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Title: Precursors For Mitochondrial DNA Replication:

Metabolic Sources and Relations to Mutagenesis and Human Diseases

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It is well known that the mitochondrial genome has a much higher spontaneous mutation rate than the nuclear genome. mtDNA mutations have been identified in association with many diseases and aging. mtDNA replication continues throughout the cell cycle, even in post-mitotic cells. Therefore, a constant supply of nucleotides is required for replication and maintenance of the mitochondrial genome. However, it is not clear how dNTPs arise within mitochondria nor how mitochondrial dNTP pools are regulated. Recent evidence suggests that abnormal mitochondrial nucleoside and nucleotide metabolism is associated with several human diseases. Clearly, to uncover the pathogenesis of these diseases and the mechanisms of mitochondrial mutagenesis, information is needed regarding dNTP biosynthesis and maintenance within mitochondria, and biochemical consequences of disordered mitochondrial dNTP metabolism.

The studies described in this thesis provide important insight into these questions. First, we found that a distinctive form of ribonucleotide reductase is associated with mammalian liver mitochondria, indicating the presence of *de novo* pathway for dNTP synthesis within mitochondria. Second, We found that long term thymidine treatment could induce mtDNA deletions and the mitochondrial dNTP pool changes resulting from thymidine treatment could account for the spectrum of mtDNA point mutations found in Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) patients. These results support the proposed pathogenesis of this disease. Third, we found that normal intramitochondrial dNTP pools in rat tissues are highly asymmetric, and *in vitro* fidelity studies show that these imbalanced pools can stimulate base substitution and frameshift mutations, with a substitution pattern that correlates with mitochondrial substitution mutations *in vivo*. These findings suggest that normal intramitochondrial dNTP pool asymmetries could contribute to mitochondrial mutagenesis and mitochondrial diseases. Last, Amish lethal microcephaly (MCPHA) has been proposed to be caused by insufficient transport of dNTPs into mitochondria resulting from a loss-of-function mutation in the gene encoding a mitochondrial deoxynucleotide carrier (DNC). We found that there are no significant changes of intramitochondrial dNTP levels in both a MCPHA patient's lymphoblasts with a missense point mutation in *Dnc* gene and the homozygous mutant cells extracted from *Dnc* gene knockout mouse embryos. These results do not support the proposed pathogenesis of this disease and indicate that the DNC protein does not play a crucial role in the maintenance of intramitochondrial dNTP pools.

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CONTRIBUTION OF AUTHORS

Chapter 2. Shiwei Song performed research, wrote this manuscript and also contributed to research design. Dr. Christopher K. Mathews contributed to research design, data analysis and editing the manuscript.

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Chapter 4. Shiwei Song carried out dNTP pool analysis and wrote the first draft of this paper. Dr. Zachary F. Pursell carried out fidelity assays and contributed to paper writing. Dr. Matthew J. Longley contributed to enzyme purification. Dr. Thomas A. Kunkel contributed to data analysis. Dr. William C. Copeland contributed to research design, data analysis and paper writing. Dr. Christopher K. Mathews contributed to research design, data analysis and paper writing.

Chapter 5. Shiwei Song performed research and wrote this manuscript. Dr. Christopher K. Mathews contributed to research design, data analysis and editing the manuscript.

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Chapter 1

General Introduction

Shiwei Song

Introduction

Mitochondria are membrane-bound organelles found in the cells of most eukaryotes. Each mitochondrion has four compartments: the outer membrane, the intermembrane space, the inner membrane, and the matrix. The primary function of mitochondria is the synthesis of ATP by oxidative phosphorylation. Besides their central role in energy metabolism, mitochondria perform many important cellular functions including tricarboxylic acid (TCA) cycle, fatty acid oxidation, and biosynthesis of amino acids, heme and steroids (Scheffler, 2000). Mitochondria also play important roles in apoptosis (Green and Reed, 1998).

One of the distinctive features of mitochondria is that they contain their own DNA — mitochondrial DNA (mtDNA). The mammalian mitochondrial genome is a double-stranded circular DNA molecule of approximately 16 kbp and usually present in hundreds to thousands of copies per cell (Shadel and Clayton, 1997). MtDNA encodes 13 protein subunits of the respiratory chain complexes, as well as two ribosomal RNAs, and 22 transfer RNAs, which are necessary for mitochondrial protein synthesis (Anderson *et al*, 1981). The nuclear DNA encodes all of the remaining mitochondrial proteins, which are targeted specifically to mitochondria by the mitochondrial protein import machinery (Schatz, 1996). Therefore, mutations in either the nuclear or mitochondrial genomes could affect mitochondrial function. In the past two decades, a large number of pathological mtDNA mutations, including both point mutations and deletions, have been identified in association with specific mitochondrial diseases such as Kearns-Sayre

syndrome, Leigh's syndrome, and mitochondrial encephalomyopathy (Wallace, 1999). In addition, mtDNA mutations have been implicated in aging and in common disorders including neurodegenerative disorders, cardiomyopathies, and cancer (Wallace, 1999; Penta *et al*, 2001).

The mitochondrial theory of aging was proposed decades ago (Harman, 1972, Linnane *et al,* 1989). The theory postulates that somatic mutations of mtDNA could result in defective mitochondrial proteins. Then, the defective mitochondrial respiratory chain could increase the production of mutagenic oxidants. These, in turn, could cause the accumulation of further mtDNA mutations. Finally, the crisis of mitochondrial biogenesis caused by the vicious cycle could lead to tissue dysfunction and degeneration. A substantial body of circumstantial evidence seems to support this theory. First, it is known that mitochondria are the major site of generation of reactive oxygen species (Chance *et al,* 1979) and there is evidence that oxidative damage of mtDNA accumulates with age (Richter *et al*, 1998; Lenaz, 1998). Second, there is considerable evidence that the oxidative phosphorylation function of mitochondria declines in various tissues with aging (Yen *et al*, 1989; Trounce *et al*, 1989). Third, there is substantial evidence demonstrating an aging-related accumulation of mtDNA point mutations and deletions (Michikawa *et al*, 1999; Cortopassi and Wong, 1999). Last, Trifunovic et al (2004) created homozygous knock-in mice expressing the mitochondrial DNA polymerase with defective proofreading function. The mutant

mice showed increased somatic mtDNA mutations and premature aging. These findings provide strong direct support for the mitochondrial theory of aging.

Mammalian mtDNA replication has long been considered to occur through an asymmetric mechanism. The "strand-asynchronous" model was proposed more than three decades ago (Robberson *et al*, 1972; Clayton, 1982). According to this model, mtDNA replication begins at the origin of the guanine rich heavy (H) strand and advances around the circle. After the newly synthesized H strand travels about two-thirds of the genome and passes the origin of the cytosine rich light (L) strand, the L strand is then synthesized in the opposite direction using the H strand as a template. Recently, Holt *et al* (2000) proposed a "strand-synchronous" model of mtDNA replication. According to this model, replication of both strands begins at the origin of H strand and continues synchronously around the mtDNA circle. Synthesis of the H strand proceeds continuously at a replication fork while the light strand replicates discontinuously. Recent evidence suggests that both mechanisms coexist in mammalian cells. The "strand-asynchronous" mechanism predominates in cells that are maintaining a steady-state number of mtDNA molecules, while the strand-synchronous mode operates to rapidly reamplify mtDNA after partial depletion (Holt *et al.*, 2000; Fish *et al*, 2004). Still unknown is the biological significance of using two different replication mechanisms under different conditions.

Although much progress has been made with respect to the understanding of the mechanism of mtDNA replication, little is known about those pathways

through which DNA precursors, the deoxyribonucleoside triphosphates (dNTPs) arise within the mitochondrion. In mammalian cells, most enzymes of dNTP synthesis are highly regulated, with activities and rates of synthesis of the enzymes being maximal during S phase (Kunz *et al*, 1994). In most cells, total dNTP pools rise dramatically during S phase. Of course, these events reflect the far greater demand for deoxyribonucleotides during times of nuclear DNA replication. In contrast to nuclear DNA replication, which is confined to S phase, mtDNA replication continues throughout the cell cycle (Bogenhagen and Clayton, 1977). So a constant supply of nucleotides is required for replication and maintenance of the mitochondrial genome. Nearly three decades ago, Bogenhagen and Clayton (1976) reported that in cultured mouse L cells, mtDNA replication was unusually resistant to treatment of the cells with either methotrexate (MTX) or 5-fluorodeoxyuridine. Because both agents act by inhibiting synthesis of DNA precursors, this observation suggested that mtDNA replication is supplied from precursor pools that are somehow distinct from those pools used for nuclear DNA replication. A study from this laboratory (Bestwick *et al*, 1982) showed that MTX treatment caused dramatic depletion of both dTTP and dGTP pools in whole cells and accumulation of all four dNTPs in mitochondria. These results indicate the existence of metabolically distinct dNTP pools within mitochondria. However, it is not clear how dNTPs arise within mitochondria. Recent evidence supports the existence of four different pathways by which dNTPs could arise within mitochondria. First, dNTPs could be synthesized in the cytosol and taken up by

specific transport systems as the triphosphate. Only one such transport system has been described. Bridges *et al* (1999) described a transport system for dCTP in human mitochondria. As studied in reconstituted liopsomes, dCTP transport was inhibited by other dNTPs, but the authors did not establish whether other dNTPs were transported by the system. Second, Dolce *et al* (2001) cloned and expressed a human cDNA encoding mitochondrial deoxynucleotide carrier (DNC), which was described as a member of the family of mitochondrial carriers. The protein showed about 22% sequence identity with mammalian adenine nucleotide transporters. As the recombinant protein was reconstituted into proteoliposomes, this system was found to transport deoxyribonucleoside diphophates (dNDPs) most efficiently, followed closely by ribonucleoside diphophates (rNDPs). Although the protein was shown to have broad distribution within human and mouse tissues, the authors did not demonstrate that the protein is located within mitochondria in any tissue. The authors proposed that the cytosolic dNTPs could be dephosphorylated and then the corresponding dNDPs could be taken up by the DNC and converted to dNTPs within the mitochondrion. Third, mitochondria could directly import deoxyribonucleosides by facilitated diffusion, then phosphorylate them within the organelle by deoxyribonucleoside kinases. To date, both thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK), two of the four known deoxyribonucleoside kinases in human cells, are found within mitochondria (Johansson and Karlsson, 1996; Wang *et al*, 1999). Human dGK efficiently phosphorylates deoxyguanosine and deoxyadenosine, whereas TK2

phosphorylates deoxythymidine, deoxycytidine and deoxyuridine. The toxicity of some antiviral nucleoside analogs such as azidothymidine (AZT) results from their conversion within the mitcochondrion to the dNTP analogs (Wang *et al*, 1999), suggesting that the salvage pathway plays a significant role in mitochondrial dNTP synthesis. Fourth, evidence for possible *de novo* synthesis of mitochondrial dNTPs comes from our laboratory. Young *et al* (1994) detected ribonucleotide reductase (RNR) activity in mitochondria purified from HeLa cells. RNR catalyzes the reduction of the four ribonucleotide substrates to the corresponding deoxyribonucleotides. In *E. coli* and vertebrate cells, the reduction occurs at the nucleoside diphosphate (rNDP) level. It is possible that rNDPs could be taken up by the DNC, then reduced within the mitochondrion by mitochondrial RNR, eventually phosphorylated by mitochondrial nucleoside diphosphate kinase (Milon *et al*, 2000). Since thymidylate synthase is the key enzyme for *de novo* synthesis of thymidine monophosphate (dTMP), it would be expected that thymidylate synthase is located within mitochondrion. In line with this expectation, Janet Leeds in our group found a significant amount of thymidylate synthase activity in purified mitochondria (Leeds, 1986). Also, a study by Neuburger *et al* (1996) showed that most of the thymidylate synthase activity in plant cells lies within the mitochondrion. These results indicated that *de novo* pathways for dNTP synthesis are active within mitochondria. To summarize, current evidence suggests that dNTPs could arise within mitochondria from several different pathways. However,

to date, there is no information available about the relative contribution of each pathway and the regulation of intramitochondrial dNTP pools.

It is well known that mtDNA has a much higher mutation rate than nuclear DNA (Marcelino and Thilly, 1999). However, the basis for this higher mutation rate in mtDNA is not well defined. Several factors could contribute to the higher mutation rate in mtDNA. First, mtDNA is subject to more oxidative damage than nuclear DNA due to its proximity to the electron transport system, a major source of reactive oxygen species (Richter *et al*, 1988). Second, mitochondria have limited DNA repair systems. Although there is substantial evidence that mammalian mitochondria possess a base excision repair system, neither nucleotide-excision repair nor mismatch repair has been demonstrated in mammalian mitochondria (Bogenhagen, 1999). Third, mtDNA mutations could also result from replication errors. A study by Khrapko *et al* (1997) showed that the same kind and position of hot spot mtDNA mutations were present in different tissue and cultured cells, suggesting that the mutations were spontaneous in origin. They proposed that mtDNA mutations most likely arise from DNA replication and /or replicative bypass of DNA adducts created by endogenous factors. DNA polymerase gamma (pol γ) is the only known enzyme responsible for mtDNA replication and repair. In animal cells, pol γ is composed of two subunits. The large catalytic subunit contains DNA polymerase activity and 3'-5' exonuclease activity. The small accessory subunit is required for highly processive DNA synthesis and increases the affinity of pol γ to the DNA (Lim *et al*, 1999).

Extensive investigations with human DNA polymerase γ have shown it to be a relatively accurate enzyme, with error rates *in vitro* below 10^{-5} (Longley *et al*, 2001; Johnson and Johnson, 2001). However, analysis of human mitochondrial mutations suggests that many arise through replication errors (Khrapko *et al*, 1997), and this finding suggests a focus upon DNA precursor pools, since unbalanced dNTP pools are known to stimulate mutagenesis *via* replication errors (Kunz *et al*, 1994). Although mitochondrial dNTP pools have been analyzed in cultured cells (Bestwick *et al*, 1982; Song *et al*, 2003; Rampazzo *et al*, 2004), no such data have previously been reported for mitochondria from animal tissues. Therefore, it is important to determine mitochondrial dNTP pool sizes in animal tissues, asking whether the pools are sufficiently asymmetric to affect replication fidelity within the organelle.

Moreover, recent evidence suggests that abnormal mitochondrial nucleoside and nucleotide metabolism is associated with several human diseases. One disease in this series is called mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), which is an autosomal recessive disorder associated with multiple deletions and depletion of mtDNA in skeletal muscle (Hirano *et al*, 1994) as well as mtDNA point mutations (Nishigaki *et al*, 2003). Nishino *et al* (1999) found that the disease is caused by loss-of-function mutations in the nuclear gene encoding thymidine phosphorylase (TP). TP protein catalyzes the reversible phosphorolysis of thymidine to thymine plus deoxyribose-1-phosphate. It has been shown that in MNGIE patients, TP enzyme activity is reduced drastically, and the levels of

thymidine and deoxyuridine in plasma are significantly elevated (Spinazzola *et al*, 2001; Marti *et al*, 2003). It has been postulated that increased levels of thymidine and deoxyuridine in MNGIE cause mitochondrial nucleotide pool imbalances, which, in turn, lead to mtDNA abnormalities (Nishino *et al* 1999; Marti *et al*, 2003).

Amish lethal microcephaly (MCPHA) is another disease in this series. The diseases comprises severe microcephaly, increased urinary α-ketoglutarate, and premature death (Kelley *et al*, 2002). Through whole-genome scanning and fine-scale genetic mapping, Rosenberg et al (2002) identified a missense mutation in the *SLC25A19* gene encoding a mitochondrial membrane deoxynucleotide carrier (DNC), which segregates with the disease and alters a highly conserved amino acid of DNC protein. The DNC protein is proposed to transport deoxynucleotides from the cytosol into the mitochondrial matrix in exchange for ATP (Dolce *et al*, 2001). Functional analysis of the mutant protein using an *in vitro* transport assay revealed that nucleotide transport activity was lost. They proposed that insufficient transport of dNTPs into mitochondria in the developing central nervous system interferes with synthesis of mitochondrial DNA, causing abnormal brain growth.

Another disease in this series, autosomal dominant progressive external ophthalmoplegia (adPEO), is associated with mtDNA mutagenesis (Kaukonen *et al*, 2000; Ponamarev *et al*, 2002). Some of the adPEO families carry heterozygous mutations in the *ANT1* gene, which encodes the muscle-heart-specific mitochondrial adenine nucleotide translocator (Kaukonen *et al*., 2000). The

adenine nucleotide translocator (ANT) normally exchanges ATP for ADP, allowing exit of newly synthesized ATP from the mitochondrion in exchange for ADP entering and becoming available for resynthesis of ATP within the organelle (Klingenberg, 1989; Fiore *et al*, 1998). Human cells have three ANT isoforms, so presumably the bioenergenetically essential function can be partially compensated for if one isoform is inactive or its function is abnormal. It is still not clear how an *ANT1* gene mutation causes mtDNA mutagenesis. Because a deficiency of ANT function could cause an ADP deficiency within the mitochondrion, which in turn could lead to a dATP deficiency, this could, through a mitochondrial ribonucleotide reductase (Young *et al*, 1994) allosteric control mechanism, lead to abnormal accumulation or deficiency of other nucleotides in mitochondrion. It has been proposed that abnormal mitochondrial nucleotide metabolism resulting from the defective adenine nucleotide translocator accounts for the mtDNA mutations seen in adPEO patients (Kaukonen *et al.*, 2000). To uncover the pathogenesis of these diseases, information is needed about dNTP biosynthesis within mitochondria, maintenance of normal pools, and biochemical consequences of disordered mitochondrial dNTP metabolism.

Four projects are included in this thesis. My first project was focused on ribonucleotide reductase, a key enzyme in the *de novo* dNTP biosynthetic pathways. I found that mammalian liver mitochondria contain a distinctive form of ribonucleotide reductase. My second project was focused on investigating the pathogenesis of a mitochondrial disease — mitochondrial neurogastrointestinal

encephalomyopathy (MNGIE). My third project was focused on the study of the relationship between mitochondrial dNTP pools and mitochondrial mutagenesis. My last project was focused on investigating the pathogenesis of Amish lethal microcephaly.

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Chapter 2

Specific Association of a Novel Form of Ribonucleotide Reductase with Mammalian Liver Mitochondria

Shiwei Song and Christopher K. Mathews

Abstract

Mitochondrial DNA synthesis is continuous throughout the cell cycle. However, the metabolic source of deoxyribonucleoside triphosphates (dNTPs) for mitochondrial DNA replication has not been identified. Earlier studies from this laboratory indicated that intramitochondrial dNTP pools are metabolically distinct from the pools that serve nuclear DNA replication and the presence of ribonucleotide reductase (RNR) activity in HeLa cell mitochondria. In the present study we have examined RNR activity in liver from rats, pigs, and mice. In all cases we found RNR in mitochondrial extracts to have specific activities two- to five-fold higher than in cytosolic extracts, indicating that the mitochondrial enzyme does not represent a cytosolic contaminant. CDP reduction in both rat liver cytosolic and mitochondrial extracts was completely inhibited by 10 mM dATP. Enzyme assays of submitochondrial fractions suggested that the enzyme was mainly located in the mitochondrial matrix fraction. The results from both immunoblotting and sedimentation analysis suggest that the mitochondrial form of ribonucleotide reductase is a distinct protein, different from the major cytosolic form of this enzyme.

Introduction

Ribonucleotide reductase (RNR) catalyzes the first metabolic reaction committed to DNA synthesis, by reducing each of the four ribonucleotide substrates to the corresponding deoxyribonucleotides (Thelander and Reichard, 1979). RNRs characterized to date include three major forms (Jordan *et al*, 1998). Type I RNRs, which include the aerobic enzymes from *E. coli* and phage T4, as well as all known mammalian RNRs, comprise an $\alpha_2\beta_2$ heterodimer of about 240,000 molecular weight. Type II RNRs are the adenosylcobalamin-requiring enzymes of some bacteria and blue-green algae, while Type III RNRs are designed to function anaerobically, and they use ribonucleoside triphosphates, rather than diphosphates, as their substrates. In mammalian cells, most enzymes of dNTP synthesis, including RNR, are highly regulated, with activities and rates of synthesis of the enzymes being maximal during S phase (Kunz *et al*, 1994). dNTP pool levels in mammalian cells vary during the cell cycle, being highest in S phase and dropping to quite low levels in other phases (Leeds *et al*, 1985). In contrast to nuclear DNA replication, which is confined to S phase, mtDNA replication continues throughout the cell cycle (Bogenhagen and Clayton, 1977). So a constant supply of nucleotides is required for replication and maintenance of the mitochondrial genome. It has been shown that dNTPs within mitochondria comprise a metabolically and physically distinct pool (Bestwick *et al*, 1982; Bestwick and Mathews, 1982), but it is not clear how dNTPs arise within mitochondria nor how mitochondrial dNTP pools are regulated. Evidence supports

the existence of several different pathways by which dNTPs could arise within mitochondria. First, dNTPs could be taken up from cytosol by specific transport systems as the triphosphate (Bridges *et al,* 1999). Second, deoxyribonucleoside diphosphates (dNDPs) could be taken up from cytosol by the mitochondrial deoxynucleotide carrier (DNC) (Dolce *et al*, 2001) and converted to dNTPs within the mitochondrion. Third, mitochondria could directly import deoxyribonucleosides, then phosphorylate them within the organelle by deoxyribonucleoside kinases (Arnér *et al*, 1995). Fourth, there is evidence indicating the possible existence of *de novo* dNTP biosynthetic pathways in mitochondria (Leeds, 1986; Neuburger *et al*, 1996). Young *et al* (1994) first detected ribonucleotide reductase activity in mitochondria purified from HeLa cells. Although such activity seen in mitochondria is too low to preclude that the mitochondrial enzyme represents a cytosolic contaminant, the mitochondrial enzyme seemed to be regulated differently from the major cellular activity. In the present study we have examined RNR activity in mitochondria from rat, mouse and pig livers, and our data suggest the existence of a novel form of ribonucleotide reductase in mammalian liver mitochondria.

Experimental Procedures

Isolation of mitochondria from HeLa cells and the livers of rats, mice and pigs -- Liver mitochondria were isolated from adult rats and mice by differential centrifugation, essentially as described by Schnaitman *et al* (1968) with some modifications. The livers were quickly removed after the animals were sacrificed and placed in cold buffer A [220 mM mannitol, 70 mM sucrose, 2mM HEPES (pH 7.4), 0.5 mM EGTA and 0.5 mg/ml BSA]. The livers were minced with small scissors and washed in cold buffer A to remove as much blood as possible. The minced tissue was resuspended in the same buffer (approximately 4ml/g of tissue) and homogenized in a pre-cooled glass-Teflon motorized Glas-Col homogenizer by three to four up and down strokes of the pestle rotating at 1500 rpm. The homogenate was spun at 1000 x g for 5 minutes, and the pellet was discarded. The supernatant was centrifuged at 8000 x g for 15 minutes, and the pellet was washed three times with buffer A. The final mitochondrial pellet was resuspended in a small volume of buffer B [220 mM mannitol, 70 mM sucrose, 2mM HEPES (pH 7.4) 0.5 mM EGTA]. All operations were carried out at 4° C. Fresh pig liver was obtained from a local slaughterhouse immediately after the animal was killed. Mitochondrial isolation was basically the same as above except for homogenizing tissue with a Waring blender. HeLa cell mitochondria were isolated from suspension cultures of HeLa S3 cell as described by Trounce *et al* (1996) with some modifications. Briefly, approximately 1 x 10^9 HeLa S3 cells were harvested from one liter of culture and washed twice in ice-cold PBS. The cell pellet was

resuspended in 5 ml cold isolation buffer, consisting of 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2, 1 mM EGTA, and 0.5% bovine serum albumin (fatty acid-free). Then 10% (w/v) digitonin solution was added to the suspension to a final concentration of 0.3 mg/ml. After 5 min incubation on ice, the suspension was diluted by adding 20 ml of isolation buffer, and the cells were pelleted by centrifugation. Then the cell pellet was resuspended in 10 ml of isolation buffer and the cells were disrupted with a Dounce homogenizer until about 80% of the cells were broken. The mitochondrial fraction was isolated by differential centrifugation as described above and subsequently washed twice and resuspended in 2 ml of isolation buffer.

Subfractionation of rat liver mitochondria -- Intact mitochondria for subfractionation were isolated by a combination of differential centrifugation and Percoll gradient centrifugation, essentially as described by Hovius *et al* (1990) with some modifications. Briefly, the mitochondrial pellet that was isolated by differential centrifugation was resuspended in 5 ml of buffer A, then loaded on the top of 20 ml of 30% Percoll in 225mM Mannitol, 1mM EGTA, 25mM HEPES (pH 7.4), 0.1% BSA (fatty-acid free). This solution was spun at 95,000 x g in a Beckman Ti 70 rotor for 40 min. Mitochondria were collected from the lower part of the dense, brown mitochondrial band and were washed three times with buffer A by centrifuging 10 min at 7500 x g in a SS34 rotor. The mitochondrial pellet was gently suspended in a small volume of buffer B. To separate mitochondria into membrane and soluble fractions, a method described by Kang *et al* (1995)

was used with some modifications. Briefly, 5 mg of the mitochondrial fraction in 1ml of buffer B was sonicated at output power 6 watts for three to four 10-second cycles using a Fisher 60 Sonic Dismembrator. Mitochondrial disruption was monitored by following the activity of citrate synthase. Sonicated mitochondrial extract was centrifuged at 320, 000 x g for 1 hour at 4°C. The supernatant was used as the mitochondrial soluble fraction. The pellet was homogenized in buffer B and served as the mitochondrial membrane fraction. Mitoplasts were prepared by a modification of the method described by Ragan *et al* (1987). Briefly, purified digitonin was dissolved in buffer B by gentle heating. An equal volume of digitonin solution was added to an equal volume of mitochondrial suspension (25 mg protein / ml) with a ratio of 1mg digitonin / 10 mg protein. After incubation for 15 min on ice the sample was diluted with 3 volumes of buffer B and centrifuged at 15,000 x g for 10 min at 4° C. The pellet (mitoplast) was gently resuspended in buffer B.

Enzyme assays -- Lactate dehydrogenase (Wroblewski *et al*, 1955), citrate synthase (Robinson, 1987), adenylate kinase (Schnaitman *et al*, 1968), and cytochrome c oxidase (Darley- Usmar, 1987) were measured according to published methods. Ribonucleotide reductase activity was determined by measuring conversion of [^{3}H] CDP to [^{3}H] dCDP as previously described (Slabaugh *et al*, 1984). Each extract was assayed in duplicate, and the data represent averages of these determinations.

Western Blots --Equal amounts of mitochondrial and cytosolic extracts from mouse liver were loaded onto 12% SDS-polyacrylamide gels and electrophoresed to resolve proteins. The proteins were then transferred to nitrocellulose membranes (OSMONICS) and blocked in 50ml blocking solution (20mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% Tween 20, 1% gelatin). The membrane was incubated with a 1:1000 dilution of rabbit antiserum to vaccinia virus RNR small subunit for 3 hours. The membrane was then washed four times in 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20. The alkaline phosphatase-linked anti-rabbit secondary antibody was incubated with the membrane for 1 h, the washes were repeated as described above, and the membrane was stained with BCIP and NBT kit (GibcoBRL). The antiserum to vaccinia virus RNR small subunit was previously produced in this laboratory (Howell *et al*, 1993).

Sedimentation Analysis – Rat liver mitochondria were incubated with 1% Trix-100 on ice for 20 minutes. After centrifugation at 18,000 x g for 10 min at 4 °C, the resulting supernatants (400 µl) were layered onto 5 to 20% linear sucrose gradients (10 ml) containing 0.1 M HEPES (pH 7.5), 1 mM DTT, and 2 mM $MgCl_2$. Following centrifugation at 4 °C for 24 h at 26,000 rpm in a Beckman SW 41 Ti rotor, fractions were collected from the bottom of each gradient by a fraction collector.

Results

Ribonucleotide reductase activities in mammalian mitochondria—A previous study (Young *et al*, 1994) from this laboratory showed that HeLa cell mitochondrial extracts had about 3% of the RNR activity seen in whole-cell extracts, on a per-cell basis. This did not preclude the possibility that the activity seen in mitochondria represents contamination with cytosol. In the present study, we turned first to rat liver. Using a standard assay for CDP reduction, we found that the specific activity of RNR in mitochondrial extracts is at least two-fold higher than that in cytosolic extract (Figure 2.1), while the specific activity of lactate dehydrogenase, a cytosolic marker enzyme, in mitochondrial extract is only 4.3% of that in cytosolic extract. To further confirm our finding, we determined the specific activities of RNR and LDH in mitochondrial extract and cytosolic extract from pig and mouse livers. We obtained similar results (Figure 2.1). We also examined RNR and LDH activities in HeLa cell mitochondrial and cytosolic extracts. By using an improved mitochondria isolation method, we found HeLa cell mitochondrial RNR activity to be about one third that of a cytosolic extract, considerably greater than the lactate dehydrogenase activity ratio. The data appear to rule out the possibility that liver mitochondrial RNR represents cytosolic contamination, and they strongly suggest that most of the HeLa activity is mitochondrial in origin as well.

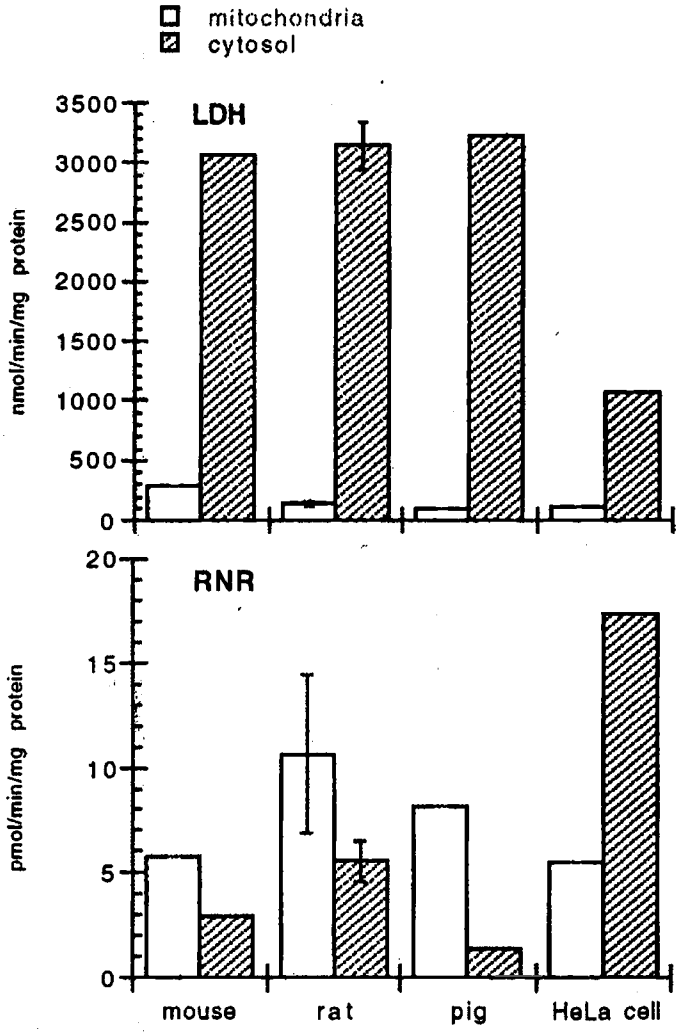

Figure 2.1. Specific association of ribonucleotide reductase with mitochondria in mammalian livers. Ribonucleotide reductase(RNR) avtivity and lactate dehydrogenase (LDH) activity were measured in the liver mitochondrial and cytosolic extracts from rats, pigs, and mice. In all cases we found RNR in mitochondrial extracts to have specific activities two- to five-fold higher than in cytosolic extracts, while the specific activity of LDH, a cytosolic marker enzyme, in mitochondrial extract is only 3% to 9% of that in cytosolic extract. The data indicate that the mitochondrial enzyme does not represent a cytosolic contaminant.

Localization of RNR in mitochondrial matrix--- To define the location of the enzyme in mitochondria, we first need to ask whether the enzyme is present in mitochondrial soluble fraction or associated with mitochondrial membrane. We subfractionated intact mitochondria isolated by the combination of differential and Percoll-gradient centrifugation into a membrane fraction and a soluble fraction. Table 2.1 shows the specific activity of three enzymes in mitochondrial fractions. Based on the specific activity of cytochrome c oxidase, a mitochondrial inner membrane marker enzyme, the soluble fraction (intermembrane space and matrix) contains about 5% of inner and outer membrane on a protein basis. It also suggests that the procedure we employed to subfractionate mitochondria did not cause membrane enzymes to dissociate with the membranes. As shown in Table 2.1, the specific activity of RNR is eleven-fold higher in the soluble fraction than that in the membrane fraction while the specific activity of citrate synthase, a matrix marker enzyme, is about nine-fold higher in soluble fraction than in membrane fraction. These findings indicate that mitochondrial RNR is present in mitochondrial soluble fraction. To further define the location of the enzyme within mitochondria, mitoplasts, which consist of inner membrane and matrix, were prepared by using digitonin to selectively dissolve mitochondrial outer membrane. As shown in Table 2.2, about 82 % of the total enzyme activity of RNR and 79% of the total enzyme activity of citrate synthase were present in mitoplast fraction while the activity of adenylate kinase, a marker enzyme for intermembrane space, in mitoplast fraction was about 2% of the total enzyme activity found in intact

mitochondria. The evidence presented here suggests that RNR is mainly localized within mitochondrial matrix.

Table 2.1

Enzyme activities in fractionated rat liver mitochondria

Enzymes	Mitochondrial extract	Mitochondrial soluble fraction	Mitochondrial membrane fraction
Citrate synthase	126.4 ±21	291.5±88.1	31.2±12.7
Cytochrome c oxidase	156.9±13.1	20.3±1.1	420.8±68.5
Ribonucleotide reductase	13.55±0.5	49.75±16.3	4.59±1.9

Enzyme activities were measured as described under "Experimental Procedures".
Each value is a mean ± SD. of two independent experiments from different rats.
Both citrate synthase activities and cytochrome c oxidase activities are recoreded in nmol/min/mg protein.
Ribonucleotide reductase activities are recorded in pmol/min/mg protein.

Table 2.2

Distribution of enzymes in intact mitochondria and mitoplast (% of total)

Fraction	Citrate synthase	Ribonucleotide reductase	Adenylate kinase
Mitochondria	100	100	100
Mitoplast	79	82	2

Enzyme activities were measured as described under "Experimental Procedures".
Each value is a mean of two independent experiments from different rats.
Citrate synthase is a marker enzyme of mitochondrial matrix.
Adenylate kinase is a marker enzyme of mitochondrial intermembrane space.

Effects of dATP on RNR activities in both mitochondrial and cytosolic extracts from rat liver – We tested the effect of dATP on the CDP reductase activity of RNR in rat liver cytosolic and mitochondrial extracts. As shown in Figure 2.2, the reduction of CDP is inhibited by dATP, as it is in most known forms of ribonucleotide reductase. This result is different from that of the previous study (Young *et al*, 1994) which reported that dATP did not inhibit CDP reduction RNR activity in HeLa cell mitochondrial extract. We have noticed that the previous studies were carried out with preparations of very low RNR activity.

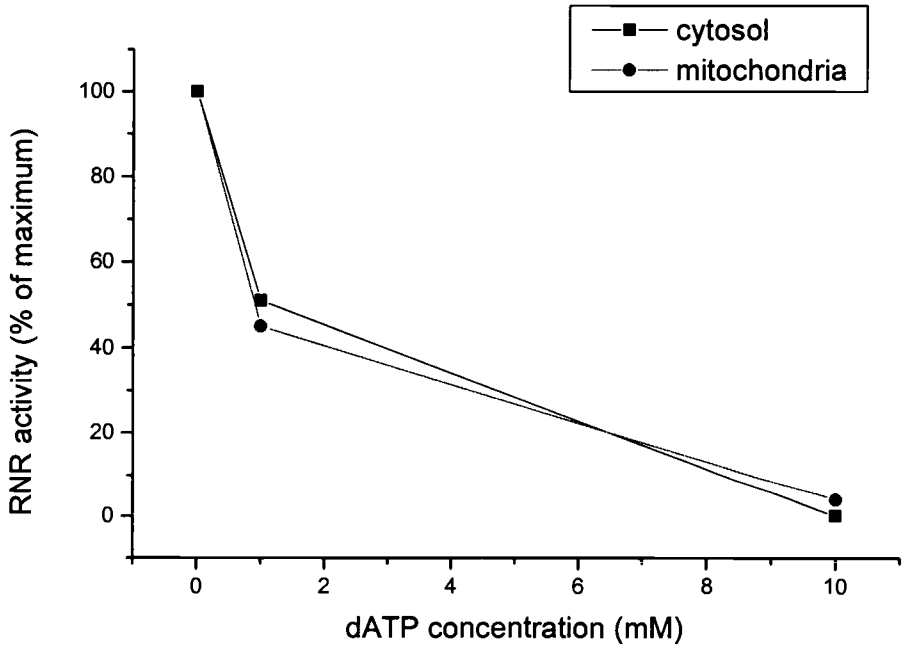

Figure 2.2. Effect of dATP on CDP reductase activity in rat liver cytosolic and mitochondrial extracts.

Mitochondrial ribonucleotide reductase is a distinct reductase – Western blot was performed with rabbit antiserum to vaccinia virus RNR small subunit, as shown in Figure 2.3, The antiserum cross-reacted with mouse liver cytosolic extract but not with mitochondrial extract, even though equal amounts of samples were loaded and the specific activity of RNR in the mitochondrial extract is two-fold that in the cytosolic extract. The result is reproducible. It indicates that mouse mitochondrial RNR is antigenically unrelated to cytosolic RNR. We also found by immunoblotting that the mitochondrial enzyme is unrelated immunologically to ribonucleotide reductase of mouse, phage T4, or *E.coli* (data not shown). Mammalian RNR is an $\alpha_2\beta_2$ tetramer, with a holoenzyme molecular weight of about 240,000 (Jordan *et al*, 1998). The holoenzyme consist of two loosely associated proteins called R1 and R2. R1 is an α_2 dimer and R2 is a β_2 dimer. Normally these proteins dissociate during gel filtration chromatography or gradient centrifugation. Therefore, we were surprised to recover 100 percent of applied activity when the rat liver mitochondrial enzyme was subjected to sucrose gradient centrifugation (Figure 2.4). More surprising was the fact that the peak of activity sedimented as though it represented a protein of 50 kDa or less, far smaller than any known ribonucleotide reductase.

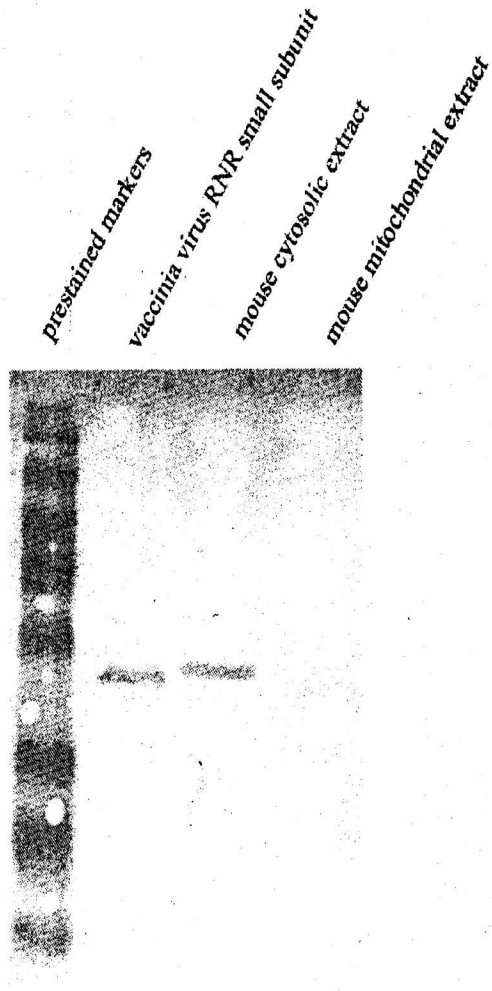

Figure 2.3. Western blot analysis. Western blot was performed with rabbit antiserum to vaccinia virus RNR small subunit. The antiserum cross-reacted with mouse liver cytosolic extract but not with mitochondrial extract, even though the same amounts of samples were loaded and the specific activity of RNR in mitochondrial extract is two-fold that in cytosolic extract. It indicates that mouse mitochondrial RNR is a distinct protein.

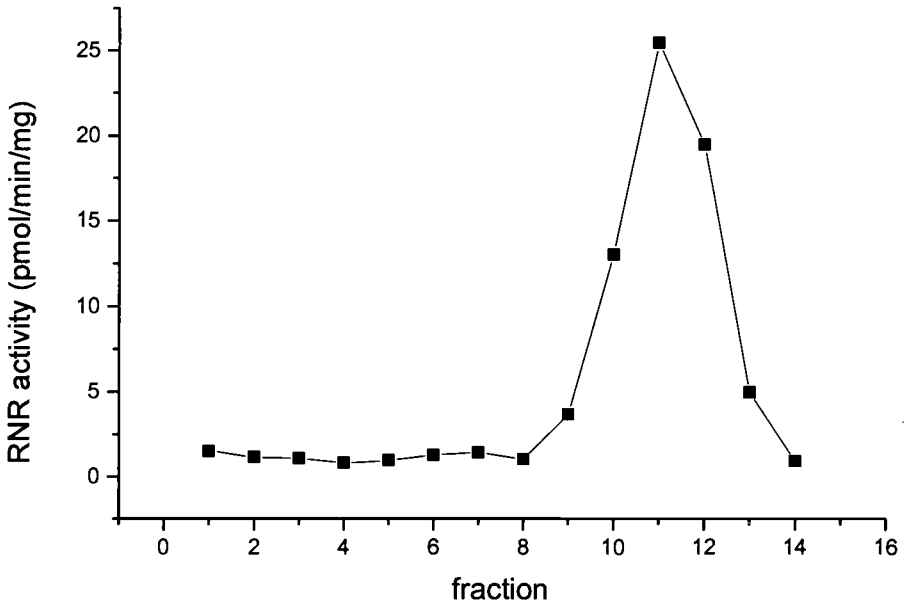

Figure 2.4. Sucrose gradient centrifugation of ribonucleotide reductase activity in a rat liver mitochondrial extract. Following treatment with Triton X-100, a mitochondrial extract was loaded on 5-20% linear sucrose gradient, then centrifuged for 24 hours at 26,000 rpm with a Beckman SW41 rotor. Sedimentation is from right to left.

Purification of ribonucleotide reductase from rat liver mitochondria - Since the enzyme is inhibited by dATP, we first tried to purify the protein with a dATP Sepharose column, and we found that α-ketoglutarate dehydrogenase, an abundant protein in liver mitochondria, can compete with the much less abundant mitochondrial RNR for the binding to dATP Sepharose column. Our continued attempts at purification focused upon other conventional processes, including chromatography on ion-exchange media, hydrophobic media and calcium phosphate. Although an extensive effort was made to purify this protein, so far the enzyme has not been purified successfully. RNR activity was completely lost during the purification process, probably due to the instability of the enzyme and possible separation of enzyme subunits. Also, it is difficult to monitor the protein during the process of purification, because we don't have any antibody recognizing mitochondrial RNR protein.

Discussion

Several significant facts and conclusions emerge from this investigation.

First, we demonstrated that ribonucleotide reductase is present in mammalian liver mitochondria. The results of the specific activity measurements of the enzyme in both mitochondrial and cytosolic extracts strongly suggest that the RNR activity described here is purely of mitochondrial origin and is not due to cytosolic contamination. And it is also interesting to observe that in nonproliferating tissue the specific activity of the enzyme is higher in mitochondria than in cytosol. A possible explanation is that mitochondrial DNA synthesis continues throughout the cell cycle, and mitochondrial regeneration is active in nonproliferating tissues, which may require more active RNR in mitochondria to supply dNTPs for mtDNA replication and repair.

Second, the data from submitochondrial fractionation indicate that mitochondrial RNR is mainly located in mitochondrial matrix. Therefore, the *de novo* synthesized dNTPs can be available directly for mitochondrial DNA synthesis which also occurs in mitochondrial matrix.

Third, the mitochondrial ribonucleotide reductase appears to be quite distinct from previously described forms of the enzyme, with regard to molecular size and immunological properties.

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Chapter 3

Deoxyribonucleotide Pool Imbalance Stimulates Deletions in HeLa Cell Mitochondrial DNA

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Abstarct

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder associated with multiple mutations in mitochondrial DNA—both deletions and point mutations—and mutations in the nuclear gene for thymidine phosphorylase. Spinazzola *et al* (*J. Biol. Chem.* **277**:4128–4133 (2001)) showed that MNGIE patients have elevated circulating thymidine levels and they hypothesized that this generates imbalanced mitochondrial deoxyribonucleoside triphosphate (dNTP) pools, which in turn are responsible for mtDNA mutagenesis. We tested this hypothesis by culturing HeLa cells in medium supplemented with 50μM thymidine. After eight months' growth, mtDNA in the thymidine-treated culture, but not the control, showed multiple deletions, as detected both by Southern blotting and by long extension polymerase chain reaction. After four hours' growth in thymidine-supplemented medium, we found the mitochondrial dTTP and dGTP pools to expand significantly, the dCTP pool to drop significantly, and the dATP pool to drop slightly. In whole-cell extracts, dTTP and dGTP pools also expanded, but somewhat less than in mitochondria. The dCTP pool shrank by about 50 percent, and the dATP pool was essentially unchanged. These results are discussed in terms of the recent report by Nishigaki *et al* (*J. Clin. Invest,* **111**:1913–1921 (2003)) that most mitochondrial point mutations in MNGIE patients involve T→C transitions in sequences containing two A's to the 5' side of a T residue. Our finding of dTTP and dGTP elevations and dATP depletion in mitochondrial dNTP pools are consistent with a mutagenic

mechanism involving T-G mispairing followed by a next-nucleotide effect involving T insertion opposite A.

Introduction

The human mitochondrial genome is a 16.6-kbp double-stranded circular DNA molecule that encodes 22 tRNAs, 2 rRNAs, and 13 polypeptides, all of which are essential proteins of the respiratory chain enzyme complexes (Anderson *et al,* 1981). Mitochondrial DNA (mtDNA), which is usually present in 2–10 copies per mitochondrion and 10^3 to 10^4 copies per cell, comprises ~1% of total cellular DNA (Shadel and Clayton, 1997). Since mtDNA replication is continuous, even in post-mitotic cells (Bogenhagen and Clayton, 1977), a constant supply of nucleotides and balanced nucleotide pools are required for replication and maintenance of the mitochondrial genome. It has been shown that dNTPs within mitochondria comprise a metabolically and physically distinct pool (Bestwick and Mathews, 1982; Bestwick *et al,* 1982; Bogenhagen and Clayton, 1976), but it is not clear how dNTPs arise within mitochondria nor how mitochondrial dNTP pools are regulated. Evidence supports the existence of four different pathways by which dNTPs could arise within mitochondria. First, dNTPs could be synthesized in the cytosol and taken up by specific transport systems as the triphosphate (Bridges *et al,* 1999). Second, cytosolic dNTPs could be dephosphorylated and then the corresponding deoxyribonucleoside diphosphates (dNDPs) be taken up by

the mitochondrial deoxynucleotide carrier (Dolce *et al,* 2001) and converted to dNTPs within the mitochondrion. Third, ribonucleoside diphosphates could be taken up by the deoxynucleotide carrier (DNC), then reduced within the mitochondrion by mitochondrial ribonucleotide reductase (Young *et al,* 1994). Fourth, mitochondria could directly import deoxyribonucleosides, then phosphorylate them within the organelle by thymidine kinase 2 and deoxyguanosine kinase (Johansson *et al,* 1997; Wang *et al,* 1999).

It is well established that dNTP pool imbalances are mutagenic to cells (Kunz *et al* 1994), with mutagenic mechanisms including both insertion errors and next-nucleotide effects (Kunkel *et al,* 1986; Goodman *et al,* 1998). *In vitro* studies have demonstrated that dNTP imbalances can induce frameshift mutations as well (Bebenek and Kunkel, 1990). Recent evidence has suggested that an imbalance of mitochondrial nucleotide pools plays an important role in the pathogenesis of several human diseases, including mitochondrial neurogastrointestinal encephalomyopathy (MNGIE; Nishino *et al,* 1999), autosomal dominant progressive external ophthalmoplegia (Kaukonen *et al,* 2000; Spelbrink *et al,* 2001), and Amish lethal microcephaly (Rosenberg *et al,* 2002). So far, however, there are no published data reporting direct analysis of mitochondrial dNTP pools in any of these conditions. Our laboratory described direct measurement of mitochondrial dNTP pools as long ago as 1982 (Bestwick *et al,* 1987). However, the measurements were laborious, and progress was slow. Recently we have modified our analytical procedures for dNTPs, allowing for reliable measurements

of mitochondrial dNTPs from as few as 2×10^8 cultured cells. Using these modified techniques, we have explored, in HeLa cells, some of the predictions made from studies on one mitochondrial disorder, MNGIE.

MNGIE is an autosomal recessive disorder associated with multiple deletions and depletion of mtDNA in skeletal muscle (Hirano *et al,* 1994) as well as mtDNA point mutations (Nishigaki *et al,* 2003). The disease is caused by loss-of-function mutations in the nuclear gene encoding thymidine phosphorylase (TP; Nishino *et al,* 1999). TP catalyzes phosphorolysis of thymidine to thymine and deoxyribose 1-phosphate. A deficiency of TP leads to increased circulating levels of thymidine (dThd; Spinazzola *et al,* 2002) and deoxyuridine (Marti *et al,* 2003). It has been postulated that increased levels of dThd in MNGIE cause mitochondrial nucleotide pool imbalances, which, in turn, lead to mtDNA abnormalities (Nishino *et al,* 1999; Spinazzola *et al,* 2002). We tested this hypothesis by culturing HeLa cells in medium supplemented with 50μM thymidine and observing the effects upon dNTP pools within mitochondria and upon the generation of mtDNA deletions.

Experimental Procedures

Cell Culture—HeLa S3 cells were routinely grown at 37 °C with 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 5% dialyzed fetal bovine serum (Hyclone, Logan, UT), with or without 50 μM thymidine. When cultures became 80–90% confluent, cells were washed twice with phosphate-buffered saline and then collected after trypsin treatment. When needed for DNA extraction, 80% of those cells were pelleted, with the remaining cells being subcultured and the culture medium being changed every 3 days.

Whole-cell dNTP Pool Extraction and Analysis—HeLa S3 cells were cultured as described above. For each analysis, the cells in two 100-mm plates were washed with 5 ml each of cold phosphate-buffered saline. Extraction was carried out with 3 ml per plate of ice-cold 60% methanol. Plates were incubated at –20 °C for 1 h, following which the fluid was recovered and each plate was washed with an additional 1 ml of 60% methanol. Following this, all suspensions and washes were pooled. The pooled suspension was heated for 3 min in a boiling water bath, followed by centrifugation for 15 min at 17,000 x *g*. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and stored at –20 °C for later analysis. Analysis of the dNTP pools in each extract was based upon the method of Sherman and Fyfe (1989). Reaction mixtures (50 μl) contained 100 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 0.1

unit of *Escherichia coli* DNA polymerase I Klenow fragment (United States Biochemical), 0.25 μM oligonucleotide template, and 0.67 μM [^{3}H]dATP (30 Ci/mmol; 1.0 μCi per assay; Amersham Biosciences) or [^{3}H]dTTP (PerkinElmer Life Sciences). Incubation was carried out for 60 min at 37 °C. Cell number per sample was determined by hemocytometer counting.

Mitochondrial dNTP Pool Extraction and Analysis—To determine the effect of thymidine treatment on mitochondrial dNTP pools, HeLa S3 cells were grown in 150-mm tissue culture dishes in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Once the cultures had reached 70–80% confluence, thymidine was added to a final concentration of 50 μM. After a 4-h treatment, the medium was removed and mitochondria were prepared by a modification of the methods of Trounce *et al* (1996). Briefly, ~2 x 10^8 HeLa S3 cells were harvested from eight 150-mm tissue culture dishes by trypsin treatment and washed twice in ice-cold phosphate-buffered saline. The cell pellet was resuspended in 5 ml cold isolation buffer, consisting of 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2, 1 mM EGTA, and 0.5% bovine serum albumin (fatty acid-free). Then 10% digitonin solution was added to the suspension to a final concentration of 0.3 mg/ml. After 5-min incubation on ice, the suspension was diluted by adding 20 ml of isolation buffer, and cells were pelleted by centrifugation. Then the cell pellet was resuspended in 10 ml of isolation buffer and the cells were disrupted with a motor-driven glass-Teflon homogenizer until about 80% of the cells were broken. The mitochondrial fraction was isolated by

differential centrifugation and subsequently washed twice and resuspended in 2 ml of isolation buffer. Immediately after that, most of the mitochondrial suspension was centrifuged to pellet mitochondria and the remaining suspension was saved for later mitochondrial protein determination. The mitochondrial pellet was immediately resuspended in 1 ml of ice-cold 60% methanol and incubated at –20 °C for 1 h. The remaining procedures were the same as described for the whole-cell dNTP pool extraction procedures. Protein concentration was determined by the Bradford method (Bradford, 1976).

Long Extension PCR—Total DNA from HeLa cell samples was isolated by using the Wizard® genomic DNA purification kit (Promega). LX-PCR was performed to amplify the whole mtDNA genome using expand long template PCR system (Roche Applied Science). Primers were forward primer (L 15148–15174) and reverse primer (H 14842–14816). The PCR protocol consisted of initial 2-min denaturation at 94 °C, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 10 min, and a final extension of 7 min at 68 °C in a thermal cycler (PerkinElmer Life Sciences). The PCR products were subjected to electrophoresis on a 1% agarose gel at 100 V for 45 min and stained with 0.5 μg/ml ethidium bromide. To exclude the possibility that the low molecular weight bands were due to PCR artifacts, a second LX-PCR was performed using nested primers (L strand: 15148–15174) and (H strand: 13980–13956).

Southern Blot Analysis—Total DNA from HeLa cell samples was isolated as described above. Approximately 5 μg of total DNA was digested to completion with restriction enzyme PvuII (Invitrogen) and the fragments separated by electrophoresis through a 0.7% agarose gel. After electrophoresis the gel was blotted onto a nylon membrane (Hybond-N^+, Amersham Biosciences). Then, after 4-h prehybridization, the membrane was hybridized with a mtDNA probe, which was generated by PCR using primers corresponding to mtDNA positions 8900–8922 and 9422–9400 and labeled with [α-^{32}P]dCTP using random primers DNA labeling system (Invitrogen). Prehybridization, hybridization, and washing were carried out as described by Sambrook *et al* (1989). The washed membrane was exposed to an x-ray film at –70 °C with an intensifying screen.

Results and Discussion

Mitochondrial dNTP Extraction and Analysis—A large body of literature exists regarding control of cellular dNTP pool sizes and the effects of pool imbalance on mutagenesis. In contrast, little information is available concerning mitochondrial dNTP metabolism, due to the technical difficulty of measuring these much smaller pools. Recent evidence indicates that an imbalance of mitochondrial nucleotide pools may play important roles in the pathogenesis of several human diseases, including MNGIE, autosomal dominant progressive external ophthalmoplegia, and Amish microcephaly. To investigate relationships between mitochondrial nucleotide metabolism and pathogenesis, it became necessary to increase the sensitivity of existing methods for dNTP measurement. By increasing the specific radioactivity of dNTPs used in the DNA polymerase-based method for dNTP analysis by some 3-fold, and by adopting the modifications to this assay of Sherman and Fyfe (1989), we have increased the sensitivity of this method by nearly an order of magnitude. By adopting the methods of Trounce *et al* (1996), we have improved our yield of mitochondria with no loss in quality. With these improvements, we can determine as little as 0.05 pmol of dNTP in a cell or organelle extract. This means that we can prepare enough mitochondria for a complete dNTP assay from 2×10^8 cells. Our earlier study (Bestwick *et al,* 1987) required more than 10^9 cells for each analysis.

Effects of Thymidine Treatment on dNTP Pools—Spinazzola *et al* (2002) showed that MNGIE patients showed elevations in circulating thymidine levels, presumably a consequence of decreased thymidine catabolism resulting from the deficiency of thymidine phosphorylase. They speculated that thymidine could be salvaged to dTTP within mitochondria, generating a pool imbalance that could account for replication errors in the mitochondrial genome, leading both to deletions and point mutations. To test this hypothesis, we cultured HeLa cells in thymidine-supplemented medium and determined the effects, both upon dNTP pools, in short term experiments, and generation of mtDNA deletions, in a long term experiment. Thymidine supplementation was at 50 μM. Figure 3.1 shows the results of dNTP analyses in whole-cell and mitochondrial extracts. We found the mitochondrial dNTP pools to become imbalanced as a result of a 4-h exposure to 50 μM thymidine but not dramatically more so than the pools in whole-cell extracts. In whole-cell extracts dTTP and dGTP pools expanded by 2.2- and 1.5-fold, respectively; the dATP pool was unchanged, and the dCTP pool decreased to half its normal value. By comparison, the mitochondrial dTTP and dGTP pools increased by 2.6- and 2.2-fold, respectively, while the mitochondrial dATP and dCTP pools decreased to 78 and 43%, respectively, of corresponding values in the untreated cells.

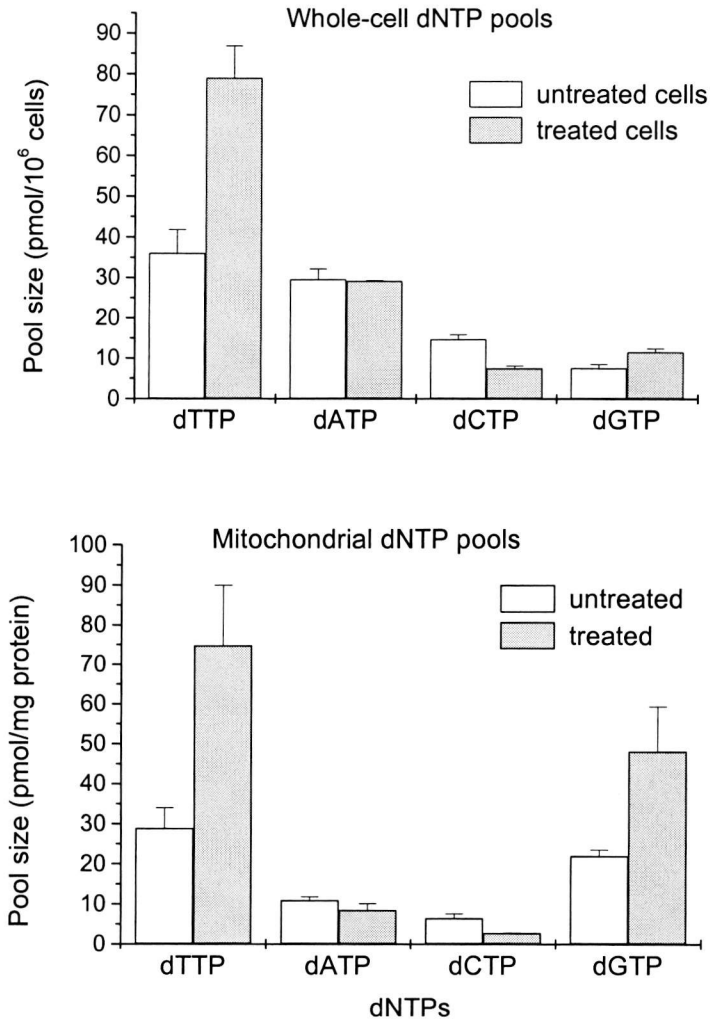

Figure 3.1. Effects of 50 μM thymidine exposure on whole-cell and mitochondrial dNTP pools. HeLa cell dNTP pools were extracted and analyzed after 4 h of thymidine treatment or without thymidine treatment, as described under "Experimental Procedures." Each data point represents a mean ± S.D. of two separate experiments in duplicate. Note the different units used for reporting mitochondrial and whole-cell values.

Several significant findings emerge from this experiment. First, as noted above, thymidine treatment did not unbalance the dNTP pools substantially more in mitochondria than in whole cells. However, factors such as a lack of mismatch repair in mitochondria (Marcelino *et al,* 1999; Bogenhagen, 1999) may render mitochondrial DNA replication more susceptible than nuclear DNA replication to modest dNTP asymmetries. Second, the composition of the mitochondrial pool is quite different from that of the much larger whole-cell pool. As we and others have observed repeatedly (Zhang *et al,* 1995; Martomo *et al,* 2002), dGTP represents only 5–10% ot the total dNTP pool in whole-cell extracts of cultured eukaryotic cells. However, in HeLa cell mitochondria, dGTP is considerably more abundant than either dATP or dCTP and is nearly as abundant as dTTP. Third, the effect of thymidine upon the mitochondrial dNTP pools suggests that ribonucleotide reductase plays a significant role in regulating dNTP pool sizes in the organelle. dTTP acts as an allosteric activator of GDP reduction by ribonucleotide reductase and an inhibitor of CDP reduction (Jordan *et al,* 1998). In recent experiments we have detected in liver mitochondria a ribonucleotide reductase activity that is similar to the major cell ribonucleotide reductase in its response to allosteric effectors. The dGTP accumulation and dCTP depletion seen in mitochondria from thymidine-treated cells are consistent with a regulatory role for mitochondrial ribonucleotide reductase.

Of greatest interest, however, is the fact that the pool changes that we observe can account for the mutation spectrum reported by Nishigaki *et al* (2003)

in mitochondrial DNA from MNGIE patients. These authors reported that most mitochondrial point mutations in MNGIE patients involve T → C transitions in sequences containing at least two As to the 5' side of a T residue. Our findings of dTTP and dGTP elevations and dATP depletion in mitochondrial dNTP pools are consistent with a mutagenic mechanism involving competition between dGTP and dATP for incorporation opposite template T, followed by a next-nucleotide effect, in which T from the expanded dTTP pool is incorporated opposite the two A residues on the 5' side of the mispairing.

Deletion Mutagenesis Caused by dNTP Imbalance—Mitochondrial gene mutations in MNGIE patients include both point mutations and deletions (Hirano *et al,* 1994; Nishigaki *et al,* 2003). As noted earlier, there are other disease states in which abnormalities in deoxyribonucleotide metabolism are associated with the generation of deletions in mitochondrial DNA (Kaukonen *et al,* 2000; Spelbrink *et al,* 2001). Although large dNTP asymmetries are known to stimulate frameshift mutations *in vitro* (Bebenek and Kunkel, 1990), it is not immediately apparent how modest pool asymmetries, of the type seen in this study, could stimulate the formation of long deletions. Nevertheless, we carried out an experiment to determine whether mitochondrial deletions could, in fact, be generated by long term exposure to pool-imbalanced conditions.

To do this we cultured HeLa cells for 8 months in medium supplemented with 50μM thymidine or without thymidine supplement. We then analyzed

mitochondrial DNA from both cultures. Using a LX-PCR method, as shown in Figure 3.2, we observed multiple deletion-containing mtDNA molecules ranging in size from 1 to 3.3 kbp in cells grown in thymidine-supplemented medium but not in cells grown for the same period in the same medium but without thymidine supplement. To exclude the possibility that the low molecular weight bands were due to PCR artifacts, a second LX-PCR was performed using nested primers. In all cases analyzed we obtained a pattern consistent with that observed in the first PCR (data not shown).

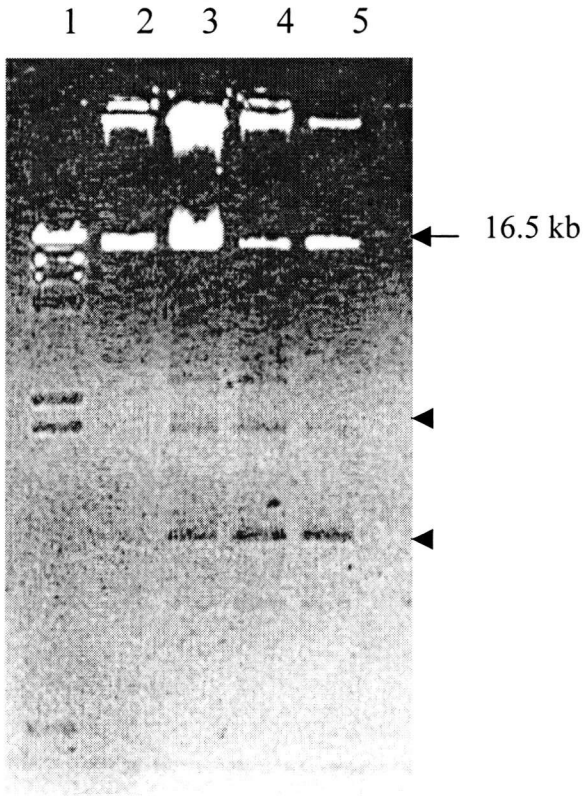

Figure 3.2. LX-PCR detection of mtDNA deletions. Total DNAs from HeLa cells grown in medium supplemented with or without 50 μM thymidine for 8 months were extracted and subjected to LX-PCR as described in the text. Reaction products were visualized in ethidium bromide-stained agarose gels and photographed with UVP ImageStore 7500 (PerkinElmer Life Sciences). *Lane 1*, DNA marker (λ DNA/HindIII fragments); *lane 2*, PCR product generated from cells grown in medium without thymidine supplement; *lanes 3–5*, PCR products generated from cells grown in medium with 50 μM thymidine supplement for 240, 247, and 254 days, respectively. The *arrow* indicates a 16.5-kbp full-length mtDNA product. The *arrowheads* indicate mtDNA molecules containing deletions.

To further confirm that long term thymidine treatment induces mtDNA deletions, we analyzed mtDNA by Southern blotting with a mtDNA-specific probe. As shown in Figure 3.3, we detected a deletion-containing mtDNA molecule about 3 kbp in size in cells grown in the thymidine-treated culture but not in the control culture.

Although these data clearly show a relationship between unbalanced dNTP pools and the creation of mitochondrial DNA deletions, they do not shed light on possible mechanisms. Several models, including a slip-replication model (Shoffner *et al,* 1989) and an illegitimate elongation model (Buroker *et al,* 1990), have been proposed. Both of these require movement of single-stranded mtDNA and have been described (Hirano *et al,* 2001) as compatible with the concept that dNTP pool imbalances can induce multiple deletions of mtDNA. In that study Hirano *et al* (2001) proposed that in the presence of unbalanced nucleotide pools, relatively low levels of one particular dNTP may lead to stalling of mtDNA replication, with the stalled replication intermediate containing a single-stranded region that would be susceptible to migration and to inappropriate annealing, particularly at direct sequence repeats. The data of Figure 3.1 show that dCTP after short term thymidine exposure represents only two percent of the total mitochondrial dNTP pool. It seems reasonable to suggest that long term exposure to such conditions could generate deletions as a result of replication fork stalling, as proposed by Hirano *et al* (2001). A prediction from this model is that long term culturing of

cells under conditions that prevent dCTP depletion should not result in the generation of deletions.

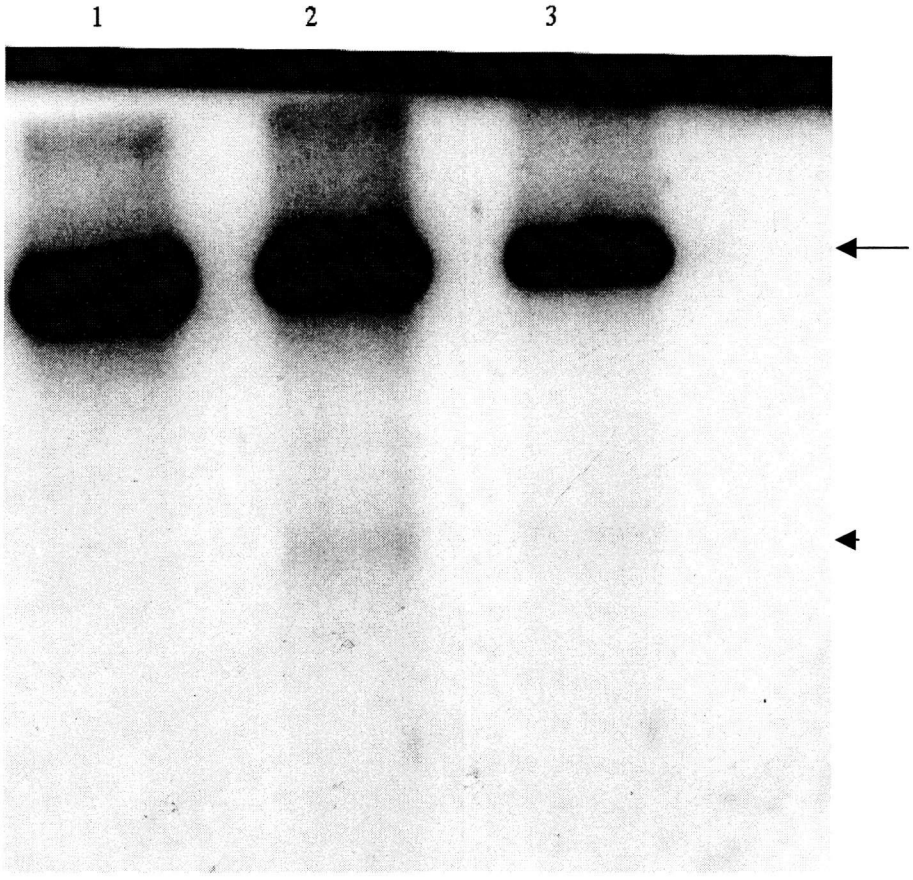

Figure 3.3. Detection of mtDNA deletion by Southern blot. Total DNAs from HeLa cells grown in medium supplemented with or without thymidine for 8 months were digested with PvuII and subjected to Southern blot analysis with a mtDNA-specific DNA probe as described under "Experimental Procedures." Lane 1, mtDNA from cells cultured without thymidine supplement; lanes 2 and 3, mtDNAs from cultures supplemented with 50 μM thymidine for 240 and 254 days, respectively. The arrow indicates a 16.5-kbp full-length mtDNA and the arrowhead indicates a deletion-containing mtDNA molecule whose size is about 3 kbp. Lanes 2 and 3 show the likely presence of larger deletion-containing molecules in smaller amounts. Note that other deletion-containing molecules may be present but not detected because of the lack of sequences represented in the probe.

The significance of this study is 3-fold. First, by improvements in methods for mitochondrial dNTP extraction and analysis, we have shown that cell culture represents a suitable model for investigating mitochondrial diseases that involve abnormalities in nucleotide metabolism. Second, although it is well known that dNTP asymmetries stimulate mutagenesis, this may be the first study to relate specific pool changes to a mutagenic mechanism that can account for actual mutations seen in affected humans. Third, even though this study does not test specific mechanisms, it strengthens the predicted causal relationship between deoxyribonucleotide pool imbalance and deletion mutagenesis.

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Chapter 4

DNA Precursor Asymmetries in Mammalian Tissue Mitochondria and Possible Contribution to Mitochondrial Mutagenesis Through Reduced Replication Fidelity

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Abstract

The mutation rate of the mammalian mitochondrial genome is higher than that of the nuclear genome. Because mitochondrial and nuclear deoxyribonucleoside triphosphate (dNTP) pools are physically distinct and because dNTP concentrations influence replication fidelity, we asked whether mitochondrial dNTP pools are asymmetric with respect to each other. We report here that the concentrations of the four dNTPs are not equal in mitochondria isolated from several tissues of both young and old rats. In particular, in most tissues examined, mitochondrial dGTP concentrations are high relative the other dNTPs. Moreover, in the presence of the biased dNTP concentrations measured in heart and skeletal muscle, the fidelity of DNA synthesis *in vitro* by normally highly accurate mitochondrial DNA polymerase γ is reduced due to increased formation of template T-dGTP mismatches that are inefficiently corrected by proofreading. These data are consistent with the hypothesis that normal intra-mitochondrial dNTP pool asymmetries may contribute to spontaneous mutagenesis in the mammalian mitochondrial genome.

Introduction

It has been shown that mitochondrial DNA (mtDNA) mutations are associated with aging and a wide variety of diseases including neurodegenerative diseases, cardiomyopathies and cancer (Linnane *et al*, 1989; Wallace, 1999). It is also known that the mitochondrial genome has a much higher mutation rate than the nuclear genome (Marcelino and Thilly, 1999). Several factors have been proposed to account for the higher mutation rate in mtDNA. First, mtDNA is subject to more oxidative damage than nuclear DNA due to its proximity to the electron transport system, a major source of reactive oxygen intermediates (Richter *et al*, 1988). Second, mtDNA does not have the protection provided by histones. Third, mitochondrial DNA repair systems are limited (Bogenhagen, 1999). Although there is substantial evidence that mammalian mitochondria possess a base excision repair system, both nucleotide-excision repair and mismatch repair have not been demonstrated in mammalian mitochondria (Dianov *et al*, 2001). There is also evidence that mtDNA mutations most likely arise from DNA replication errors (Khrapko *et al*, 1997). Several factors, including the properties of the replicative DNA polymerase and concentrations of dNTP DNA precursors could influence replication fidelity (Kunkel, 2004; Kunz *et al*, 1994). DNA polymerase gamma (pol γ) is the enzyme responsible for replication and repair of mtDNA. This enzyme is composed of two subunits. The catalytic subunit contains DNA polymerase activity and 3'-5' exonuclease activity. The accessory subunit is required for highly processive DNA synthesis and increases the affinity of pol γ to

the DNA (Longley *et al*, 2001). The pol γ is a high fidelity polymerase with an average of fidelity of one error in every 280,000 base polymerized (Johnson and Johnson, 2001). Because it is well established that deoxyribonucleoside triphosphate (dNTP) pool imbalances are mutagenic to cells (Kunz *et al*, 1994), and recent evidence has suggested that an imbalance of mitochondrial nucleotide pools plays an important role in the pathogenesis of several human diseases (Nishino *et al*, 1999; Kaukonen *et al,* 2000), attention should be paid to investigate whether normal mitochondrial dNTP levels have any impact on mtDNA mutagenesis. Also, because mitochondrial mutations accumulate with age (Wang *et al*, 2001; Michikawa *et al*, 1999), it could be worthwhile to determine whether mitochondrial dNTP pools change significantly with age in a way that could influence replication fidelity and possibly the mutation rate of the mitochondrial genome. However, so far there are no published data regarding tissue mitochondrial dNTP levels from either young or old aminals .

Cardiac muscle contains two distinct populations of mitochondria: Subsarcolemmal mitochondria (SSM) are located beneath the sarcolemma, whereas interfibrillar mitochondria (IFM) are located between the myofibrils (Palmer *et al*, 1977). Previous studies showed a decline in oxidative phosphorylation with age in IFM (Fannin *et al*, 1999), as well as age-related decreases of reduced glutathione levels and glutaredoxin reductase and glutathione (GSSG) reductase activities (Suh *et al*, 2003). Because glutaredoxin is a glutathione-dependent electron donor for ribonucleotide reductase, an enzyme

found in mitochondria (Young *et al*, 1994), an age-related decrease of glutaredoxin and GSSG reductase activities in IFM could affect mitochondrial dNTP pools and mtDNA replication, with an eventual effect upon IFM function. To address this issue, information about cardiac muscle mitochondrial dNTP pools is needed.

In this study we measured mitochondrial dNTP pool levels in various tissues from young and old rats. These data were used to estimate molar dNTP concentration in mitochondrial fluid spaces, and these values in turn were used as the basis of a series of fidelity assays with purified recombinant DNA polymerase γ.

Materials and Methods

Isolation of Mitochondria. Young male Wistar and Fischer 344 rats (3-7 months of age) were purchased from Simonsen Laboratories, Inc. Old male Fischer 344 rats (26-28 months of age) were purchased from the National Institute of Aging. All animal procedures were approved by the Institutional Animal Care and Use Committee of Oregon State University. Animals were anesthetized with diethyl ether and killed by decapitation, one animal per analysis. Tissues (one gram or more) of heart, liver, forebrain, and skeletal muscle were immediately removed, washed and minced in cold isolation buffer containing 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4, 2 mM EGTA and 0.2 mg/ml BSA. Liver and brain were homogenized in cold isolation buffer with a glass-Teflon motorized homogenizer. The mitochondrial fraction was isolated by differential centrifugation and subsequently washed twice and resuspended in 2 ml of isolation buffer. Immediately after that, most of the mitochondrial suspension was centrifuged to pellet mitochondria and the remaining suspension was saved for determination of mitochondrial respiratory control ratio and protein concentration. The skeletal muscle mitochondria isolation procedure was the same as described above except for using more vigorous homogenization and a different isolation buffer consisting of 0.1 M KCl, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA. Two subpopulations of cardiac mitochondria were isolated as described by Suh *et al* (2003). Lactate dehydrogenase and respiratory control ratio were determined as described (Wroblewski and LaDue, 1955; Rickwood, 1987). Mitochondrial protein was estimated by the Bradford method. The purity of the mitochondrial preparation was monitored by assaying for contaminating lactate dehydrogenase activity. The preparations routinely showed lactate dehydrogenase

specific activity less than one percent the specific activity of corresponding cytosolic extracts. Functional integrity of the preparations was routinely monitored by determining the respiratory control ratio and the P/O ratio for succinate. All P/O ratios measured were found to be above 1.7, close to the theoretical value of 2. The respiratory control ratios varied with the tissue analyzed, but all values determined were in the range expected for functionally intact mitochondria (Rickwood, 1987).

Mitochondrial dNTP Pool Extraction and Analysis. Freshly isolated mitochondria were immediately resuspended in 1 ml of ice-cold 60% methanol and incubated at –20 °C for 1 hour to extract nucleotide pools. The suspension was heated for 3 min in a boiling water bath followed by centrifugation for 20 min at 17,000 x g. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and stored at –20°C for later analysis. Analysis of the dNTP pools in each extract was carried out by the DNA polymerase-based method as previously described (Song *et al,* 2003) with some modifications. Reaction mixtures (25 µl) contained 100 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 units of *Escherichia coli* DNA polymerase I Klenow fragment (U.S. Biochemical), 0.25 µM oligonucleotide template, 5 µg of bovine serum albumin (New England Biolabs) and 1.25 µCi [^{3}H]dATP (Amersham Biosciences) or [^{3}H]dTTP (Perkin Elmer Life Sciences). Incubation was carried out for 45 min at 37 °C.

Fidelity Assays. His_6 affinity-tagged recombinant human DNA polymerase γ catalytic (p140) subunit (exonuclease-proficient and –deficient forms) and accessory subunits were purified separately to homogeneity and reconstituted as described previously (Longley *et al*, 1998; Lim *et al*, 1999). Human DNA

polymerase γ fidelity was measured as described (Longley *et al*, 2001). Briefly, pol γ was used to copy a single-stranded region of the M13 *lacZ* α-complementation gene. Gap-filling reaction mixtures (25 µl) contained 25 mM HEPES•KOH (pH 7.6), 2mM dithiothreitol, 2 mM $MgCl_2$, 50 µg/ml BSA, 0.1 M NaCl, approximately 150 ng gapped M13mp2 DNA, 40 ng of Exo^+ or Exo^- p140 pol γ, a 1.3-fold molar excess of the p55 accessory subunit, and dNTPs at the indicated concentrations. Gap-filling reactions were run to completion as monitored by agarose gel electrophoresis. Products containing completely filled gaps were introduced by electroporation into the host strain and replication errors were scored by plating as described (Bebenek and Kunkel, 1995). M13 DNA from independent mutant M13 plaques was isolated and sequenced to determine the types of polymerization errors. Error rates were calculated from these data as described (Bebenek and Kunkel, 1995).

Results

Mitochondrial dNTP Pool Analysis. We measured mitochondrial dNTP pools from heart, brain, liver and skeletal muscle from both young and old rats as described in Materials and Methods. Values recorded as pmol/mg mitochondrial protein were converted to molar concentrations using the estimated aqueous volume of rat tissue mitochondria. For this purpose, a value of 0.82 µl/mg for rat heart mitochondrial protein was used (Vinnakota and Bassingthwaighte, 2004). This is close to the value of 0.84 that we calculated as an average of values reported for rat heart and liver mitochondria in three earlier reports (Whipps and Halestrap, 1984; Lim *et al*, 2002; Das *et al,* 2003); these earlier values range from 0.67 to 1.04 µl/mg mitochondrial protein). The calculated molar concentrations of

mitochondrial dNTPs (Figure 4.1; Table 4.1) revealed no significant difference in mitochondrial dNTP levels between young and aged rats or between heart subsarcolemmal mitochondria and interfibrillary mitochondria. This result suggests that the age-related decrease of glutaredoxin reductase and oxidized glutathione (GSSG) reductase activities in IFM does not affect mitochondrial dNTP levels. The results further show that mitochondrial dNTP pools in all tissues analyzed are highly asymmetric. Surprisingly, dGTP was the most abundant dNTP present in most tissues. In heart and skeletal muscle mitochondria, dGTP comprises between 85% and 91% of total dNTPs. By contrast, dTTP only accounts for 0.5% of total dNTPs. In brain mitochondria, dGTP comprises about 62% of total dNTPs, whereas dTTP accounts for only about 4% of total dNTPs. In liver mitochondria, dGTP, dCTP, dATP and dTTP account for 37%, 51%, 9% and 3% of total dNTPs respectively. These values contrast sharply with whole-cell measurements that primarily reflect cytosolic dNTP pools that supply nuclear DNA replication. As measured in cultured cells, dGTP usually comprises just 5 to 10% of the total dNTP pool (Mathews and Ji, 1992). The differences in relative dGTP abundance in total versus mitochondrial dNTP pools is not simply due to the use of rat tissues versus cultured cells, because we have found that even in HeLa cells, mitochondrial dGTP accounts for 33% of total dNTPs, while the corresponding value is just 7.6% for total HeLa cell extracts (Song *et al*, 2003), and just 10% in extracts of whole rat embryos (Mole *et al*, 1998).

Table 4.1. Estimated mitochondrial dNTP concentrations in rat tissues.

Source of mitochondria	dNTP concentration (μM)			
	dATP	dTTP	dCTP	dGTP
Young rats				
Heart, subsarcolemmal (3)	3.6 ± 1.0	0.7 ± 0.21	13 ± 4.4	110 ± 48
Heart, interfibrillary (3)	4.0 ± 0.43	0.8 ± 0.46	12 ± 6.6	140 ± 62
Liver (2)	3.8 ± 0.23	1.3 ± 0.87	23 ± 3.9	16 ± 4.4
Brain (3)	11 ± 5.4	3.4 ± 2.1	16 ± 9.1	49 ± 7.4
Skeletal muscle (2)	2.8 ± 1.4	0.27 ± 0.22	5.3 ± 1.8	82 ± 13
Old rats				
Heart, subsarcolemmal (2)	4.9 ± 0.63	0.9 ± 0.23	14 ± 1.0	87 ± 4.1
Heart, interfibrillary (2)	6.1 ± 2.0	1.2 ± 0.43	15 ± 6.4	130 ± 18
Liver (2)	3.3 ± 1.0	1.9 ± 1.6	19 ± 1.1	14 ± 6.3
Brain (1)	7.7	2.5	21	50

dNTP pools were determined as described in Materials and Methods. Values are mean ± S.D. The number of rats used is in parentheses.

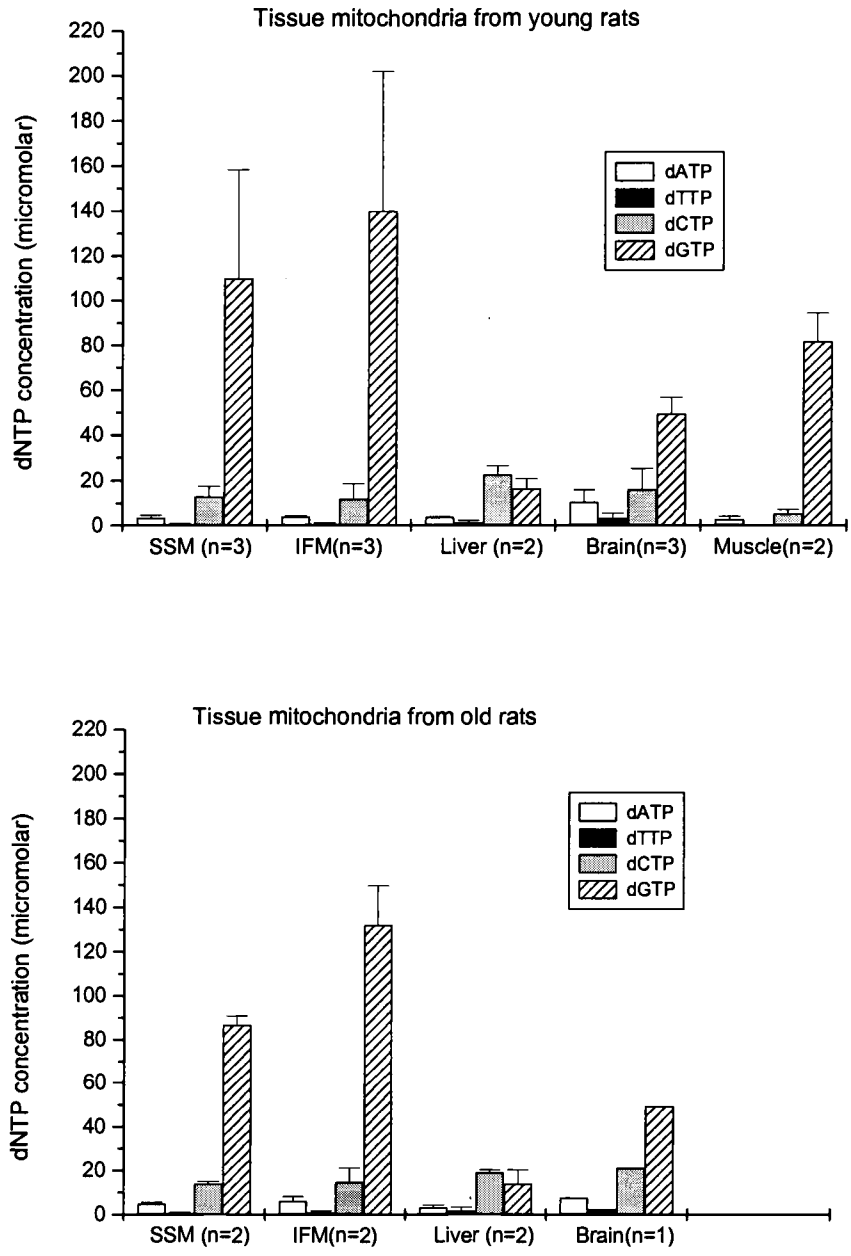

Figure 4.1. Estimated mitochondrial dNTP concentrations in rat tissues. dNTP pools were determined as described in Materials and Methods. Values recorded as pmol/mg mitochondrial protein were converted to molar concentrations using the estimated aqueous volume of rat tissue mitochondria. For this purpose, a value of 0.82 µl/mg for rat heart mitochondrial protein was used (Vinnakota and Bassingthwaighte, 2004). Values are mean ± S.D. The number of rats used is in parentheses.

Pol γ Fidelity with Biased dNTP Pools. The high dGTP concentration in mitochondria and the fact that dGTP is generally more abundant than the other three dNTPs, particularly in heart and skeletal muscle mitochondria, suggests that dNTP asymmetries could be a contributor to the high mutation rate for the mitochondrial genome. To test this idea, we measured the fidelity of human DNA polymerase γ during synthesis *in vitro* to fill a 407-nucleotide gap containing the 275-nucleotide *LacZ* α-complementation gene sequence in M13mp2. We compared results for reactions containing equimolar dNTPs at 1, 10, 100 and 1000 μM versus those containing intramitochondrial dNTP concentrations measured in rat tissues. A forward mutation assay for loss of α-complementation function (blue to light blue and colorless M13 plaques on indicator plates) was used in order to score substitution, deletion and addition errors in a variety of sequence contexts. In order to determine the extent to which the dNTP pools asymmetries affected the nucleotide selectivity of the polymerase and the proofreading efficiency of the intrinsic 3′ exonuclease, fidelity results were obtained for both wild-type (i.e., exonuclease-proficient) and exonuclease-deficient forms of pol γ.

For reaction mixtures containing equimolar dNTPs at concentrations of 1 and 1000 μM, which largely span the range of concentrations in tissues reported in Table 4.1, the products of gap filling reactions by wild-type pol γ yielded *lacZ* mutant frequencies of 7.8×10^{-4} and 11×10^{-4}, respectively (Table 4.2). These values are consistent with previous studies (Longley *et al*, 2001; Johnson and Johnson, 2001; Kunkel and Soni, 1988) indicating that wild type pol γ is an

accurate polymerase. The products of gap filling reaction by exonuclease-deficient pol γ yielded *lacZ* mutant frequencies that were higher, with similar frequencies obtained at equimolar dNTPs concentrations ranging from 1 to 1000 μM (values from 45×10^{-4} to 62×10^{-4}). These values are all greater than for the wild type polymerase due to loss of proofreading activity, thereby reflecting the nucleotide selectivity of the polymerase alone. Importantly, in comparison to results with equimolar dNTP concentrations, the *lacZ* mutant frequencies are a 2-fold to 6-fold higher (Table 4.2) for reactions performed in the presence of dNTP concentrations estimated in heart (SSM and IFM) and skeletal muscle mitochondria. These are the two tissues having the largest dNTP pool asymmetries and the highest dGTP concentrations. The *lacZ* mutant frequencies for reactions performed in the presence of dNTP concentrations estimated in liver and brain mitochondria, which are less asymmetric, are similar to those observed at equimolar dNTPs.

In order to determine which nucleotide changes led to the pool asymmetry-dependent increases in *lacZ* mutation frequencies, we sequenced DNA from independent *lacZ* mutant plaques obtained from reactions performed with equimolar (1 μM) dNTPs and with the three dNTP pool asymmetries observed in mitochondria of rat heart and skeletal muscle tissues. Base substitution errors comprise the majority of errors made by wild type and exonuclease-deficient pol γ for all conditions examined (Table 4.3). Among these, T to C substitutions resulting from stable misincorporation of incoming dGTP opposite template T

were the most common. With equimolar dNTPs, among 45 and 15 total substitutions recovered from wild type and exonuclease-deficient pol γ reactions, respectively, 5 (11%) and 2 (13%) substitutions were T to C. In contrast, in reactions containing the biased dNTP pools, approximately half the base substitutions by exonuclease-deficient pol γ were T to C. These errors are predicted by the excess dGTP present in these reactions. Moreover, in reactions mimicking intramitochondrial dNTP pools in heart mitochondria (both SSM and IFM), 25% of the base substitutions made by wild type pol γ were T to C. This implies that some dG misinsertions opposite template T promoted by the excess dGTP escaped proofreading by this highly accurate polymerase. This interpretation is supported by the observation that among 13 T to C substitutions by the wild type polymerase (Table 4.3, five with SSM conditions, eight with IFM conditions), 10 had a template C 5′ to the T (Table 4.3, column labeled $T_{(C)}$ to C). This sequence context bias in base substitution specificity is predicted by the high dGTP concentrations (110 and 140 μM respectively, for SSM and IFM, Table 4.1), because extension of T-dG mismatches would be promoted by more frequent correct incorporation of dG opposite the next template C, thus reducing proofreading efficiency.

Table 4.2. *lacZ* mutant frequencies for products of Pol γ gap filling.

dNTP Pool Used	Wild-type Pol γ Mutant Freq. ($\times 10^{-4}$)	Relative Freq.	Exo-deficient Pol γ Mutant Freq. ($\times 10^{-4}$)	Relative Freq.
Equimolar dNTP pools:				
1000 μM	11		62	
100 μM	N.D.		56	
10 μM	N.D.		48	
1 μM	7.8	1.0	45	1.0
Intramitochondrial dNTP pools:				
Heart, SSM	23	2.9	160	3.6
Heart, IFM	21	2.7	170	3.8
Liver	≤22	–	42	0.9
Brain	N.D.	–	60	1.3
Skeletal muscle	13	1.7	270	6.0

N.D. means not determined. Relative frequencies were normalized to 1 μM equal dNTP pools for each enzyme. The data for the 1000 μM reaction mixture are from a reference (Longley *et al*, 2001).

Table 4.3. Sequence analysis of *lacZ* mutants.

dNTP Pool	Number of Mutants Observed			
	T to C	$T_{(C)}$ to C	Substitution	Total
Exonuclease-deficient Pol γ:				
Equimolar (1 μM)	5	2	45	70
Heart (SSM)	20	10	27	38
Heart (IFM)	28	16	44	57
Skeletal Muscle	27	14	58	65
Wild type Pol γ:				
Equimolar (1 μM)	2	1	15	15
Heart (SSM)	5	4	21	23
Heart (IFM)	8	6	32	35
Skeletal Muscle	1	0	16	18

* T to C substitution with a C 5′ to the T

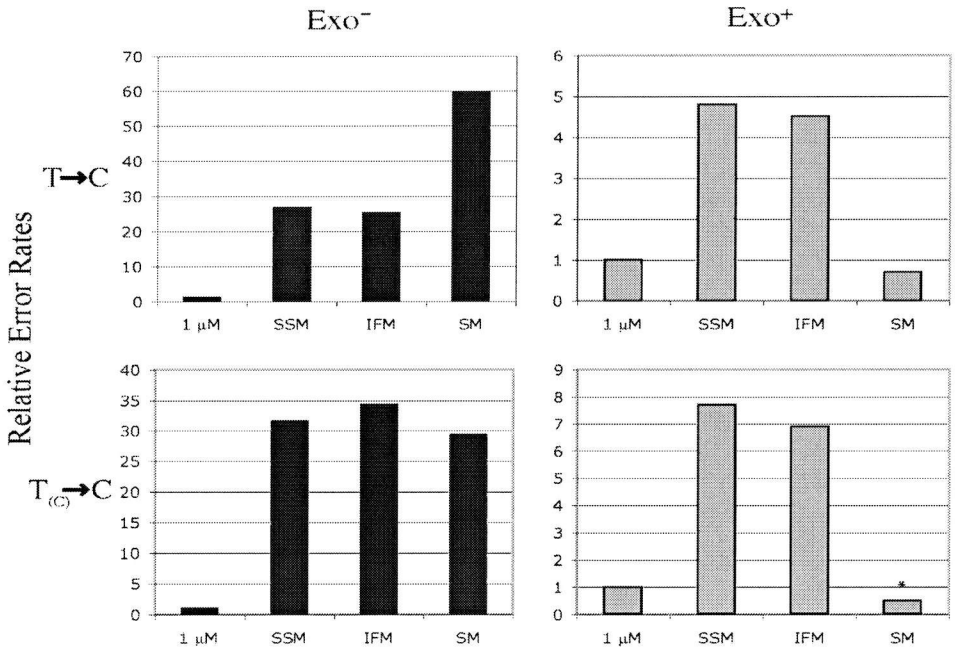

Figure 4.2. Relative error rates for T to C substitutions by Pol γ with varying dNTP pools. Wild type and exonuclease-deficient pol γ were used to fill M13mp2 gapped DNA as described in Materials and Methods, using the dNTP concentrations indicated in Table 4.1. Error rates were calculated from the data in Table 2 and 3 for all T to C changes and for $T_{(C)}$ to C changes for T having template C as a 5′neighbor. The values plotted are rates relative to error rates observed using 1 μM of each of the four dNTPs, which were assigned a value of 1. For exonuclease-deficient pol γ, the actual rates are 2.1×10^{-5} and 3.0×10^{-5} for T to C and $T_{(C)}$ to C errors, respectively. For wild type pol γ, the actual rates are 0.64×10^{-5} and 1.1×10^{-5} for T to C and $T_{(C)}$ to C errors, respectively. The (*) in the lower right panel indicates the theoretical value if at least one mutant plaque had been detected. SSM, subsarcolemmal mitochondria; IFM, interfibrillary mitochondria; SM, skeletal muscle.

To quantify the effects of the pool asymmetries on pol γ nucleotide selectivity and proofreading, we used the mutant frequency (Table 4.2) and sequencing data (Table 4.3) to calculate, as described by Bebenek and Kunkel (1995), the error rates for T to C substitutions for the four dNTP pool conditions. When dNTP pools are balanced and low, the error rates of both wild type and exonuclease-deficient pol γ are low (Fig. 4.2, legend). This is so when considering T to C substitutions at all 27 different detectable template T positions in the *LacZ* template, or when considering only those 8 template Ts flanked by a 5′ template C. In reaction mixtures containing dNTPs at concentrations intended to mimic the approximate 30:1 ratio of dGTP:dATP seen in mitochondria of young rats (Table 4.1), the T to C error rate for exonuclease-deficient pol γ is approximately 30-fold higher (Fig. 4.2, left panels). This is a remarkably close match between the predicted and observed effect on error rate for this specific mismatch when made by a polymerase whose fidelity depends only on nucleotide selectivity. Of potentially greater biological relevance, the dNTP pool imbalances found in heart mitochondria even reduce the fidelity of wild type pol γ (Fig. 4.2, right panels). When all sequence contexts are considered, fidelity is reduced by 4- to 5-fold (upper right), indicating that proofreading is still operational but that some dGMP misinsertions opposite T are not edited. When the $T_{(C)}$ sequence context alone is considered, fidelity is reduced even more by the pool imbalances (7- to 8-fold, lower right), consistent with even less efficient proofreading driven by mismatch extension due to the high concentration of the immediate next correct nucleotide.

Curiously, the fidelity of wild type pol γ remained high using the pool imbalance found in skeletal muscle. This may possibly reflect the slightly lower concentration of each of the four dNTPs for skeletal muscle as opposed to heart mitochondrial pools (Table 4.1), which would less effectively promote mismatch extension at the expense of proofreading.

Additional analysis of pol γ error specificity indicates that next nucleotide effect of dNTP pool asymmetries is not limited to base substitutions. In studies of the large Klenow fragment of *E. coli* DNA polymerase I (a family A homolog of pol γ), dNTP pool imbalances were found to initiate single base deletions that were suggested to result from nucleotide misinsertion followed by strand realignment to create correct termini for extension (Bebenek and Kunkel, 1990). This model can explain single base deletion errors observed in the current study, where biased dNTP pools elevated the rate of single nucleotide deletions by exonuclease-deficient pol γ by 14- to 30-fold over equimolar dNTPs (Table 4.4). These errors include loss of template bases whose 5′ neighbor was a C (as observed for substitution errors), consistent with the high dGTP concentration. As for Klenow polymerase, proofreading by pol γ can apparently edit most of these errors, but a few of these frameshift intermediates appear to have escaped proofreading (Table 4.4, bottom).

Table 4.4. Single base deletions generated by Pol γ.

dNTP Pool	Number of Mutants		Error rate	Fold
	-1_{total}	$-1_{(C)}$*	(x 10^{-4})	difference
Exonuclease-deficient Pol γ:				
Equimolar (1 μM)	10	1	0.19	1.0
Heart (SSM)	5	2	2.6	14
Heart (IFM)	10	5	3.5	18
Skeletal Muscle	4	2	5.7	30
Wild type Pol γ:				
Equimolar (1 μM)	1	1	2.5	1.0
Heart (SSM)	1	1	9.6	3.8
Heart (IFM)	1	1	8.8	3.5
Skeletal Muscle	0	0	–	–

* Single nucleotide deletion where next templating base is C

Discussion

In this study we measured mitochondrial dNTP pools of several tissues from both young and aged rats. The tissue mitochondrial dNTP pools are highly asymmetric and quite distinct from what has been repeatedly been seen in whole cell and nuclear extracts. To date there have been no published data with respect to mitochondrial dNTP levels in tissues. Therefore, we need to establish the validity of our data about the estimated intramitochondrial dNTP concentrations before further discussing the fidelity assay results, which were based on these estimations. In this study, we extracted dNTPs from mitochondria by using a method in common use for extracting dNTPs from cells (Angus *et al*, 2002). We used the DNA polymerase-based assay to measure mitochondrial dNTPs. The DNA polymerase-based assay is a sensitive, reliable and widely used method to measure dNTP pools (Sherman and Fyfe, 1989). We believe that the observed highly asymmetric dNTP pools in mitochondria are unlikely to be artifacts resulting from either the method to extract dNTPs or the method to measure the pools. Besides the issues as we have discussed above, we also need to know whether the measured pool asymmetries resulted from the cytosolic dNTP contamination during the mitochondrial isolation process. The following evidence argues against this notion: First, we measured the specific activity of lactate dehydrogenase (a cytosolic marker enzyme) and mitochondrial respiratory control ratio (RCR) to establish the purity and functional integrity of mitochondrial preparations. Our data indicated that the mitochondria preparations were largely intact and contain

very limited cytosolic contamination. Second, it has been well documented that dGTP is the least abundant dNTP in cultured cells and also in rat embryos (Mathews and Ji, 1992; Mole *et al,* 1998), usually comprising only 5 to 10 % of the total of the four dNTPs. If there is significant contamination of our mitochondria preparations by cytosolic dNTPs, the amounts of the contaminating dNTPs would correspond to the ratios of those dNTPs in cytosolic pools. Therefore, we would expect the amount of the contaminating cytosolic dGTP in mitochondrial dNTP pools would be the least. Based on this evidence, we believe the observed highly asymmetric dNTP pools in mitochondria are unlikely to be experimental artifacts.

The extent of the mitochondrial dNTP pools' asymmetries in rat heart and muscle tissues reported here was unexpected. These asymmetries provide conditions that should strongly increase misinsertion during mitochondrial DNA replication. For example, the 30:1 dGTP:dATP ratio and the 19:1 dCTP:dTTP ratio in heart muscle (SSM) of young rats (Table 4.1) predicts increased formation of T•dGMP and A•dCMP mismatches that, if not corrected by proofreading or DNA repair, would result in A•T to G•C transition mutations. This prediction is confirmed by our *in vitro* studies showing an approximate 30-fold increase in the to T to C error rate for exonuclease-deficient pol γ (Fig. 4.2). This raises the biologically relevant question of whether these mitochondrial dNTP pool asymmetries detectably affect the fidelity of wild type mitochondrial pol γ, whose accuracy is normally strongly enhanced by proofreading. Our *in vitro* data suggest

a positive answer to this question as well, since the dNTP pool asymmetries observed in heart mitochondria reduce the fidelity of wild type pol γ for T to C substitutions by 4- to 8-fold (Fig. 4.2). The *in vitro* data suggest that A•T to G•C transitions should represent a significant proportion of spontaneous mitochondrial mutations *in vivo*. Indeed, Ponamarev *et al* (2002) reported that A•T → G•C transitions were the most common mutation (8 of 14 cases listed in the MitoMap data base) in mtDNA from Progressive External Ophthalmoplegia (PEO) patients. PEO is a heritable mitochondrial disorder characterized by the accumulation of multiple point mutations and large deletions in mtDNA. Recent evidence indicates that autosomal dominant PEO is associated with a mutation in the polymerase γ active site, which could promote error-prone DNA synthesis. In addition, a report by Marin-Garcia *et al* (2001), which summarized published data on specific mtDNA point mutaions in cardiomyopathy, showed A•T → G•C transitions to comprise 62% of total mutations, much more than the abundance of G•C → A•T transitions (19% of total mutations) or of A•T → C•G and G•C → T•A transversions (14% of total mutations). A similar conclusion was reached by Khaidakov *et al* (2003), who surveyed several studies of mtDNA point mutations in mice and found A•T → G•C transitions to account for 55% of the total mutations, with G•C → A•T transitions accounting for only about 36%. The latter could arise either as a result of cytosine deamination to uracil or to preferential dAMP incorporation that could occur opposite an abasic site created as the result of DNA damage. As investigations on this subject continue, it is important to keep

in mind that tissue-specific variations in dNTP pool asymmetries, such as those already seen here (Table 4.1), may preferentially promote different mutational pathways in different tissues.

As noted earlier, mtDNA point mutations have been shown to accumulate with age (Wang *et al*, 2001; Khaidakov *et al*, 2003), and it was of interest to determine whether mitochondrial dNTP pools change with aging in a way that could account for an age-related increase in mitochondrial mutation rate. Our data clearly show no significant aging-related changes in mitochondrial dNTP pools. Whether the mutation rate actually does increase with age is still an open question, but it is also possible that the accumulation of mitochondrial mutations with age is simply the consequence of a spontaneous mutation rate that is high at birth and remains so throughout life.

It is believed that mtDNA is more susceptible to oxidative damage than nuclear DNA. 8-Oxoguanine is the most common oxidized base modification found in mtDNA and it has been shown that age-related accumulation of 8-oxoguanine occurs in the mitochondrial genome (Ames *et al*, 1993). Oxidative DNA damage also occurs at the level of free nucleotides. Oxidized deoxyguanosine nucleotide, 8-oxo-dGMP, is incorporated against adenine in DNA as efficiently as against cytosine during DNA replication, thus causing A to C and T to G transversion mutations (Maki and Sekiguchi, 1992). A recent report by Simon *et al* (2004) showed that the aggregate levels of G/C to T/A and T/A to G/C transversions increase with age in the frontal cortex of human brain. Since we

found substantial amounts of dGTP in heart, skeletal muscle and brain mitochondria, this raises the possibility that all of that dGTP creates a large target for oxidation, and that mitochondria may have substantial 8-oxo-dGTP pools, which could be incorporated in mtDNA, subsequently resulting in A to C and T to G transversion mutations in the mitochondrial genome. Therefore, analysis of the 8-oxo-dGTP pools in tissue mitochondria could provide insight into mtDNA mutations.

The dGTP excess over the other dNTPs in these tissues is so high as to suggest the possibility of metabolic roles other than its function as a DNA precursor. The literature on non-DNA-related functions of dNTPs is sparse. There is one report (Angelastro and Purich, 1992) of dGTP accumulation in the microtubule cytoskeleton of neuronal cells cultured with nerve growth factor, but no biological function for this dGTP was proposed. Also, dATP and cytochrome c released from mitochondria have been proposed to activate a chain of caspase reactions in an apoptotic cascade (Li *et al*, 1997). More recently, dATP was reported to be much more effective than ATP in stimulating contraction of rat heart muscle (Regnier *et al*, 2000). These results and the present study suggest possible metabolic functions of dNTPs other than as DNA precursors.

In summary, we find that normal intramitochondrial dNTP pools in rat tissues are highly asymmetric, and *in vitro* fidelity studies show that these imbalanced pools can stimulate base substitution and frameshift mutations, with a substitution pattern that correlates with mitochondrial substitution mutations *in vivo*. These findings suggest that normal intramitochondrial dNTP pool

asymmetries could contribute to mitochondrial mutagenesis and possibly to the origins of mitochondrial diseases.

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Chapter 5

Effects of Mutations Affecting Deoxyribonucleotide Carrier Upon Mitochondrial Nucleoside Triphosphate Pools

Shiwei Song and Christopher K. Mathews

This study is part of a collaboration with Dr. Leslie G. Biesecker's laboratory in the National Human Genome Research Institute, NIH.

Introduction

Amish lethal microcephaly (MCPHA) results in severe microcephaly, increased urinary α-ketoglutarate excretion, and premature death. The disorder is inherited in an autosomal recessive pattern and has been observed only in Old Order Amish families whose ancestors lived in Lancaster County, Pennsylvania (Kelley *et al*, 2002). Through whole-genome scanning and fine-scale genetic mapping, Rosenberg *et al* (2002) identified a missense mutation in the *SLC25A19* gene encoding a mitochondrial membrane deoxynucleotide carrier (DNC), which segregates with the disease and alters a highly conserved amino acid of DNC protein. The DNC protein is proposed to transport deoxynucleotides from the cytosol into the mitochondrial matrix in exchange for ATP (Dolce *et al*, 2001). Functional analysis of the mutant DNC protein using an *in vitro* transport assay revealed that nucleotide transport activity was lost (Rosenberg *et al*, 2002). Those authors proposed that insufficient transport of dNTPs into mitochondria in the developing central nervous system interferes with synthesis of mitochondrial DNA, causing abnormal brain growth.

Dolce *et al* (2001) cloned and expressed a human cDNA encoding mitochondrial deoxynucleotide carrier, which was described as a member of the family of mitochondrial carriers. The protein showed about 22% sequence identity with mammalian adenine nucleotide transporters. As the recombinant protein was reconstituted into proteoliposomes, this system was found to transport deoxyribonucleoside diphophates (dNDPs) most efficiently, followed closely by

ribonucleoside diphophates (rNDPs). Although the protein was shown to have broad distribution within human and mouse tissues, the authors did not demonstrate that the protein is located within mitochondria in any tissue. The authors proposed that the cytosolic dNTPs could be dephosphorylated and then the corresponding dNDPs could be taken up by the DNC and converted to dNTPs within the mitochondrion.

To understand how loss of function of this protein results in MCPHA, Lindhurst *et al* (Personal communication) have created a knockout mouse that has lost three exons of the *Dnc* gene. Homozygous mutant embryos fail to develop past 11.5 days p.c. Mutant embryos are small and most fail to complete neural tube closure in the mid and hindbrain portion of the embryo, resulting in disorganization of developing brain structures. The neural folds have ruffled edges with convolutions extending into the neural tube of the trunk and tail. In addition, 10.5-day mutant embryos had few circulating erythrocytes, indicating that yolk sac erythropoiesis requires DNC. Preliminary functional analyses of mitochondria from cultured cells isolated from 10.5-day embryos were unremarkable. No evidence of decreased mitochondrial DNA levels has been found. Immunohistochemistry for both mitochondrial HSP70 and subunit I of the cytochrome c oxidase complex was normal. These data raise a question whether the DNC protein really acts as a nucleotide transporter *in vivo*. To address this issue, we measured mitochondrial dNTP pools of embryonic cells from *Dnc*

knockout mice. We also measured mitochondrial dNTP pools of lymphoblasts from an MCPHA patient.

Experimental Procedures

Cell culture — Homozygous mutant cell line S-3H-F2-4 was isolated from the *Dnc* gene knockout mouse embryos and cell line S-1J-F2-9 was isolated from wild-type mouse embryos. Both cell lines were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SH6-3, SH8-3 and SH9-3 are lymphoblast cell lines from Amish lethal microcephaly patients with a homozygous missense point mutation in the *Dnc* gene. The control lymphoblast lines came from anonymous donors. Lymphoblasts were grown in RPMI media supplemented with 10% FBS. All the cell lines used in this study were obtained from Dr. Leslie G. Biesecker's laboratory in the National Human Genome Research Institute, NIH.

Mitochondrial nucleotide pool extraction — Mitochondria were prepared by a modification of the methods of Trounce *et al* (1996). Briefly, mouse embryonic cells for each cell line were harvested from twenty 150-mm tissue culture dishes by trypsin treatment and human lymphoblasts for each cell line were collected from suspension culture in five T100 flasks. After washing twice in ice-cold PBS, the cell pellet was resuspended in 3 ml cold isolation buffer, consisting of 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2, 1 mM EGTA, and 0.5% bovine serum albumin (BSA, fatty acid-free). Then 10% digitonin solution was added to the suspension to a final concentration of 0.3 mg/ml. After about 4 to 5 min

incubation on ice, the suspension was diluted by adding 20 ml of isolation buffer, and cells were pelleted by centrifugation. Then the cell pellet was resuspended in 5 ml of isolation buffer and the cells were disrupted with a Dounce homogenizer until about 80% to 90 % of the cells were broken. The mitochondrial fraction was isolated by differential centrifugation and subsequently washed twice and resuspended in 1 ml of isolation buffer without BSA. Immediately after that, most of the mitochondrial suspension was centrifuged to pellet mitochondria and the remaining suspension was saved for later mitochondrial protein determination. The mitochondrial pellet was immediately resuspended in 1 ml of ice-cold 60% methanol and incubated at –20 °C for 1 h. The suspension was heated for 3 min in a boiling water bath, followed by centrifugation for 15 min at 17,000 x g. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and stored at –20 °C for later analysis. Protein concentration was determined by the Bradford method.

dNTP pool analysis — Analysis of the dNTP pools in each extract was based upon the method of Sherman and Fyfe (1989). Reaction mixtures (50 µl) contained 100 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 units of *Escherichia coli* DNA polymerase I Klenow fragment (U.S. Biochemical), 0.25 µM oligonucleotide template, and 1 µCi [^{3}H]dATP (Amersham Biosciences) or [^{3}H]dTTP (Perkin Elmer Life Sciences). Incubation was carried out for 45 min at 37 °C.

rNTP pool analysis — The rNTP pools were determined by high-performance liquid chromatography (HPLC) by using a HP1050 system with

detection at 260 nm. The extracts were injected into an Altex Partisil 10 SAX ion exchange column equlilibrated in 75 mM ammonium phosphate, pH 3.6. rNTPs were eluted with a gradient of ammonium phosphate buffer ranging in concentration from 0.075 M to 1.0 M (pH 3.6) at a flow rate of 1.2 ml/min. The ribonucleotides were identified on the basis of retention times and quantitated on the basis of peak area by comparison to authentic standards.

Results and Discussion

We first measured mitochondrial dNTP pools in fibroblast-like cells isolated from both wild-type mouse embryos and from homozygous *Dnc* mutant mouse embryos. As shown in Table 5.1, there is no significant difference in mitochondrial dNTP pool levels between wild-type and homozygous mutant cells except for a less than two-fold increase in mitochondrial dTTP level in mutant cells. It also showed that mitochondrial dNTP pools in mouse embryonic cells were highly asymmetric and dGTP accounted for 54% and 48% of total mitochondrial dNTP pools in wild-type and mutant cell lines, respectively. This result is consistent with our previous observation with regard to normal intramitochondrial asymmetries seen in rat tissues (Chapter 4).

Table 5.1

Mouse embryonic cell mitochondrial dNTP pool size
(pmol/mg mitochondrial protein)

Cell lines	dTTP	dATP	dCTP	dGTP
S-1J-F2-9 (*Dnc* +/+)	2.86 ± 1.20	2.98 ± 1.00	4.96 ± 0.19	12.84 ± 0.75
S-3H-F2-4 (*Dnc* -/-)	5.52 ± 0.53	3.18 ± 0.16	6.40 ± 0.65	13.85 ± 0.12

dNTP pools were determined as described in the Experimental Procedures. Values are expressed as mean ± SD of two separate experiments in duplicate.

To confirm our results seen in mouse embryonic cells, we also measured mitochondrial dNTP pools in both lymphoblasts (SH6-3 and SH9-3) from MCPHA patients and control lymphoblasts from anonymous donors. As shown in Table 5.2, there is no significant difference in mitochondrial dNTP levels between patient lymphoblasts and control lymphoblasts except for a slight increase in mitochondrial dTTP level in MCPHA patient lymphoblasts. The present data with respect to the mitochondrial dNTP pools in human lymphoblasts are consistent with previously reported results regarding the mitochondrial dNTP levels in HeLa cells (Song *et al*, 2003).

Table 5.2

Human lymphoblast mitochondrial dNTP pool size
(pmol/mg mitochondrial protein)

Cell lines	dTTP	dATP	dCTP	dGTP
MCPHA	19.5 ± 4.5	4.9 ± 0.8	7.0 ± 1.7	18 ± 1.7
Control	14.6 ± 1.8	4.4 ± 0.9	5.9 ± 0.7	20.2 ± 2.5

dNTP pools were determined as described in the Experimental Procedures. The values in the cell line of MCPHA are the average of the values of two cell lines (SH6-3, SH9-3) from MCPHA patients. The values in Control cell line are the average of the values of two control cell lines from anonymous donors.

Since it has been shown that ribonucleoside diphosphates (rNDPs) are also good substrates for DNC *in vitro*, this raises the possibility that DNC may act mainly as a mitochondrial transporter for rNDPs, which could be converted to triphosphates within the organelle by nucleoside diphosphate kinase. Hence, we measured mitochondrial rNTP levels in lymphoblasts from both MCPHA patients and normal controls. Because the HPLC/UV detection that we use for rNTPs is much less sensitive than the enzymatic method we use for dNTPs, with one-liter cultures of the lymphoblast cell lines, the rNTPs extracted from mitochondria generated ATP and GTP peaks large enough to quantitate, but UTP and CTP pools too small to measure with any reliability. As shown in Table 5.3, there is no significant difference in either mitochondrial ATP levels or GTP levels between patient lymphoblasts and control lymphoblasts.

Table 5.3

Human lymphoblast mitochondrial rNTP pool size
(nmol/mg mitochondrial protein)

Cell lines	CTP	UTP	ATP	GTP
Control	ND	ND	2.83 ± 0.38	1.27± 0.01
MCPHA	ND	ND	2.69 ± 1.19	1.23 ± 0.18

rNTP pools were determined as described in the Experimental Procedures.
ND = undetectable or less than 0.5 nmol/ mg protein. The values in the cell line of MCPHA are the average of the values of two cell lines (SH8-3, SH9-3) from MCPHA patients. The values in control cell line are the average of the values of two control cell lines from anonymous donors.

To summarize, our data demonstrated that there are no significant changes of both intramitochondrial dNTP levels in both MCPHA patients' lymphoblasts with a missense point mutation in the *Dnc* gene and the homozygous mutant cells extracted from *Dnc* gene knockout mouse embryos. And there is no significant difference in lymphoblast mitochondrial rNTP levels between MCPHA patients and controls. These results suggest that the DNC protein does not play a crucial role in the maintenance of intramitochondrial nucleoside triphosphate pools and that Amish lethal microcephaly does not result from the depletion of mitochondrial dNTP pools as previously proposed (Rosenberg *et al*, 2002). Interestingly, a recent study showed that down- regulation of DNC by small interfering RNA did not cause mtDNA depletion or affect the rate of TTP uptake into isolated mitochondria (Lam *et al*, 2005). Those results are consistent with our observation. Thus, further investigation with respect to the physiological role of the DNC protein is needed.

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Chapter 6

General Conclusion

Shiwei Song

Mitochondria are essential organelles found in the cells of most eukaryotes. Mitochondria contain their own genomes. It is well known that the mitochondrial genome has a much higher spontaneous mutation rate than the nuclear genome. mtDNA mutations have been identified in association with many diseases and aging.

mtDNA replication continues throughout the cell cycle, even in post-mitotic cells. Therefore, a constant supply of nucleotides is required for replication and maintenance of the mitochondrial genome. However, it is not clear how dNTPs arise within mitochondria nor how mitochondrial dNTP pools are regulated. Recent evidence suggests that abnormal mitochondrial nucleoside and nucleotide metabolism is associated with several human diseases. Clearly, to uncover the pathogenesis of these diseases and the mechanisms of mitochondrial mutagenesis, information is needed regarding dNTP biosynthesis and maintenance within mitochondria, and biochemical consequences of disordered mitochondrial dNTP metabolism.

My first project was focused on ribonucleotide reductase, a key enzyme in the *de novo* dNTP biosynthetic pathways. I found that mammalian liver mitochondria contain a distinctive form of ribonucleotide reductase that does not represent cytosolic contamination. Physical and immunological properties suggest the mitochondrial form of ribonucleotide reductase is different from the major cytosolic form of this enzyme. Current evidence suggests that dNTPs could arise within mitochondria from several different pathways. However, there is still

uncertainty regarding the relative contribution of each pathway and the regulation of intramitochondrial dNTP pools. Our data (Chapter 5) indicate that directly importing deoxyribonucleotides from cytosol through the DNC does not play a crucial role in the maintenance of intramitochondrial dNTP pools. The presence of both ribonucleotide reductase and thymidylate synthase in mitochondria indicates that *de novo* pathways for dNTP synthesis are active within mitochondria. It seems clear that the salvage pathway is also active in mitochondria, because of the presence of deoxyribonucleoside kinases in mitochondria and the mitochondrial toxicity of several antiviral nucleoside analogs. Our data also show that quite different dNTP pools are present in different tissues and different cell lines, suggesting that those different pathways might function to different extents in different mitochondrial populations.

My second project was focused on investigating the pathogenesis of a mitochondrial disease — mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). MNGIE is caused by loss-of-function mutations in the nuclear gene encoding thymidine phosphorylase and is associated with mtDNA deletions and point mutations. It has been hypothesized that increased levels of thymidine resulted from defective thymidine phosphorylase cause mitochondrial nucleotide pool imbalances, which, in turn, lead to mitochondrial mutagenesis. We tested this hypothesis by culturing HeLa cells in medium supplemented with thymidine and observing the effects upon dNTP pools within mitochondria and upon the generation of mtDNA deletions. We found that (1) Thymidine treatment could

cause mitochondrial dNTP pool imbalance. (2) Long term thymidine treatment could induce mtDNA deletions. (3) The mitochondrial dNTP pool changes resulting from thymidine treatment could account for the spectrum of mtDNA point mutations found in MNGIE patients.

My third project was focused on the study of the relationship between mitochondrial dNTP pools and mitochondrial mutagenesis. It is known that the mutation rate of the mammalian mitochondrial genome is higher than that of the nuclear genome. Because mitochondrial and nuclear dNTP pools are physically distinct and because dNTP concentrations influence replication fidelity, we asked whether asymmetry in mitochondrial dNTP pools might affect mutagenesis within the mitochondrial genome. Indeed, we found dNTP pools in mitochondria of several rat tissues to be highly asymmetric. Most striking was the high level of dGTP, which in heart and skeletal muscle comprises nearly 90 percent of total mitochondrial dNTP. Our *in vitro* fidelity assays show that these imbalanced pools can stimulate base substitution and frameshift mutations, with a pattern that corresponds to patterns of mitochondrial mutations *in vivo*. These findings suggest that normal intramitochondrial dNTP pool asymmetries may contribute to spontaneous mutagenesis in the mammalian mitochondrial genome.

My last project was focused on investigating the pathogenesis of Amish lethal microcephaly (MCPHA). It has been proposed that the disease is caused by insufficient transport of dNTPs into mitochondria due to a loss-of-function mutation in the gene encoding a mitochondrial deoxynucleotide carrier (DNC). We

found that there are no significant changes of intramitochondrial dNTP levels in either a MCPHA patient's lymphoblasts with a missense point mutation in the *Dnc* gene or the homozygous mutant cells extracted from *Dnc* gene knockout mouse embryos. Our data do not support the proposed pathogenesis for this disease.

My thesis study not only provides some insights into the metabolic sources of dNTPs within mitochondria and the relations of mitochondrial dNTP pools to mitochondrial mutagenesis and human diseases, it also raises some important questions for future investigation.

First, it is important to further purify and characterize the intramitochondrial form of ribonucleotide reductase, to identify its genetic origin, to describe its mode of regulation and identify its role in mitochondrial dNTP synthesis. Purifying this enzyme is very difficult because the activities of this enzyme are completely lost during fractionation steps carried out so far, probably due to the instability of the enzyme and possible separation of enzyme subunits. It might be worthwhile to try some genomic approaches, but it could be challenging, because there exist at least three classes of RNRs with completely different primary sequences.

Second, it is believed that mtDNA is more susceptible to oxidative damage than nuclear DNA and that 8-oxoguanine is the most common oxidized base modification found in mtDNA. Because oxidative DNA damage may also occur at the level of free nucleotides, and because mitochondria have substantial amounts of dGTP, this raises the possibility that high intramitochondrial dGTP pools create a large target for oxidation. Therefore, analyzing the 8-oxo-dGTP pools in

mitochondria could provide information regarding mitochondrial mutagenesis.

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Appendices

Appendix A

Analysis of an unidentified sequence similar to the human ribonucleotide reductase large subunit

Ribonucleotide reductase (RNR) catalyzes the first metabolic reaction committed to DNA synthesis, by reducing each of the four ribonucleotide substrates to the corresponding deoxyribonucleotides. RNRs characterized to date include three major forms. Although the three classes of RNRs differed significantly in their primary sequences, within each class the enzymes show clear amino acid sequence homologies. The aerobic form of *E. coli* RNR and all known mammalian RNRs belong to the same class enzyme (type I enzyme). By searching the National Center for Biotechnology Information (NCBI) databases with several homologous sequences of RNR large subunit (R1) from mouse, human and the aerobic form enzyme of *E. coli*, an unidentified sequence (Figure A1) in the database for human Expressed Sequence Tags was found. This unidentified sequence (GenBank accession number: BQ935605) is highly similar to the sequence of the human ribonucleotide reductase large subunit (R1). Because the source of this cDNA sequence is a human carcinoma cell line, I tried to clone the putative mitochondrial R1 by reverse transcriptase PCR (RT – PCR) using human colon tumor total RNA (Clontech) as a template. The forward and reverse primers are 5'-ATGCATGTGATCAAGCGAGATGG and 5'-CAGCCACTTTTCCATTGATCTTC, respectively. And the RT-PCR product is expected to be 493 bp in length, corresponding to the unknown sequence at

positions 271 to 763 or the human R1 sequence at positions 233 to 725. As shown in Figure A 2 (Lane 3), The RT-PCR product is about 500 bp in length, which is matched to the expected size. Since the unknown sequence is highly similar to that of human R1, the PCR product could be either the unknown sequence and/or human R1. To uncover the nature of the PCR product, restriction digestion analysis was performed on the PCR product. The restriction enzyme Bgl I does not cut human R1, but is supposed to cut the unknown sequence at position 499 generating a 264 bp product and a 230 bp product; The restriction enzyme Hind III does not cut the unknown sequence, and is supposed to cut human R1 at position 302 generating a 423 bp product and a 70 bp product. As shown in Figure A 2, Bgl I did not cut the PCR product (Lane 2), but Hind III cut the PCR product into smaller bands with right size (lane 4). This result suggested that the RT-PCR product is a partial sequence of human R1. Thus, continued effort to clone the unknown sequence is needed.

SEQUENCE:

GTCCGGAATTCCCGGGATGTCAAGAACGTCATTCGAATTCCGTCCGTCG
CGTTGCTCTGCACGTCACGGGTGGCGGGAGCGGGAAGGAGTTCGTAAT
TCGGTTAGTCTGCTCTGGTGAGGAAAGTGCTGTCTATCGCGCAGCTTCC
ATCCCTCCGTCCGAGCAGCCTCTCGGAGTCCAACCCTTCACATCTGACA
GTCGTCTCTGTCCCTTCTTCGCCTCGGAGCTGCTAACTGGTCTCGAACC
TCTCAGCACTTCAGCTTCTAGCGGCGATGCATGTGATCAAGCGAGATG
GCCGCCAAGAGCGAGTTATGTTTGACAAAATTACATCACGAATCCAGA
AACTCTGTTATGGACTCAACATGGACTTTGTTGATCCTGCTCAGATCAC
CATGAAAGTAATCCAAGGCCTATATAGTGGGGTCACCACAGTGGAACT
GGACACCCTGGCTGCTGAGACAGCCGCGACCTTGACCACGAAGCACCC
TGACTATGCCATCCTGGCAGCAAGGATAGCCGTCTCTAACTTGCACAA
AGAAACAAAGAAAGTGTTCAGTGATGTGATGGAGGATCTCTACAACTA
CATAAATCCGCACAACGGCAGACACTCTCCCATGGTGGCCAGCTCAAC
ACTCGACATTGTTATGGCCAATAAGGATCGCCTGAATTCTGCCATTATC
TATGACCGAGATTTCTCTTATAACTACTTTGGCTTTAAGACACTGGAAC
GGTCATATTTGTTGAAGATCAATGGTAAAGTGGCTGAAAGAACACAGC
ATATGTTGATNGAGGGTTTCTGTGNGGGATTCACAAAGAAGATATTGA
TGCTGCAATTGGAACCTACAACCTACTTTTCTGAGGAGTGGTTCACTCA
TGCCTCTCCTACTCTCTTCAATGCTGGGGACAACCCGCCACAGGCTGTC
TAGCTGTTTCCTCTTGNAGTATGAAAGATGACAGGCATTGGA

Figure A 1. An unidentified sequence highly similar to the human ribonucleotide reductase large subunit. dbEST Id: 13214770; GenBank Acc: BQ935605; EST name: AGENCOURT_8802625; GenBank gi: 22350988.

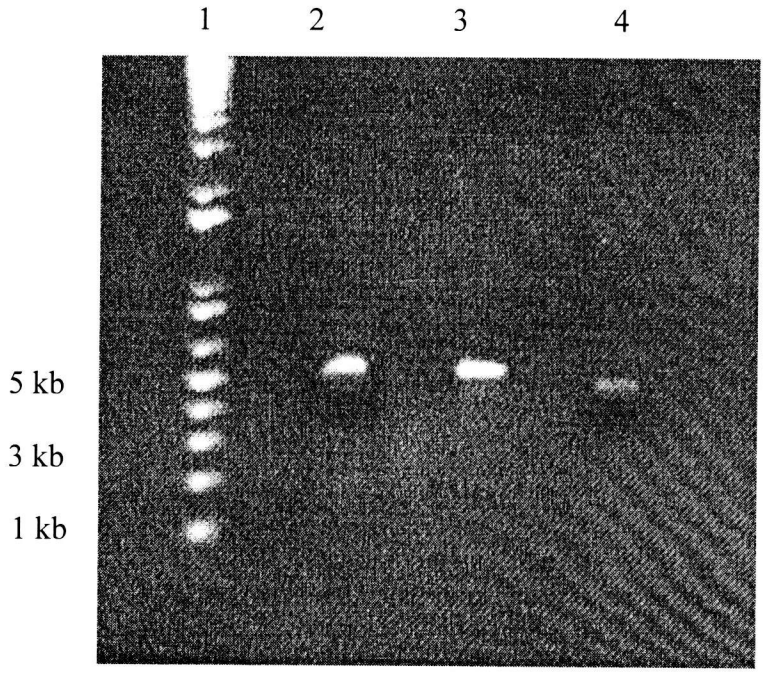

Figure A 2. Restriction digestion analysis of the RT-PCR product.
Lane 1, DNA markers; Lane 2, digestion of the RT-PCR product with restriction enzyme Bgl I; Lane 3, the RT-PCR product without restriction enzyme treatment; Lane 4, digestion of the RT-PCR product with restriction enzyme Hind III.