. Normal SSCP



B. Normal SSCP



C. Mutation in Rett Patient
And in Control



D. Mutation in Rett Patient

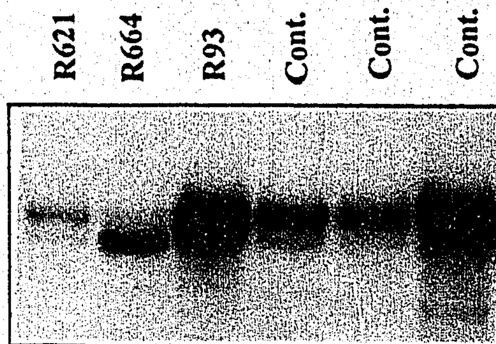


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FIGURE 26: SSCP analysis of the 7_1-COI_2 region and the 3_1-$COIII_2$ region in Rett Syndrome and control patients. **A.** and **B.**, ser-asp tRNA region; **C.** and **D.**, gly tRNA. **A.** and **C.**, PCR fragments from lanes 1, and 2, control DNA; lane 3, blood DNA, RS#1; **B.** and **D.**, lanes 1-3, RS brain samples; lanes 4-6, control brain samples.

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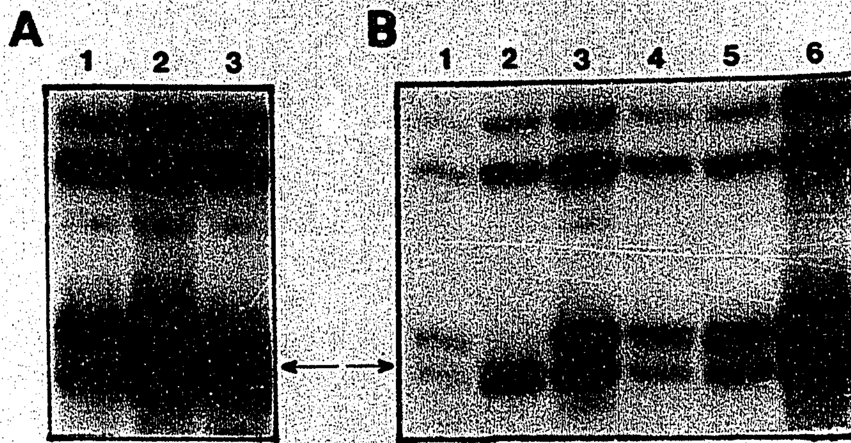

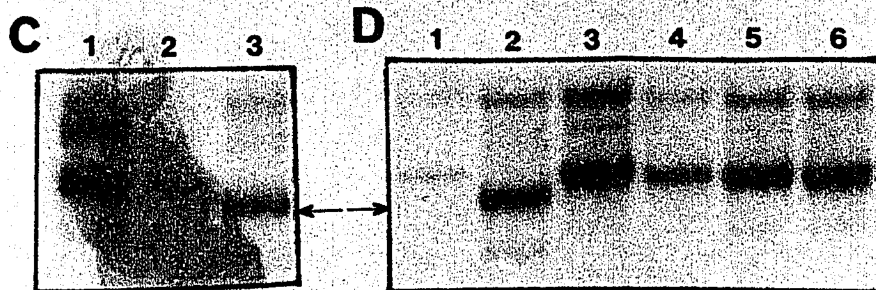

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(Promega). The primer COI_2 was used for the analysis. Sequence was read from nucleotides 7558 to 7311 encompassing the tRNA regions at nucleotides 7445-7516 ($tRNA^{Ser}$) and 7518-7585 ($tRNA^{Asp}$). A single G->A mutation was found at position 7476 in the $tRNA^{Ser}$ gene (Figure 27A). This mutation occurs in the stem structure of the anticodon loop of the tRNA (Figure 27B). Note that in the normal $tRNA^{Ser}$, a G|U basepair occurs at this position. The mutation to an A|U basepair would only enhance the stability of the stem structure as it does in the bovine tRNA (Figure 27B). This mutation is not only unconserved in bovine but also a normal variant by Marzuki et al standards.

The second SSCP variant appearing in the two RS patients, again in patient one and R664, occurs in the region encompassing the $tRNA^{Gly}$ (9991-10058). Primers used for this analysis were 3_1 (nts. 9731-9750) and $COIII_2$ (nts. 10145-10125) producing a 414 bp fragment that was screened using two SSCP pattern analyses (see Table 8, $tRNA^{Gly}$). The PCR product was sequenced using the $COIII_2$ primer. No mutations were detected in nucleotides 10119-9902 and, therefore, no mutation is present in the $tRNA^{Gly}$ gene region (data not shown). Because the SSCP analysis detects all mutations existing between the *SfaNI* site (nt. 9778) and the end of the amplified fragment (nt. 10145), the mutation could be within the Cytochrome c Oxidase III coding region. However, the multicomplex effect seen in the respiratory chain enzymes of Rett Syndrome would not result from an alteration of the function of one component of complex IV, therefore this mutation was not considered disease-specific.

All other areas analyzed by SSCP showed band alterations in no more than one

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FIGURE 27: **A.** Sequence analysis showing the G -> A change found in the $tRNA^{Ser}$ gene region. Sequence was performed using the primer COI_2. **B.** tRNA map showing the position of the mutation relative to the $tRNA^{Ser}$ structure. Note that the mutation alters a G|U basepair to an A|T basepair in the stem of the anticodon loop.

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A. Sequence Analysis of 7_1 - COI_2 Region Rett Mutation at 7476 np

A C G T

t t t c g→a a c c a a a

B. Map of the tRNA^{Ser} Showing Position of Rett Mutation

```
                  T
                T+G
                T T
                G+T
                A-T
                A-T
                A-T
                A-T          CTT
               A     CCTTC
 AGG          G      !!++!      A
        TACT         GGGGG      G
        !!!+         G     TTC
G       ATGG            T
 GCC        G
              G-CT   T
              T+G
              T-A    \
              G-C     A
              G-C
             C    A
             T    A
              TGA
```


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patient indicating that the mutations were not disease-specific. To confirm our results, we decided to sequence a number of tRNA regions. In only one instance was a mutation found within an SSCP analyzed area. Figure 28A is the SSCP analysis of the three brain and three control samples. Note that no band mobility changes are apparent. Sequence analysis of the amplified region, J_1 (nts. 8188-8210) to 8438_2 (nts. 8438-8418), with the three prime primer, 8438_2, identified a single base mutation at position 8241 in the cytochrome c oxidase II region (Figure 28B). Note the appearance of the band in the C lane of the sequence. This data indicates that the possibility exists that a pathogenic mutation may not be identified using SSCP. A single analysis of a DNA region by SSCP has been shown to be 80% accurate. We have increased this percentage by analyzing the tRNA regions three times using the SSCP - each time with a different band pattern to allow placement of the mutation in an area involved in the formation of secondary conformation. Subsequently, in the seven regions we chose to sequence, our SSCP data has proven reliable.

A compilation of all the SSCP analyses and sequencing data is shown in Table 9. The tRNAs are separated into the analyzed regions and the results from each patients' SSCP analysis is indicated. Changes in band mobility as compared to controls are indicated by a 'Y' while band patterns equivalent to controls are represented by an 'N'. Any additional sequencing is indicated as are the results of that analysis. As expected by our SSCP analysis, no significant mutation in seven of the sequenced tRNA regions was found. Based on this data, no disease-specific mutation correlating with the presentation of Rett Syndrome exists within the tRNA regions of the mitochondrial genome.

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FIGURE 28: **A.** SSCP analysis of the J_1-8438_2 region (nts. 8188-8438) showing no changes in band migration relative to the control lanes. R### indicates the RS brain sample in that lane. C indicates the control brain samples. **B.** Sequence analysis of R93 (Rett Syndrome brain sample) showing an A -> C mutation at position 8240 in the COII gene region not detected on the SSCP analysis.

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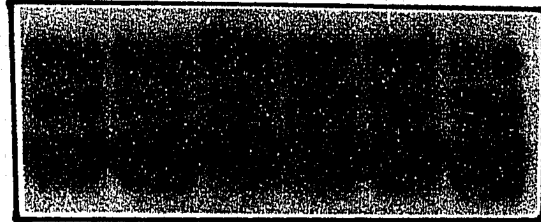
A



B

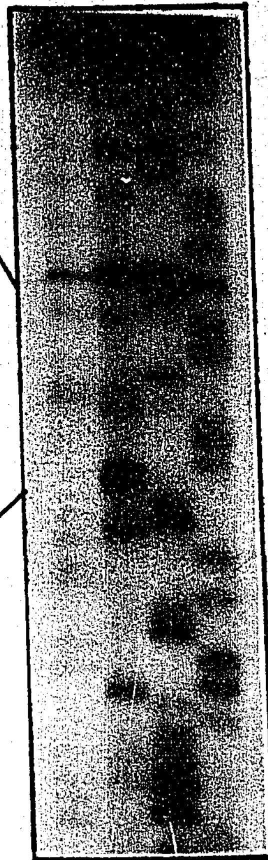


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TABLE 9: Results of the tRNA Rett Syndrome analysis by regions analyzed. Each Rett Syndrome patient is indicated along with the results of the SSCP analysis. Regions that were sequenced are also indicated by a 'Y' for sequenced, or an 'N' for not sequenced. If mutations were found in the tRNA region, their positions are also indicated.

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TABLE 9 - RETT SYNDROME tRNA ANALYSIS

REGION and tRNAs	Pt. 1	Pt. 2	R621	R664	R93	SEQ'D	COMMENTS
1 - Phe	Y	N	N	N	N	Y	No mutation in tRNA
2 - Val	Y	N	N	N	N	Y	No mutation in tRNA
3 - Leu(UUR)	N	Y	N	N	N	Y	No mutation in tRNA
4 - Iso, Glu, Met	Y	N	N	N	N	N	
5 - Trp, Ala, Asn, Cys, Tyr	N	N	N	N	N	N	
6 - Ser, Asp	Y	N	N	Y	N	Y	G->A @ 7476; Unconserved
7 - Lys	N	N	N	N	N	Y	No mutation in tRNA
8 - Gly	Y	N	N	Y	N	Y	No mutation in tRNA
9 - Arg	N/A	N/A	Y	N	N	N	
10 - His, Ser(AGY), Leu(CUN)	Y	N	N	Y	N	Y	No mutation in Ser or Leu tRNAs
11 - Glu (L-strand)	N	N	N	N	N	N	
12 - Thr, Pro	N	N	N	Y	N	N	

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CHAPTER IV. DISCUSSION

This project investigated three different aspects with which mitochondrial DNA may be involved in the disease state. As the Results chapter was presented in sections to accommodate the variety of analyses, the Discussion will also be addressed as such. The preliminary work involved an investigation of PCR analysis of white blood cell mtDNA in order to develop a rapid method of investigating patient samples. From this stemmed an investigation into the sensitivity of the PCR in mtDNA analysis and, finally, into an attempt to understand the distribution of the heteroplasmic mtDNA in diseased tissue. The second section began an investigation of a variety of encephalomyopathies and the possible mtDNA involvement in pathogenesis. Two cases were studied in some detail based on the biochemical evaluations available. From this generic myopathic evaluation we became aware of an encephalopathy that demonstrated many of the factors common to the mitochondrial encephalomyopathy family - Rett Syndrome. This disorder became the focus of our evaluation in the final section of this project.

A. Single Cell Project

This preliminary analysis investigated the effects of hemoglobin and blood anticoagulants on the amplification efficiency of PCR. The rapid protocol described in

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the Results was initially developed to permit a rapid PCR evaluation of patient samples for possible pathogenic mutations. Its advantage resulted from the speed and efficiency with which a DNA isolate could be prepared. The procedure could be performed in any lab in a matter of minutes and required only the capacity to boil the sample, centrifuge and collect the supernatant. As many as 15-20 reactions could be amplified from the initial 30 μL of anticoagulated blood following template preparation, and PCR from this prepared template could efficiently amplify both nuclear and mitochondrial gene sources. Earlier blood DNA isolation protocols required a variety of reagents as well as extensive purification times to separate the white blood cells from the hemoglobin component. In determining the effect of hemoglobin on the PCR reaction, we found that the reaction could tolerate very low levels of hemoglobin but the tolerance level declined very rapidly. In our protocol, there is a 1200-fold reduction in the hemoglobin content (1.2 g/mL to 0.001 g/mL). At this concentration, the hemoglobin effect was negligible.

Most blood samples are collected in anticoagulants to prevent clumping prior to analysis. Many standard DNA amplification protocols avoid the inclusion of heparin in their blood samples due to its proven effect on amplification efficiency (129). EDTA, which is a more potent inhibitor due to its ability to chelate ions, is suggested for these protocols because it can be separated more easily from blood than heparin. Beutler et al (129) demonstrated that heparin interferes with the amplification efficiency of the PCR reaction and proposed that heparin binding to the DNA was responsible for this inhibition. Further investigation of this heparin effect, however, showed that at low levels, the amount of inhibition of the PCR is negligible. Our developed protocol

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involves a strong heat denaturation step which may effectively separate the heparin into solution or denature its structure in such a way that its effect on the PCR is greatly reduced. Additionally, the use of small volumes of blood may also restrict the effect heparin demonstrates on amplification. Our protocol carried-over only 25 mU of heparin to the DNA isolate and amplification appeared to be unaffected.

Use of this protocol for long term evaluation and storage of blood samples was also investigated. In many instances, testing from an isolated blood sample must be performed within 48 hours post-collection. For genetic analysis, however, we found our procedure to be efficient at isolating the DNA for PCR as much as 25 days post-collection. In fact, our results showed that heparin proved more effective at inhibiting DNA degradation over time than did the EDTA indicating, for our purposes, that heparin was the more stable anticoagulant.

This analysis indicates that blood collected in either of two common anticoagulants could be used for DNA analysis. And, because of the minimal sample size required for the procedure, it reduced the necessary volume of blood collected from patients. This procedure is quick, easy to use and generates a patient sample that is viable in both the standard and multiplex protocols previously described for six months.

With our success in amplifying mtDNA sequences from a single drop of blood, we decided to investigate the possibility of identifying mtDNA lesions from even smaller samples, e.g. amniotic fluid, single cells. A variety of diseases of the OXPHOS pathway have been correlated with single nucleotide lesions on the mtDNA. Such diseases include LHON, MERRF, MELAS, NARP, and FIC. From a single cell isolated from cultured

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LHON lymphoblasts, we have demonstrated the feasibility of using the polymerase chain reaction (PCR) and the appropriate restriction enzyme to detect the presence of the mutation at nt 11778 on the mtDNA which is diagnostic for certain forms of this mitochondrial myopathy. In a similar fashion, PCR amplification of mtDNA from a single cell could be used to provide enough material for restriction enzyme digestion and detection of lesions associated with MERRF (and A->G transition at nt 8344 in the tRNA^{Lys} gene), MELAS (an A->G transition at nt 3243 of the tRNA$^{Leu(UUR)}$ gene), NARP (a T->G transversion at nt 8993 in the ATPase subunit six gene), and FIC (an A->G transition at nt 4317 in the tRNA^{Ile} gene). The present study indicates the potential for using PCR and restriction enzyme digestion to detect the presence of mutations in mtDNA from as little starting material as five microliters of amniotic fluid or even a single cultured cell. We have successfully amplified single mtDNA gene loci from five microliters of amniotic fluid and individual HL60 and mouse blastomere cells. We have also simultaneously amplified two genetic loci in a PCR duplexing reaction using HL60 cells. This latter result suggests that two distinct mtDNA regions could be scanned for the presence or absence of a known mutation from a single reaction. In this respect, we conducted experiments to amplify three regions of the mtDNA, encompassing the LHON, MERRF and MELAS mutation sites, from a LHON single cell isolated from a cultured lymphoblast cell line. In cases where the exact mutation may not be known (i.e., LHON which may result from six different mutations as well as various combinations of synergistic mutations) or the clinical expression is divergent enough not to indicate a specific disease, the ability to amplify multiple target regions from limited sample could

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prove to be very advantageous. Additionally, following other mutation detection techniques such as DGGE and SSCP, the ability to amplify multiple target regions would result in the capability to do extensive investigation even from a limited sample. Because exact identification of a mutation is critical for diagnostic efforts, we chose to specifically sequence the LHON fragment from our milieu of mixed fragments. We used sequencing because it is a technique that is sensitive to a variety of artifactual contaminants and identification of the LHON mutant would indicate that the all the fragments had specifically amplified their target region. We found that, although there was a mixture of target fragments, the primer specifically amplified the template of choice without any interference in sequence quality. The LHON mutation was also clearly detected and enough template remained for at least two additional sequence analyses. Additionally, the clarity of our sequence indicated that the target volume could be reduced thereby permitting even more analyses. In the event that available template was low, PCR could be performed on the amplified material to reamplify the target regions.

The drain on source DNA would be low with the ability to amplify multiple target regions in a single reaction. In instances where target DNA is extremely limited (i.e. crime scene analysis, archeological DNA sources, preimplantation IVF analysis), such a capability could have far reaching effects. For example, multiple highly variable regions of the mtDNA could be amplified and subsequently sequenced in an attempt to link a perpetrator to a crime; presumably both nuclear and mtDNA regions could be amplified in archeological DNA sources to identify species and link populations; and a variety of gene sources could be amplified from a single blastomere removed from a

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preimplantation embryo to screen for sex as well as specific diseases in instances where this may be a factor in disease expression. However, even though the analysis of mtDNA from a single cell is shown here to be quite feasible, diagnosis, either preimplantation or prenatal, of a mitochondrial defect would have to be made very carefully. First, in regards to LHON, although the disease initially manifests itself in a heteroplasmic state, over generations, the disease establishes a mtDNA homoplasmy and, since the disease is transmitted maternally, it would not require the analysis of fetal tissue for diagnosis. We have used the LHON mutation merely to demonstrate the capability of detecting single base mutations within the mtDNA and from multiple targets amplified from a single cell. Application of prenatal diagnosis to mitochondrial defects would be more appropriate in the heteroplasmic disorders such as MERRF, MELAS and NARP. In these diseases, possession of mutant mtDNA above a threshold level leads to the manifestation of mitochondrial dysfunction with the pathology and severity of the disease correlating with the proportion of the mutant mtDNA present (2,163). Second, due to the apparently random distribution of mitochondria into daughter cells during cell division, the distribution and proportion of the mutant mtDNA may change both during embryogenesis and later in life. Third, in some instances, high proportions of mutant mtDNA within a tissue may not result in the phenotypic consequences without the influence of a second genetic or environmental factor (i.e., LHON which appears to require an additional mutation on the X-chromosome for phenotypic expression). And finally, analysis of a single blastomere from a preimplantation embryo may result in an increase in the mutant proportion for diseases resulting from heteroplasmic expression. In this case, removal of

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a blastomere containing only normal mtDNA would result in an increase in the mutant proportion among the remaining cells within the embryo. Thus, the detection and quantitation from a single cell of a mtDNA mutation that is associated with a particular encephalomyopathy may not be sufficient to formulate a clinical decision.

Prenatal diagnosis of the mitochondrial gene defect which leads to NARP has been reported by Harding et al (163), but in this case a chorionic villus (CVS) sample obtained transcervically was used as a source of mtDNA. The high proportion of mutant mtDNA detected by these authors from the CVS sample was also found in the placenta, brain, muscle, lung, limb and, to a lesser degree in liver and kidney. The only previous accounts of single cell analysis of mtDNA lesions have been reported in skeletal muscle fibers (155,156). However, these myofibers are multinucleate single cells resulting from the fusion of multiple precursor myoblasts. Hence, the potential exists that the mitochondrial contingent may be heteroplasmic due to the mixing of organelles derived from various myoblasts containing different mitochondrial genomes. In this vein, the procedures described in this study were utilized to investigate individual lymphoblasts cells from patients with MELAS, a heteroplasmic mitochondrial encephalomyopathy, to determine whether the heteroplasmy associated with this disease is due to normal and mutant mtDNAs coexisting within a single cell or whether it is due to a mixing of cells which are homoplasmic for either the normal or mutant mtDNA.

Previous studies by Chomyn et al (164) in the myoblast clones of a MERRF pedigree predicted that heteroplasmy would be largely intercellular (a mixture of homoplasmic mutant and normal cells). Studies conducted by Boulet et al (165)

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investigating the distribution and expression of mutant MERRF mtDNAs in myoblast clones found that the heteroplasmy was primarily intercellular, at least in 74%-89% of the clones tested. The remaining clones showed a low level of heteroplasmy and wildtype homoplasmy. In fibroblast clones of the same patient, however, only heteroplasmic (44%) and homoplasmic wildtype (56%) clones were found. Boulet postulated that this heteroplasmy was, therefore, cell-type specific. However, in studies of individual MELAS skeletal muscle fibers (166) and single MELAS cells from primary lymphoblast cell lines (conducted in this study), no homoplasmic mutant cells were found. In both analyses of heteroplasmy associated with single cells or single muscle fibers, it was found that both mutant and wild-type mtDNAs coexisted within the same cell, not between cells. What could explain the difference in these results? Possibly, the variation could be a result of the differential effects of the mutation on the function of the different tRNAs. Because the mutation in MERRF is found in the TψC loop and the MELAS mutation is found in the DHU loop, this could result in a variation in translational capacity. For example, the MELAS mutation may result in a total loss of transcriptional capability which would be lethal for a homoplasmic mutant cell. Phenotypic complementation of mutant genotypes can occur at wildtype concentrations of as low as 6% (138,139). Possibly, as low as 1% wildtype, as seen in the MELAS myofibers, provides enough translational capability to permit cell survival. In the MERRF genotype, the tRNA mutation may still allow retention of some translational capacity such that a homoplasmic mutant cell would not be lethal. A low level of mitochondrial translation was found in the homoplasmic MERRF myoblasts analyzed by Boulet. Although homoplasmic mutant

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MELAS cells have not been identified, similar studies in tissue culture have shown a partial impairment of respiratory chain activity and a reduction in mitochondrial protein levels (167). Analysis of mutant levels in other heteroplasmic mutated-tRNA cell lines should be explored to determine if this variation in heteroplasmy is due to a biochemical disease-specific effect or is the result of various inhibitions of tRNA function.

How can one explain the increase in the mutant proportion relative to the wildtype that occurs with time? During our culturing of the MELAS cell lines, we found an increase in the mutant population proportion from 28% to 62% over the period of one month. This effect is not an uncommon occurrence. In an individual, the LHON mutation is known to establish itself over generations as a homoplasmic mutation and a specific criteria for mitochondrial encephalomyopathies is that the clinical expression of the disease increase along the maternal lineage, probably as result of a mutant increase toward threshold expression. Boulet (165) proposes three possible mechanisms for the increased mutant proportion in his patients. The first is some form of intracellular replicative advantage for the mutant mtDNA. This is also suggested by Yoneda et al (138) who adds that a feedback mechanism, possibly due to decreased ATP levels, may result in the clonal expansion of the mutant mitochondria: a selective propagation of dysfunctional mitochondria. However, no increase in mtDNA copy number is associated with mutant genotypes. This might be a result of simultaneous organellar turnover. In this instance, the selective propagation of mutant mitochondria and the removal of the older, and therefore wildtype, mitochondria would result in a rapid increase of the mutant population. This could possibly explain why many mitochondrial encephalomyopathies

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become phenotypically expressed as the patient ages.

The second possibility is that a high proportion of mutant mtDNAs already exist in the ovum. Because the ova develop during the embryological development of the mother, this indicates that the mother may have harbored a mitochondrial mutation which became resident in the gamete stem-cell tissue. This is clearly demonstrated in both the LHON and MERRF lineages which show an increase in mutant proportion with each successive generation.

The third mechanism is a nonrandom sampling of the mtDNA population in early embryological development. A nonrandom sampling would result in the varied levels of mutant mtDNA seen in many tissues of myopathy patients. As an independent mechanism, however, it does seem to correlate with the increasing mutant population over generations. All three mechanisms working in conjunction during different periods of development would seem to explain the increase in mutant mtDNA population. It would also correlate with the variation in heteroplasmy seen within our individual cells and Moraes (166) myofibers.

Using this understanding of heteroplasmy, both at the generational and individual level, we adopted a set of criteria (described in Results Section B) that could be used to identify individuals harboring mitochondrial encephalomyopathies. From this identified patient population, we began to investigate the mtDNA for new mutations that would correlate with the phenotypic expression of the disease.

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B. Mitochondrial Myopathy Investigation

The mitochondrial genome has a very high mutation rate, at least ten times that of the nuclear genome. Since the mitochondrial genome has no introns with only a single small non-coding region, these randomly incorporated mutations most commonly alter coding bases within the genome. And, since every cell in the body harbors mitochondria, any tissue, somatic or germline, can be affected by deleterious mutations. The implications of the mutations, however, are determined by the distribution between cells and the oxidative capacity of the tissue affected. In recent years, mitochondrial diseases have been associated with point mutations, insertions and deletions of the mitochondrial genome with many of these neurologic disorders being maternally inherited, indicating mutations arising in germline tissues. We began an investigation of the mitochondrial genome and its involvement in maternally inherited oxidative phosphorylation diseases. Nine patient samples were acquired and, through the reported criteria (Results Section B), screened for mtDNA-associated mutations. Thorough analysis of two patient samples was conducted.

In reviewing the records of patient four, the biochemical analysis revealed a significant depression of the cytochrome c oxidase levels as well as an associated lactic acidosis, probably resulting from an inhibition of complete glucose metabolism within the OXPHOS pathway.

The cytochrome c oxidase subunit (Complex IV) of oxidative phosphorylation is a complex metalloprotein that functions to catalyze the reaction between hydrogen ions,

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electrons and molecular oxygen to form water. The three mitochondrial-encoded subunits form the catalytic core of the enzyme while the nuclear-encoded components function in the strict functional regulation of the enzyme. Mutations to either mitochondrial- or nuclear-encoded subunits could result in an alteration of the enzyme function as seen in this patient. We investigated the possible mtDNA involvement for three reasons:

1. There was evidence of maternal inheritance of the phenotype.
2. The phenotype was more severe in the proband than in the mother, characteristic of a heteroplasmic mutation.
3. Highly oxidative tissues showed more disease-related symptoms (i.e. brain function involvement; skeletal muscle dysfunction).

Based on this information, we began a thorough sequence analysis of the regions of mtDNA encoding the three subunits of cytochrome c oxidase (COX). Our sequence analysis, however, revealed no significant mutations within these three regions. We did, however, identify a number of mutations that, on further examination, appeared to be normal nucleotide variants. Our criteria in this determination were based on those previously defined by Wallace (40). Wallace indicated that a disease-associated mutation should affect the amino acid sequence, should change a highly conserved amino acid, and should be disease-specific and not found in controls. One additional criterion we added was that the mutation not be found in the database assembled by Marzuki et al (151) from ten different analyzed patients with a defined mitochondrial myopathy. All our mutations failed at least one of the defined criteria. However, in assembling all of our mutations, we found that two different COX regions appeared to be more divergent than

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the third region. Within COI and COIII, we identified a total of seven mutations while in COII we found no changes. To substantiate our findings, we compared the mutations found within the Marzuki database. In the ten patients, Marzuki identified only three mutations within the COII region while COI and COIII showed a total of 21 mutations (fourteen and seven, respectively). Because the sizes of these genes are different, we then compared the ratio of the mutations to nucleotide number. The COIII gene proved to be the most variable with a 1.4% mutation rate in 783 nucleotides. The COI gene was next with a 1.1% variability in 1545 nucleotides. The COII gene was the most conserved with only a 0.44% variability in 676 nucleotides - a 60% reduction in the mutation rate. Further investigation reveals that the COII gene appears to be the more highly conserved complex IV gene, even across species lines, and is considered one of the more slowly evolving mitochondrial genes (168). This may be the result of the functional responsibility of the proteins from each gene region. COIII apparently functions in the capacity of proton pump, translocating protons to the intermitochondrial space although the precise role of the subunit is unclear. COI and COII both function as the redox centers for the enzyme but COII also functions as a high affinity binding site for cytochrome c. In the disease state, only recently have mutations in the COX genes been correlated. In one case a termination codon was eliminated in the COI gene resulting in a three amino acid addition to the protein and the encephalomyopathy LHON (169). In another instance, two mutations also correlating with a LHON presentation, were found in COIII, each altering a highly conserved residue (170). In a later communication, however, one of these mutations was found to have no pathological significance due to

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its association with ethnic populations (171). Perhaps, because this complex provides a critical function in cellular respiration, deleterious mutations in the COX II gene result in a lethal state, due to its multiple roles in complex IV function. This would explain the low evolutionary rate of the COII gene as well as the lack of corresponding gene mutations. (NADH dehydrogenase subunit five of complex I is also a very slowly evolving gene and no mutations associated with any disease state have as yet been identified in its coding region.)

The fact that no disease-specific mutation was found in our patient indicates that the mutation may occur within another gene. Cytochrome c oxidase deficiency is seen in many diseases but is usually accompanied by additional enzymatic deficiencies. Possibly, the mutation lies in a nuclear-encoded COX gene which alters enzymatic regulation. A recessive X-chromosome or autosomal inheritance pattern through a single generation could be interpreted as a maternal inheritance pattern and, thereby, warrant a mtDNA analysis. Therefore, a full generational pedigree is necessary in this case to confirm or deny any mitochondrial involvement.

Adherence to the defined guidelines (Results Section B) is essential for mitochondrial investigations. Additionally, biochemical evaluation is an integral part of the analysis which can focus efforts into specific gene areas. A broad approach to even this small genome can result in an extensive evaluation of null mutations. In order to decrease our analysis time, we developed a rapid strategy (using one of the patient samples) to find mutations in more closely defined regions of the mtDNA. The use of denaturing gradient gel electrophoresis (DGGE) for gene screening seemed to be a rapid

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and feasible technique that would reliably identify single base mutations. Verification of the base change and subsequent localization by SSCP led to a sequence analysis of only a small genomic region. The major disadvantage to use of this method, however, was the large quantity of genomic DNA required for the DGGE analysis (four aliquots of five to ten micrograms each). In many instances, the amount of sample is strictly limited and, therefore, the genomic DNA recovery is also limited. Use of the DGGE in such situations can severely curtail the amount of analysis that can be performed. In such instances, a focusing of the gene regions to be analyzed and analysis of multiple SSCP fragment patterns is recommended.

Finally, analysis of disease-specific mutations on the mtDNA is dependent on accurate initial evaluation of the disease as mitochondrial-specific. The ability to prescreen these diseases for mitochondrial involvement prior to genomic analysis may be a useful alternative. Following biochemical analysis, these studies could be performed using the rho zero (ρ^o) cell line established by King and Attardi (172). These cells lack mitochondrial DNA due to longterm exposure to low levels of ethidium bromide. The cells rely exclusively on glycolysis for their energy requirements and are both pyrimidine auxotrophs and pyruvate-dependent. Transformation of these ρ^o cell lines with human cytoplasts results in a transfer of the cytoplast mitochondria to the ρ^o cells. Following transformation, complementation of either of the metabolic requirements (screened by pyruvate or uridine deprivation) could be used as a selectable marker. Subsequently, a new biochemical analysis of the cybrid cell line would indicate if the mutation were nuclear- or mitochondrial-specific. For example, in patient four, biochemical assays

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revealed a low cytochrome c oxidase level. Following cytoplast fusion with the ρ° cell line, a depressed cytochrome c oxidase analysis would indicate that the mutation segregated with the cytoplast mitochondria and, therefore, must be mtDNA specific. A cytochrome c oxidase level within normal ranges would indicate a nuclear-encoded mitochondrial protein mutation. Although the complete analysis would take approximately three weeks, many patient cell lines could be screened together and only those showing a positive mtDNA involvement pursued.

During this analysis, we did not have access to the ρ° cell line. Therefore, using the knowledge we had gained on a random sampling for mitochondrial diseases, we began to search for a specific disease with a large patient population that exhibited morphologic and biochemical evidence of an OXPHOS deficiency and a demonstrated maternal inheritance. Rett Syndrome (RS), a neurodegenerative disorder of unknown etiology, appears to meet all these criteria.

C. Rett Syndrome Analysis

Rett Syndrome is characterized by progressive loss of intellectual functioning and fine and gross motor skills as well as development of stereotypic hand abnormalities following about six to eight months of normal development. It has previously been reported almost exclusively in female children but the possibility of the syndrome existing in male children cannot be excluded. Although the syndrome is thought to be relatively common (one in 10,000-12,000 live births), it wasn't until 1983 that the disease was

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characterized by Hagberg et al in the english literature (52). The disease is defined by four clinical stages describing the sequentially appearing features which involve a general delay of developmental progress followed by a rapid deterioration of acquired skills and social withdrawal finally leading to muscle wasting, scoliosis, and growth retardation. Microcephaly developing after birth and stereotypic hand movements are two of the major characteristics found in this disease.

At present, there are no biological markers for this disease and diagnosis depends on an accurate assessment of the child's early growth and development. In an effort to characterize the etiology of the syndrome, studies have examined the morphologic, biochemical, and genetic evidence. In the last ten years, many studies have been published but still no defined marker has been identified. Genetic emphasis has focused largely on the X-chromosome due to the preponderance of data indicating Rett Syndrome is genetically determined and specific for females. Even an understanding of how the disease progresses, its morphologic traits and common biochemical characteristics have not been found. Therefore, a major emphasis in the research has been placed upon acquiring information regarding the status and progress of the disorder. This data has shown that the microcephaly characteristic is due to a lack of brain development in the afflicted child. Without the expansion of the central nervous system, the associated cranial growth does not occur. Hagberg proposed that RS is therefore a developmental disorder resulting from a failure of the elaboration of critical neuronal networks during the later stages of gestation and early infancy (173). This, however, could result in microcephaly at birth, a non-Rett characteristic. Armstrong postulates that this

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developmental delay occurs not at the first developmental period (28 weeks gestation to birth), but at the second, transpiring from approximately two to nine months (65). This would account for the initially normal development followed by a later microcephaly as seen in Rett Syndrome. In fact, Armstrong and Hagberg (173) postulated that a failure of the elaboration of critical neuronal networks during the secondary increase in synaptic density that occurs after two months post-gestation could be the principal neurobiological event resulting in this developmental disorder. This 'growth spurt' is demonstrated by a dramatic increase in RNA synthesis which is accompanied by a corresponding increase in the production of ATP, primarily by the mitochondria. A deficiency in the OXPHOS capability at this critical developmental point would have catastrophic consequences on brain development. Thus, a mutation within the mitochondrial genome that affects the production capability of the OXPHOS pathway could result in a slow or negligible ATP replacement. This second developmental burst would, therefore, be dependent on a defective energy supply system, ultimately resulting in little or no increase in synaptic density - a common feature of Rett Syndrome brains.

If, perhaps, the mitochondrial genome were involved in the development of this disorder, why wouldn't the ATP deficiency affect the first developmental period? In most mammals, especially primates, the brain is highly underdeveloped and relatively undifferentiated at birth (174), and a major portion of its anatomical, chemical and functional differentiation is achieved postnatally. Although the energy demands of the developing brain are low (175), energy must still be provided during the early organizational processes such as neurogenesis, cell migration, and differentiation. And,

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if ATP synthesis via the electron transport chain is inhibited, why does the Rett-affected child present normally at birth? During the course of brain development, a variety of different energy substrates are available in plentiful supply to the cells in the brain. This metabolic environment ensures that substrates in addition to glucose are available as fuels. Both glucose and the ketone bodies, acetoacetate and D-(-)-3-hydroxybutyrate, are taken up by the brain and used as primary fuels for energy production and as a carbon source for lipogenesis (for review see 176). The blood concentration of ketone bodies and free fatty acids varies considerably in response to dietary conditions and when circumstances to promote their production are favored. Neurons, astrocytes and oligodendrocytes, the major cell populations of the brain, use glucose and ketone bodies for energy production. Additionally, astrocytes can β-oxidize fatty acids to produce acetoacetate which is then converted to acetyl-CoA. The versatility of astrocytes makes these cells metabolically multifunctional. This versatility of substrate use for energy production may initially compensate for the dysfunction of the respiratory chain. This adaptability extends through the neonatal nursing period wherein the milk-rich diet continues to provide substrates for alternate energy sources. When the availability of these alternate sources decreases, the brain cell populations become dependent on glucose metabolism as the primary energy supplier. If this pathway is dysfunctional due to a mtDNA mutation, the 'brain growth spurt', experienced at six-plus months of age, would be dramatically affected. This lack of brain development from the sixth month on would deleteriously affect language comprehension, auditory association, speech, motor activity and coordination, and intellect while not altering vision, hearing, sensory sensations or

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basic muscular control (as dictated by the precentral (primary motor cortex) and postcentral (somatic sensory cortex) gyrus). However, the premotor area of the brain would be affected due to its late development (16 months) and a variety of associated functional fields would also be altered. These include: Broca's Area, a speech area which, when damaged, results in a loss of word-forming capability but the retention of vocalizations and rudimentary sounds (i.e. 'yes' and 'no'); damage to this area may also affect the respiratory function associated with speech; the contralateral eye movement field, superior to the Broca's area in the premotor cortex, controls the ability to scan multiple objects with dysfunction resulting in a 'locking on' of the eyes onto a single object; and finally, the hand skills area which, when damaged, results in incoordinate and nonpurposeful hand movements (177). All of these dysfunctions appear during the four clinical stages of Rett Syndrome. Additionally, morphologic evaluation of Rett patient brains identifies the frontal lobe, housing the premotor cortex, as demonstrating the greatest loss of cortical neurons (64,80).

If this lack of brain development does involve a mitochondrial genome dysfunction, enzymatic evidence and precedence indicate that the most likely sources are the tRNAs. Enzymatic evidence (48,49) indicates a multicomplex effect which reflects a translational insufficiency. A mutation within a single protein might affect the complex it is associated with but other complexes will usually function normally. Therefore, only a tRNA or rRNA gene would have to harbor the mutation due to their coding on the maternally-inherited mitochondrial genome. Inheritance of the nuclear-encoded rRNA-associated proteins would follow a Mendelian inheritance pattern. However, we did not

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find a mutation associated with any of the 22 tRNAs encoded. Although we recommend sequencing of the five remaining tRNAs to verify these results, it seems we must turn our attention to the ribosomal RNA genes for a possible source mutation. Because the rRNAs are involved in translation, an alteration of their function would affect all the complexes encoded on the mtDNA. Such an effect could result from dysfunctional or inhibited tRNA binding or release, altered message binding capacity, start codon recognition or 12s/16s rRNA association. Although disease-specific mutations in the rRNA genes are rare, deletions (178), insertions (179) and point mutations (154) have been identified. A mitochondrial DNA involvement in Rett Syndrome is too strong a possibility to ignore. A thorough analysis of these genes is therefore indicated. Any additional analysis of the mitochondrial genome should involve first, the D-loop region due to the transcriptional promoters and Heavy-strand replication origin, the cytochrome c oxidase subunits because, enzymatically, they are most severely affected subunit, and then the remaining protein complexes (Complex V, then III, then I). Although we have provided strong evidence to indicate against protein-coding sequences, there is some small precedence for a multicomplex effect (180).

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APPENDIX I

PLACENTAL PREP FOR MTDNA RECOVERY

****HUMAN PLACENTA MUST BE PREPARED AT 0-4°C****
****WITHIN 30 MINS OF DELIVERY****

1. Blood was drained from the placenta; remove membranes and obtain tissue and weigh.
2. Rinse tissue 3x's in Buffer A (0.9% NaCl, 5 mM EDTA, 10 mM Tris, pH 7.4).
3. Rinse tissue 2x's in Buffer B (0.25 M sucrose, 5 mM EDTA, 10 mM Tris, pH 7.4).
4. Grind tissue in meat grinder.
5. Suspend in isolation media (Buffer B).
6. Blend in waring blender for two 10 sec bursts.
7. Filter homogenate through three layers of surgical gauze.
8. Centrifuge 2300xg for 10 minutes. Discard pellet.
9. Sediment supernatant at 16,000xg for 10 min. [MITOCHONDRIA]
10. Resuspend pellet in 70 mL Buffer C (0.25 mM sucrose, 10 mM EDTA, 10 mM Tris, pH 7.4).
11. Centrifuge at 500xg for 10 minutes. Discard pellet.
12. Sediment supernatant at 7000xg for 10 minutes.
13. Wash pellet once with 20 mL of Buffer C.
14. Sediment at 7000xg for 10 minutes.
15. Resuspend in Buffer D (25 mM Tris, pH 7.5, 50 mM EDTA, 75 mM NaCl).
16. Lyse with 1.7 mL 10% SDS at 37°C for 5 Minutes.
17. Add 3 mL 7 M CsCl and cool lysate on ice for 10 minutes.
18. Remove caesium dodecyl sulphate precipitate by centrifugation - 20,000xg for 10 minutes.
19. Add EtBr to 500 ug/mL.
20. Adjust refractive index to 1.39 with solid CsCl.
21. Centrifuge in Vti 65.1 rotor for 16 hours at 55 k at 20°C.
22. Collect lower band and extract with CsCl-sautrated isopropanol.
24. Transfer to 30 mL Corex tube and add 1x volume of water and 2x final volume of ice-cold ETOH. Precipitate on ice for 30 min.
25. Centrifuge at 12,000xg for 30 minutes at 4°C.
26. Pour off supernatant (and save temporarily) dry pellet.
27. Dissolve mtDNA pellet in 10 mM Tris-HCl (pH7.9)/1 mM EDTA and determine concentration spectophotometrically. Store at 4°C.

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APPENDIX II

SUBJECT CONSENT FORM

"Neuromuscular Disease and Defects in Mitochondrial DNA"

INVESTIGATORS: Dr. Frank J. Castora
Dr. L. Matthew Frank

SPONSOR: Muscular Dystrophy Association

DESCRIPTION: In order to gain further understanding into the nature of my/my child's illness, I am/my child is being asked to occasionally provide a sample of blood (equal to about 2 teaspoons each time). Over the next three years I/my child may be asked to donate this blood only once or twice more. I/my child may also be asked to allow a small amount of muscle tissue to be taken by a simple procedure called a biopsy. This biopsy involves the insertion of a small needle into an arm or leg muscle and the removal of a tiny piece of muscle. This biopsy will only be necessary if the information the doctors are trying to get cannot be obtained from the blood samples. By studying the blood and muscle samples with a new technique called the Polymerase Chain Reaction, which creates many copies of a specific region that exists on my/my child's genetic material, it is hoped that the cause of my/my child's illness can be found. If so a test for diagnosis using these new techniques will be developed and, in time, new and better treatments may become available.

RISKS AND BENEFITS: I/my child understand(s) that there is a chance of bruising, pain, or infection at the site of blood drawing or muscle biopsy. There may be other risks as yet unidentified. However, the benefit from my/my child's donation may provide a better understanding of my/my child's disease and an improvement in the treatment of my/my child's illness.

NEW INFORMATION: Any new information obtained during the course of the research that may affect my/my child's willingness to continue participation in the study will be provided to me/my child.

CONFIDENTIALITY: I/my child understand(s) that any information obtained about me/my child from the research, including answers to questionnaires, history, laboratory data findings, or physical examination will be kept strictly confidential. I/my child also understand(s) that the data derived from this study could be used in reports, presentations, and publications but that I/my child will not be individually identified. I do/my child does understand however, that my/my child's records may be subpoenaed by court order or may be inspected by federal regulatory authorities. I/my child understand(s) that in order to ensure the Food and Drug Administration (FDA) regulations are being followed, it may be necessary for a representative of the FDA to review my/my child's medical records.

WITHDRAWAL PRIVELEGE: I/my child understand(s) that I am/my child is free to refuse to participate in this study or to withdraw at any time and that my/my child's decision will not adversely affect my/my child's care at this institution or result in any penalty or loss of benefits to which I am/my child is otherwise entitled.

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COMPENSATION FOR ILLNESS OR INJURY: I/my child understand(s) that in the unlikely event of a physical injury or physical illness resulting from the research protocol no monetary compensation will be made but any immediate emergency medical treatment which may be necessary will be made available to me/my child without charge by the investigators. I am/my child is advised that if any injury should result from my/my child's participation in this research project, the Medical College of Hampton Roads (MCHR) provides no insurance coverage, compensation plan or free medical care plan to compensate me/my child for such injuries. In the event that I/my child believe(s) that I have/my child has suffered an injury as a result of my/my child's participation in any research program I/my child may contact Dr. William J. Cooke, (804)446-6015, an employee of the MCHR who will be able to review the matter with me/my child.

VOLUNTARY CONSENT: I certify/my child certifies that I have/my child has read the preceding or it has been read to me/my child so that I/my child understand(s) its contents. If I have/my child has any questions pertaining to the research or my/my child's rights as a research subject, I/my child may contact Dr. Frank J. Castora whose phone numbers are (804)446-5657 or (804)446-5757. A copy of this consent form will be given to me/my child. My signature below means that I have/my child has freely agreed to participate in this experimental study.

______________	______________________________
DATE	PATIENT SIGNATURE
	PARENT OR GUARDIAN

______________	______________________________
DATE	SIGNATURE OF WITNESS

I certify that I have explained to the above individual the nature and purpose of the potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

______________	______________________________
DATE	INVESTIGATOR"S SIGNATURE

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AUTOBIOGRAPHICAL STATEMENT

Name: Catherine Erickson Burgess
Birth: Pensacola, Florida 06/29/62
Nationality: United States Citizen

Education

1989-1994 Eastern Virginia Medical School. Norfolk, Virginia.
Biomedical Sciences Ph.D. - Molecular and Cellular Biology Track.

1980-1987 Old Dominion University. Norfolk, Virginia.
B.S. Degree - Biological Sciences, Chemistry Minor.

Research Work

1990-1994 Mitochondrial DNA analysis in Single Cells, in Rett Syndrome and in Other Neurodegenerative Disorders. Dept. of Biochemistry, Eastern Virginia Medical School. Norfolk, Virginia.

1990-1991 Adapting the Polymerase Chain Reaction to Cystic Fibrosis diagnostics from small quantities of blood with minimal processing time; Analysis of the incidence of the CF ΔF508 mutation in clinically diagnosed patients. Jones Institute Research Laboratories, Norfolk, Virginia.

1989-1990 The effect of ATP Analogs and oligo-adenylates on the activity of Nuclear Topoisomerase I. The effect of oligoadenylates on the activity of Mitochondrial Topoisomerase I. Dept. of Biochemistry, Eastern Virginia Medical School, Norfolk, Virginia.

1988 Preparation and use of human bone as a surgical replacement in dental and orthopedic treatments. Lifenet (Virginia Tissue Bank), Norfolk, Virginia.

Other

1/93-12/94 Adjunct Faculty - Anatomy and Physiology. Department of Math and Sciences, Biology Division. Tidewater Community College, Frederick Campus, Portsmouth, Virginia.

Techniques

DNA: Polymerase Chain Reaction (Standard, Multiplex Protocols); Agarose, Acrylamide Gel Electrophoresis; Micromanipulation; DNA Isolation and Purification from cultured cells, human and animal tissues; Plasmid Purification; Oligo DNA Synthesis and Purification; Southern Blot Analysis; Chemiluminescence; DNA Cycle Sequencing; Dideoxy DNA Sequencing; Denaturing Gradient Gel Electrophoresis (DGGE); Single-Stranded Conformational Polymorphism (SSCP); Dideoxy Fingerprinting (ddF); Ligase Chain Reaction (LCR); RT-PCR.

Protein: Protein Isolation from animal tissues; Denaturing Polyacrylamide Gel Electrophresis; Subcellular Fractionation; Ultracentrifugation; Ion

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Exchange, Affinity and Gel Permeation Chromatography; Enzyme kinetics.

Awards and Honors

1990-94 BiomedicalSciences Ph.D. Assistantship competitively renewed each year. Eastern Virginia Medical School, Norfolk, Virginia.

1993-94 Full Member - Sigma Xi Scientific Honor Society

1991 **PCR Amplification of mitochondrial DNA from organelles in a single cell**; Oral presentation - Best Graduate Student Abstract. EVMS

1991 **PCR Amplification of mitochondrial DNA from organelles in a single cell**; Selected for oral presentation and expense paid trip to Atlanta; FASEB, PCR Symposium, Atlanta, Georgia.

1990 **A Study of the Polymerase Chain Reaction on Anti-Coagulated Blood for Genetic Diagnosis**; Oral Presentation - Best Graduate Student Abstract. EVMS

Publications

-Erickson, C.E., Lewis, D.W., Naidu, S, and Castora, F.J. (1994) **In Search of a Genetic Marker for Rett Syndrome: SSCP Analysis of Mitochondrial tRNA genes.** To be submitted to American Journal of Medical Genetics.

-Erickson, C.E. and Castora, F.J. (1994) **Mitochondrial Heteroplasmy in MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Strokelike symptoms) Results from Mixed Populations of Mutant and Wild-Type mtDNA Within a Single Cell.** To be submitted - American Journal of Human Genetics.

-Erickson, C.E. (1994) **An Analysis of Mitochondrial DNA in Rett Syndrome and Other Neurodegenerative Disorders.** Dissertation.

-Erickson, C.E. and Castora F.J. (1994) **Multiplex Amplification and Sequencing of the LHON mtDNA Mutation from a Single Cell.** Miami Short Reports 4:Suppl (W29a).

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