AN ABSTRACT OF THE THESIS OF

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Dr. Christopher Mathews

One of the distinctive features of mitochondria is that they have their own DNA. When compared to nuclear DNA, it is known that mitochondrial DNA (mtDNA) not only has a much higher rate of spontaneous mutation, but also arises from metabolically distinct precursor pools. Others from our laboratory have demonstrated that abnormalities in DNA precursor pools (dNTPs) can be mutagenic, and that mitochondrial dNTP pools can be highly asymmetric with respect to their genomes. However, at present, neither the metabolic source of mitochondrial dNTPs nor the mechanisms of their regulation have been identified.

In all cells, ribonucleotide reductases (RNRs) are the enzymes that synthesize the four deoxyribonucleotides required for DNA synthesis. Through their role in the biosynthesis of deoxyribonucleoside triphosphates (dNTPs), RNRs play a crucial role in the regulation of their accumulation. At present, the existence of a mitochondrial RNR (mtRNR) in the mitochondrial matrix is intensely debated.

In this study, we show that mitochondria in a variety of tissues contain RNR activity. We compared mtRNR activity to cytoslic RNR activity in the presence of inhibitors and found that the activities were different. Given that mtRNR activity was several-fold higher than cytosolic RNR activity, it was unlikely that the mitochondrial activity was the

result of a cytosolic contamination. Although unfinished at present, we have also made progress in attempts to isolate the putative mtRNR gene. Thus, our present results suggest that there is indeed a form of ribonucleotide reductase associated with the mammalian mitochondrion that is enzymatically distinct from the major cytosolic form of this enzyme.

Keywords: Ribonucleotide Reductase, Mitochondria, Deoxyribonucleotides
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The Search for

Mitochondrial Ribonucleotide Reductase

by

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Daniel Helin Bai, Author

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To my parents, Jia Zhu Yang and Barry Bai, I thank you both very much for your love and support, for you were my inspiration on those long tedious nights of research.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
dGK	Deoxyguanosine kinase
dNDP	Deoxyribonucleoside diphosphate
dNMP	Deoxyribonucleoside monophosphate
dNTP	Deoxyribonucleoside triphosphate
EGTA	Ethylene glycol tetraacetic acid (chelating agent)
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer
MDDM	Mitochondrial DNA depletion myopathy
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MOPS	(N-morpholino) propanesulfonic acid buffer
NdR	Deoxyribonucleoside
mtDNA	Mitochondrial DNA
mtRNR	Mitochondrial ribonucleotide reductase
p53R2	p53-dependent RNR R2 subunit
PCR	Polymerase chain reaction
PEO -	Progressive external ophthalmoplegia
Pol γ	Polymerase γ
RNR	Ribonucleotide reductase
rNDP	Ribonucleoside diphosphate
TLC	Thin layer chromatography
TK2	Thymidine kinase 2

INTRODUCTION

Deoxyribonucleotides are the building blocks of DNA. In cells, the four deoxyribonucleoside triphosphate (dNTP) pools (dATP, dTTP, dCTP, and dGTP) are strictly regulated because genome-disproportionate dNTP pools can be highly mutagenic [Mathews, 2006].

Ribonucleotide reductases (RNRs) are enzymes that regulate dNTP pools through participating in their biosynthesis. The purpose of this thesis is to present evidence for a novel RNR that operates independently from the cytosol, i.e. a mitochondrial RNR (mtRNR).

Mitochondria, also known as the powerhouses of eukaryotic cells, are organelles which contain genomes of their own independent of the nucleus. These relatively small genomes have mutation rates two orders of magnitude greater than nuclear genomes [Vigilant, 1991]. Others in our laboratory have shown that mitochondria contain highly asymmetric dNTP pools with respect to their genomic composition [Mathews and Song, 2007]. It is possible that these highly unbalanced dNTP pools are what contribute to the high mutation rates. Through characterizing mtRNR, we hope to gain insights into how dNTP pools arise and how they are regulated in the mitochondrial matrix. Through better understanding how dNTP imbalances contribute to mitochondrial mutations, we hope to provide pharmaceutical researchers with the knowledge necessary to combat mitochondrial diseases.

RIBONUCLEOTIDE REDUCTASES EXIST IN ALL LIVING CELLS

All RNRs synthesize deoxyribonucleotides through reducing the 2'-hydroxyls on ribonucleotides to hydrogen atoms (Fig. 1).

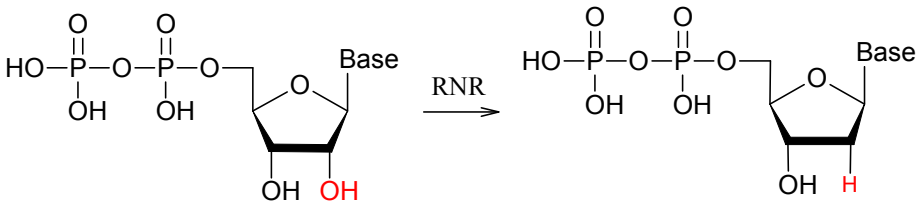

Figure 1. The above figure illustrates the reduction of an rNDP to a dNDP, which can be catalyzed by either class I or II RNRs. Class III enzymes however, act on rNTPs.

At present, RNRs are grouped into three classes. Each class has its own unique mechanism for radical generation as well as distinctive structural features [Thelander and Reichard, 1979].

Class I RNRs are considered to be the most abundant in that they are found in the greatest number of biological domains. To date, class I RNR genes have been sequenced in: eukaryotes, eubacteria, bacteriophages, and viruses. The class I enzymes are unique in that their catalytic activity strongly depends on oxygen. From solved x-ray crystallographic structures, we know that these aerobic enzymes are each composed of an $\alpha_2\beta_2$ heterodimer. The large α chain harbors both binding sites for allosteric effectors and the catalytic site. The small β chain contains an oxygen-linked diferric center which when active, generates a stable tyrosyl free radical essential for catalytic function [Jordan et al, 1998].

Class II RNRs are considered to be oxygen indifferent. Isoforms of class II genes have been found in both aerobic and anaerobic organisms, which include: eubacteria, archaebacteria, and bacteriophages. These enzymes are structurally simpler than their

class I relatives in that they are composed of only α subunits. In terms of catalytic activity, the class II enzymes are unique because they require an adenosylcobalamin moiety for radical generation [Jordan et al, 1998].

Class III RNRs share many common features with their class I and II relatives. They are similar to class II RNRs in that they are found in the same biological domains. Structurally, they are similar to class III enzymes in that they are also composed of $\alpha_2\beta_2$ heterodimers. However, their catalytic activity is unique in that they are strictly anaerobic enzymes which use ribonucleoside triphosphates rather than diphosphates as substrates [Jordan et al, 1998].

Now that we have been introduced to the three classifications of RNRs, we will explore an RNR that is not well understood, a mitochondrial RNR. Given such an RNR would function in oxygen rich environments, we will provide rationale in the later sections for why a mitochondrial RNR would likely resemble class I and II RNRs.

IMPORTANCE OF MITOCHONDRIA

Mitochondria play an indispensable role in mammalian metabolism. In addition to providing over 90% of the body's energy needs in the form of ATP, mitochondria also function in the regulation of cellular membrane potentials, proliferation and apoptosis, heme synthesis, steroid synthesis, and detoxification [Scheffler, 2000]. Today, we know that mitochondrial defects are associated with a variety of diseases. In addition to causing congenital defects in children, mitochondrial abnormalities are also correlated with: aging, type 2 diabetes, Parkinson's, Alzheimer's, atherosclerotic heart disease, stroke, and cancer [Wallace, 1999; Penta et al, 2001].

MITOCHONDRIAL DISEASES

Every fifteen minutes in the United States, a child is born who will develop a mitochondrial disease before the age of 10. [UMDF, 2008] Since mitochondrial diseases are often misdiagnosed as seizures, cerebral palsies, atypical pediatric and gerontological diseases, the exact number of worldwide sufferers is unknown. Recent studies estimate that 1 in 4000 infants in the United States each year are born with a mitochondrial disease. [Cleveland clinic, 2008]. As pediatric-diagnosing techniques improve, we now know that the disease is approaching the frequency of childhood cancers [UMDF, 2008].

Although mitochondria have relatively small (16 kilobase) genomes which code for only 13 proteins, their genetic diseases have proven to be difficult to diagnose. This is partly because most mitochondrial proteins are encoded on various nuclear chromosomes [Wallace, 1999]. For people at high risk for mitochondrial mutational diseases, preventive diagnoses have been especially difficult to perform. At present, a preventive diagnosis not only involves screening a patient's mitochondrial genome but also their

nuclear genome, and given that not all of the mitochondrial proteins have been located on the human nuclear genome, performing such tests has been difficult [Chinnery, 2008].

During cellular replication, not all of the mitochondria within a cell will be evenly distributed amongst mother and daughter cells. Thus, when pre-natal exams are performed, not all of the cells collected may have defective mitochondria. After fertilization, an abnormal maternal mitochondrion may divide only within particular cells and go undetected until birth [Holt *et al.*, 2000]. However, through understanding mtRNR abnormalities, we can make diagnoses for mitochondrial dNTP pool-associated disorders easier to perform.

Julie Manley, the mother of 5 year old Jude Manley tells of her son's experiences with mitochondrial OXPHOS disease:

> "People don't see the disease. The organs are what get damaged not necessarily the looks... [So], kids can look completely normal. After Jude was born, he had eating problems and he had developmental delays. He was anemic, he vomited frequently, he always had low muscle tone, and he ended up needing tube feedings. In August of 2006, we got a report that said he had a mitochondrial disorder and all of the other problems that he had experienced with his brain, his stomach, and his muscles stemmed from the mitochondrial disorder." [Save Jude.com]

Although defective mitochondria have been associated with complications in hundreds of diseases, this discussion will only address diseases which arise directly from abnormalities in mitochondrial dNTP pools. Since not much is known about mitochondrial dNTP metabolism, only a handful of such diseases have been documented [Rampazzo, 2000; Taylor, 2005].

MECHANISMS OF MUTAGENESIS FROM dNTP POOL IMBALANCES

Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE) is a mitochondrial mutational disease which involves the accumulation of both point mutations and large-scale DNA deletions. As such, it has been a model for studying both mutagenic mechanisms. When Hirano *et al.* in 2001 reported that a large number of MNGIE patients had abnormally high circulating plasma thymidine, others in our laboratory began investigating the mutagenic mechanisms.

To study the effects of dNTP pool imbalances on large-scale mitochondrial DNA deletion, Song *et al.* from our laboratory cultured HeLa cells in a medium containing 50μM thymidine [Song, 2005]. Over a period of eight months, the mtDNA was isolated at intervals and checked for deletions via PCR and Southern blotting. After eight months, it was observed that the mtDNA had undergone kilobase-pair-long deletions. A "stalling-DNA-polymerase" mechanism was proposed in which Pol γ would stall due to the limitation of one or more dNTPs, allowing for the 3' sequence to fray by partial unwinding. The separated strand would then inappropriately pair with a downstream homologous template sequence and continue to replicate [Mathews, 2006]. Consequently, after a few rounds of replication, the stalled site would end up being deleted as shown below:

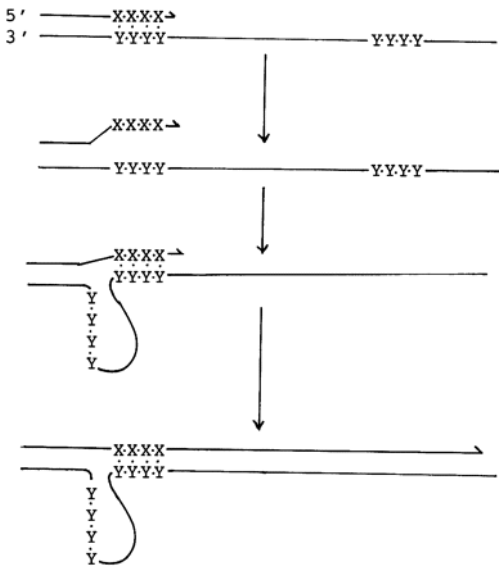

Figure 2. A proposed mechanism for large-scale mitochondrial DNA deletion created by dNTP deficiency. First, a deficiency in dNTPs causes Pol γ to stall and fray the 3' terminal end. Next the XXXX sequence incorrectly pairs downstream with a homologous YYYY sequence which causes a deletion of template sequence between YYYY repeats. Figure from [Mathews, 2006].

There are currently two known mechanisms by which pool imbalances cause mtDNA point mutations. The most straightforward mechanism is through inappropriate dNTP substitution during replication [Nishino, 1999; Spinazzola, 2002]. When one dNTP pool near the replisome is high, DNA Pol γ will readily substitute that dNTP in place of correct dNTP. In 2003, Nishigaki *et al.* sequenced the mitochondrial DNA of a number of MNGIE patients. Through sequence analysis, they found that the most common MNGIE point mutation occurs through a G substitution for A during replication in template sequences where Ts are followed by at least two As [Nishigaki, 2003]. At these sites, enlarged dGTP and dTTP pools during replication can produce the mutagenic next-nucleotide effect, where Gs can be substituted for As, and the mismatch sealed in place by T incorporations at the two downstream As. In addition, our laboratory also found that symmetric increases in all four dNTP pools can produce the same mutagenic results

[Wheeler, 2005]. Thus, it should be noted that any form of variation from homeostatic dNTP pools can be mutagenic.

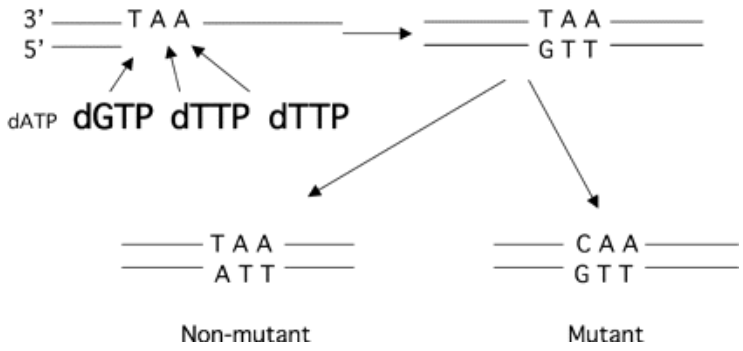

Figure 3. A proposed mechanism by which G to A substitutions occur in MNGIE patients. The abnormally high dGTP and dTTP pools are predicated to cause the next-nucleotide effect during replication, which causes such a point mutation. Figure from [Mathews, 2006].

Another pathway by which point mutations may accumulate in mitochondria is through decreased Pol γ proofreading. Our laboratory has also shown that both symmetric and asymmetric increases in dNTP pools can affect polymerase proofreading activity [Lee, 2003]. It is possible that abnormally high mitochondrial dNTP pools near Pol γ could indirectly affect its ability to remove improperly base paired dNTPs, leading to increased mitochondrial mutagenesis.

MITOCHONDRIAL DNA MUTATIONAL DISEASES

Now that we have used MNGIE to discuss some of the mechanisms of mutagenesis, we will explore the physiological symptoms of a few mitochondrial mutational diseases which arise from dNTP pool abnormalities, beginning with MNGIE.

Mitochondrial Neurogastrointestinal Encephalomyopathy (MNGIE)

MNGIE is an autosomal recessive disease caused by mutations in the gene encoding for thymidine phosphorylase. MNGIE is clinically characterized by severe

gastrointestinal dysmotility, cachexia, peripheral neuropathy, and evidence of mitochondrial dysfunction [Pontarin, 2006]. Thymidine phosphorylase is an enzyme that catalyzes the reversible breakdown of thymidine to thymine. Patients with this condition display elevated levels of circulating thymidine, which has been suggested to cause excessive thymidine uptake into mitochondria. The excessive thymidine levels were proposed to stimulate salvage synthesis of dTTPs, which unbalances dNTP pools to stimulate mutagenesis [Spinazzola, 2002]. Should mtRNR selectively favor the synthesis of dTTP, it is probably that a similar condition could develop.

Mitochondrial DNA Depletion Myopathy (MDDM)

Defects in mitochondrial dNTP metabolism enzymes create a condition known as MDDM. In MDDM patients, low dNTP pools stimulate mitochondrial mutagenesis which causes a variety of symptoms. Specifically, a deficiency in mitochondrial thymidine kinase, TK2, causes muscle weakness, as well as both liver and kidney failures [Wang, 2003]. Likewise, a deficiency in mitochondrial deoxyguanosine kinase is known to cause liver failure and severe neurological abnormalities. To test the relationship between dNTP pool abnormalities and MDDM, Saada et al measured mitochondrial dNTP pools from the fibroblasts of two TK2 deficient patients. As expected, they found that the TK2 patients had 30 and 60 percent lower dTTP pools compared to control subjects [Wang, 2003]. Through studying mitochondrial dNTP pools, it has become clear that pool abnormalities can have direct physiological effects. Thus, if mtRNR were to significantly reduce its specificity for any of its four ribonucleotide substrates, it would be reasonable to presume that a similar physiological result could ensue.

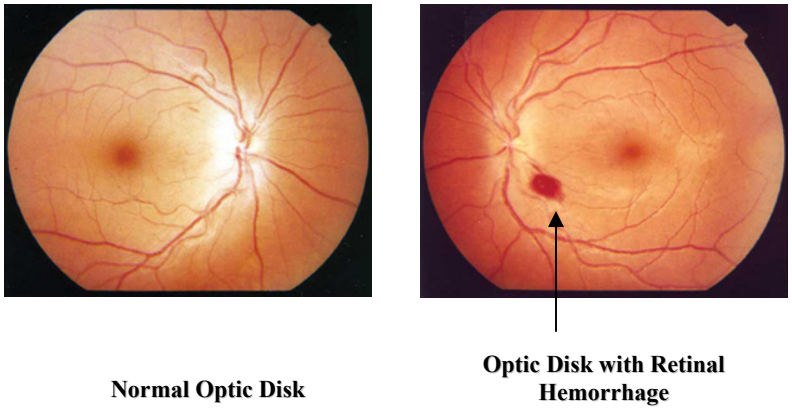

Figure 4. A comparison between the left and right optic disks of an MDDM patient. Note the optic disk hemorrhage on the right. Figure from [MDA, 2007].

Progressive External Ophthalmoplegia (PEO)

PEO is a condition characterized by large scale mitochondrial DNA deletions in the ocular muscles. Although genomic analyses of patients have shown that a large number of PEO cases have been caused by low-fidelity mitochondrial Pol γ, it has been proposed that mutations in the mitochondrial ATP/ADP carrier can also be a contributor [Kaukonen, 2000; Ponamarev, 2002]. The mitochondrial ATP/ADP carrier exchanges intra-mitochondrial ATP for extra-mitochondrial ADP (an RNR substrate). It is possible that a defect in the latter carrier can cause dNTP pool imbalances through providing mtRNR with either too little or too much substrate. Similarly, if a defective mtRNR were to increase dATP pools near the replisome (as mentioned earlier), it is likely that Pol γ will lose both replication and proofreading accuracy as well.

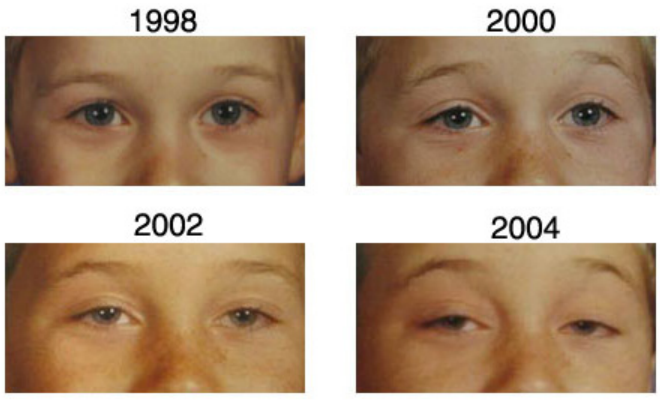

Figure 5. Four photographs showing the progression of pediatric PEO. The large scale mitochondrial DNA deletions have led to severe losses in ocular muscle control. Figure from [MDA, 2007].

Mitochondrial DNA Depletion Syndrome from Mutant p53-Dependent RNR

Mammalian cytosolic RNR has two non-identical subunits named R1 and R2. The R1 subunit harbors the catalytic site for ribonucleotide reduction while the R2 subunit supplies an essential tyrosyl radical for catalysis to occur [Jordan, 1998]. In 2000, Nakano *et al.* discovered an additional R2 subunit whose intracellular accumulation is dependent on the p53 tumor suppressor protein. This p53-dependent R2 subunit (p53R2) associates with R1 in the same manner as generic R2s, and is up-regulated 30-fold prior to DNA replication [Nakano, 2000]. It is thought that in nonproliferating cells, generic R2s are down regulated so that p53R2s will serve in supplying dNTPs for mtDNA replication and nuclear DNA repair [Bourdon, 2007]. This notion has been supported by experiments with p53R2 knockout mice, as 14 months after birth, the knockout mice all experienced significant renal failure attributed to decreased DNA repair and increased apoptosis [Nakano, 2000]. Subsequently, this hypothesis was extended to diagnose children suffering from mitochondrial DNA depletion syndrome. And as expected, a

large number of the children tested positive for mutations in the homologous p53R2 protein [Powell, 2005].

Although p53R2 is considered to be a largely cytosolic protein, it has been reported to translocate into the nucleus during times of DNA damage. It is proposed that p53R2 would associate with R1 in the nucleus and saturate repair polymerases with dNTPs [Tanaka, 2000].

If p53R2 must be translocated into the nucleus for repair polymerases to function appropriately, then it is reasonable to believe that mitochondrial Pol γ must also have an RNR to supply it with enough substrates in the mitochondrial matrix for replication.

Although it seems plausible that the mtRNR we observed contains a translocated p53R2 subunit, we will discuss later evidence for why the mtRNR we have described is indeed unique.

JUSTIFICATIONS FOR A MITOCHONDRIAL RIBONUCLEOTIDE REDUCTASE

Although the existence of a mtRNR has not been widely accepted, evidence for distinct mitochondrial dNTP pools has been presented for several decades. The existence of cytosol-independent mitochondrial dNTP pools was first proposed by Bogenhagen *et al.* in 1976 when they compared mitochondrial and nuclear DNA synthesis rates in the presence of thymidine triphosphate-inhibiting drugs. When it was observed that nuclear DNA synthesis was knocked down 96% while mtDNA synthesis was unaffected, Bogenhagen *et al.* proposed that mitochondria must have a dTTP pool independent of the cytosol [Bogenhagen, 1976].

Since dNTP pool asymmetries can have mutagenic effects, we are particularly interested in how mitochondrial dNTP pools accumulate. There are currently four proposed mechanisms by which mitochondria obtain dNTPs for DNA synthesis (Fig. 6).

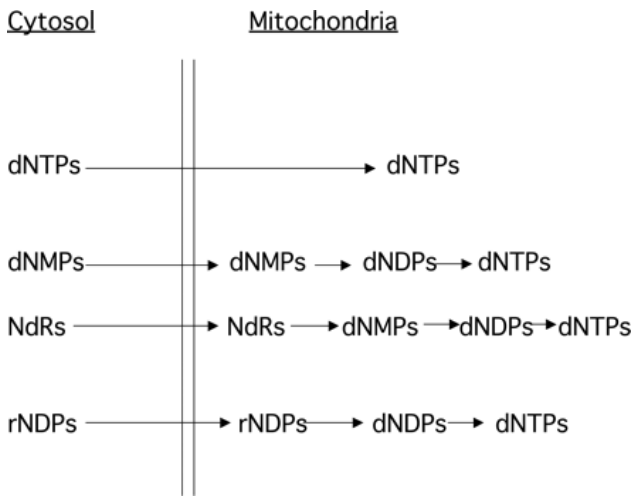

Figure 6. Possible Pathways for Mitochondrial dNTP Accumulation. Figure from [Mathews and Song, 2007].

Through isolating leukemia cell mitochondria and testing for the transport of radiolabeled dCTP, Bridges *et al.* in 1999 showed that mitochondria can import DNA-synthesis-ready dNTPs. Although it was reported this transport system had a

physiologically reasonable K_m of 3μM for dCTP, its transport of the three other dNTPs has not been studied yet [Bridges, 1999]. Moreover, it was reported that the system's transport of dCTP was not inhibited by the other dNTPs. Thus, unless there are three other specific dNTP transporters, it is unlikely that this is a system for dNTP pool regulation.

In 2006, Ferraro *et al.* reported that mouse liver mitochondria could import dTMP. Through culturing mouse liver cells in a dTMP rich medium, it was observed that the mitochondria concentrated dTMP 100-fold, with some of the dTMP phosphorylated to dTDP and dTTP. Since the matrix dTDP and dTTP pools were significantly lower than the dTMP pools, it was proposed that mitochondria could perhaps use a series of monophosphate and diphosphate kinases, as well as nucleotidases to regulate dNTP pools [Ferrano, 2006]. However, this study also found that the dTMP transporter was unaffected by other dNMPs, suggesting that this system may not be a bottleneck for dNTP regulation. Furthermore, the question of whether dNMPs other than dTMP could be transported has been left unanswered.

Although it is known that mitochondrial dGK and TK2 can phosphorylate NdRs to their corresponding dNMPs, not much is known about the subsequent phosphorylation step in which dNMPs are converted to dNDPs [Lacombe, 2000]. It is, however, known that once dNDPs are formed, they can be readily converted to dNTPs via the mitochondrial isoform of nucleoside diphosphate kinase [Arnér, 1995]. Since mutations in dGK and TK2 can be lethal in mice, we know that this pathway is significant [Desler, 2006]. However, since not much is known about the dNMP kinases, we do not at present have a quantitative understanding for the overall magnitude of this pathway.

Since nucleotide kinase and phosphatase activities occur readily inside of mitochondria, we wanted to explore the possibility of a *de novo* dNDP synthesis pathway from rNDP. The enzyme that catalyzes this conversion is of course RNR. Because the reduction of rNDP to dNDP is the first committed step of DNA synthesis, and the reverse reaction from dNDP to rNDP does not readily occur, the existence of a mtRNR would make good metabolic sense.

Although biological systems do not always favor simplicity, the existence of an mtRNR for dNTP pool regulation would be metabolically simpler than other regulatory systems. A system that utilizes many selective dNTP-precursor channels and pumps would be complex and unwieldy. Likewise, a dNTP synthesis pathway that is regulated by many kinases and phosphatases would also be complex, and given that ribonucleotides are substrates for oxidative phosphorylation, this would lead to competition with other major metabolic pathways.

In 2005, Song *et al.* reported measurements for mitochondrial dNTP pools in a variety of cells types (Fig. 7). In rat heart and liver mitochondria, it was found that the dNTP pools were highly asymmetric, such that the dGTP pools could be over nine times higher than dCTP pools [Mathews and Song, 2007]. This finding suggests two possible scenarios. One scenario is that the mitochondrial dNTP pools are very poorly regulated, and the other is that these pools are actually tightly regulated to be asymmetric. Since dNTP pool asymmetries are mutagenic, the latter scenario seems to be less plausible. In lieu of the fact that mitochondrial dNTP pools are observed to be poorly regulated, they can be theoretically well regulated via mtRNR.

Having allosterically regulated mtRNRs which would be selective for ribonucleotides makes sense. Once ribonucleotides are converted to deoxyribonucleotides, there would be no competition with other metabolic pathways. Given that RNR transcription, translation, and posttranscriptional modifications are all highly regulated, RNRs would be prime candidates for mitochondrial dNTP pool regulation [Leeds, 1987]. And since RNR products can only be used for DNA synthesis, regulation of RNR would mean regulation of a dedicated pathway.

If dNTP pools were to be regulated in mitochondria via either phosphorylation or membrane transport, all of the associated proteins would have to have different dNTP precursor selectivity. With a mtRNR however, regulation of dNTP pools in varying tissues would only require changes in RNR.

The fact that mitochondrial dNTP pools are highly asymmetric in a number of mammalian tissues suggests several metabolic possibilities. First, it is possible that these dNTP pool-imbalanced tissues lack mtRNR activity and depend on salvage pathways for obtaining dNTPs. Second, it is possible that these tissues have very nonspecific mtRNR, which do not play an appreciable role in dNTP pool regulation. Third, it is possible that mtRNRs have mutated throughout evolutionary time to be specific for certain ribonucleotides. Given that most mitochondrial diseases are late onset, it is plausible that these mutations were not selected against in early mammals [Wallace, 2005]. Thus, it is likely that these unfavorable mutations were retained during evolution and are now responsible for the dNTP pool asymmetries that we see.

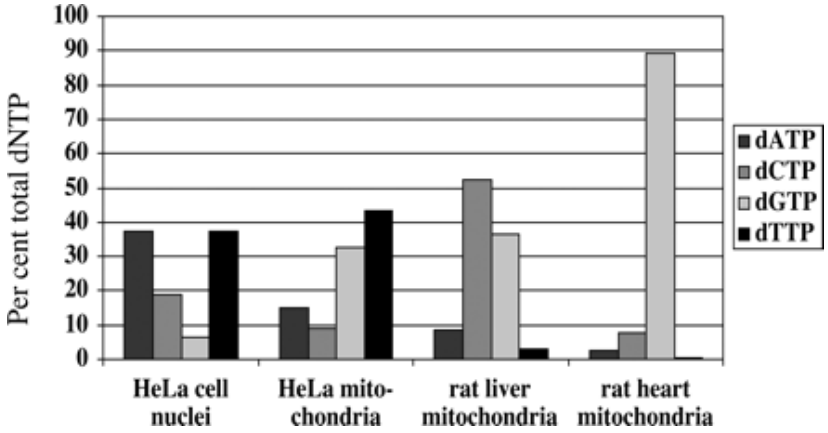

Figure 7. A comparison of mitochondrial dNTP pools between various tissues. Note the large asymmetry in rat heart mitochondria. Figure from [Mathews, 2007].

Finally, another basis for the existence of mtRNR could be to prevent dNTP pool depletion. As we have discussed earlier, deficiencies in mitochondrial dNTP pools can be a source of mutagenesis. Since abnormally large ribonucleotide pools do not have genotoxic effects, it is possible, in theory, for mitochondria to accumulate large ribonucleotide pools in response to dNTP depletion. Thus, through accumulating a large reserve of ribonucleotides and having mtRNR serve as a bottleneck for dNTP synthesis, it is possible for dNTP depletions to be alleviated.

BACKGROUND

Evidence for the possible existence of a mtRNR was first presented by our laboratory fourteen years ago [Young *et al*. 1994]. Through analyzing mitochondria from HeLa cells, it was observed that the mitochondrial enzyme was regulated differently from the cytosolic form [Young *et al*, 1994]. This interesting discovery was not widely accepted at the time because the mtRNR activity was lower than the cytosolic activity, which led to the belief that mtRNR activity was the result of cytosolic contamination.

In 2005, our laboratory demonstrated that rat liver had mtRNR activity [Song *et al.* 2005]. In contrast to HeLa cells, the nonproliferating rat hepatocytes showed a higher mtRNR activity in comparison to the cytosolic RNR. This higher activity in mtRNR suggested that contamination was not an issue, and that mtRNR may indeed be a distinct enzyme form. Through checking the mitochondrial fractions for cytosolic enzymes, he showed that there was in fact low cytosolic RNR contamination [Song *et al.* 2005].

To further prove that mtRNR was indeed a distinct enzyme from the major cytosolic form, Song *et al.* performed a series of immunoblotting experiments using rabbit antiserum for the vaccinia virus RNR R1 subunit. The vaccinia virus subunit was used because previous genomic analyses had suggested possible homology between it and mtRNR [Song *et al*, 2005]. When it was observed that the rabbit antiserum had reacted with the vaccinia virus RNR and not the mtRNR, it became increasingly clear that mtRNR was indeed a unique enzyme with a distinct molecular weight.

In our present study, we have extended our examination of mitochondrial activity to a variety of tissues. This thesis discusses the activities of mtRNR in rat brain, heart, kidney, liver, skeletal muscle, and in *Saccharomyces cerevisiae*.

OBJECTIVES

The purpose of this study is to further examine mtRNR in three ways. First, the primary goal of this thesis is to measure and compare mtRNR activities in a variety of tissues. Through studying these variations in mtRNR activity, we hope to gain physiological insights into why mitochondria in different tissues have different DNA precursor pool requirements. Using the latter data, we would also like to investigate the possibility that different tissues could have different metabolic pathways for mitochondrial dNTP accumulation. Secondly, we are interested in how mtRNR responds to known inhibitors of cytosolic RNR. Young *et al.* reported that mtRNRs could be stimulated by millimolar concentrations of dATP, a known cytosolic RNR allosteric inhibitor [Young *et al*., 1994]. In addition to testing this observation, we were also interested in how mtRNR responds to radical-scavenging inhibitors such as hydroxyurea, which readily neutralizes the essential radical for catalytic activity in known RNRs. Through measuring mtRNR activity in the presence of inhibitors, we would like to gain an understanding of how mtRNR is regulated *in vivo*. Also, through performing these inhibitor studies, we could test the distinctiveness of mtRNR. Our third aim is to amplify and sequence the mtRNR gene. Through obtaining a transcript of the presumed mtRNR gene, we hope to understand the mechanisms of mtRNR activity, and provide pharmaceutical researchers with the information they need to treat mitochondrial dNTP pool abnormality associated diseases.

MATERIALS AND METHODS

Isolation of Rat Mitochondria

The procedure for isolating mitochondria from rat tissues was performed roughly as described by Song *et al.* (2005).

All animal procedures were approved by the OSU Institution of Animal Care and Use Committee. Young male Wistar and Fischer rats were used to study mitochondrial RNR activity in kidney, heart, cardiac muscle, brain, and skeletal muscles. The rats were anesthetized with isoflurane and killed by decapitation.

Tissues were removed immediately, washed and minced in cold isolation buffers containing 220 mM mannitol, 70 mM sucrose, 5 mM MOPS (pH 7.4), 2.0 mM EGTA, and 0.2 mg/ml BSA. The tissues (excluding skeletal muscle) were homogenized in cold isolation buffer with a motorized homogenizer.

The mitochondrial fractions were isolated by differential centrifugation and subsequently washed twice and resuspended in 2 ml of isolation buffer. Immediately afterwards, most of the mitochondrial suspension was centrifuged to pellet mitochondria, and the remaining suspension was saved for cytosolic RNR analysis. Prior to the RNR assays, the mitochondrial preparations were gently sonicated for 30 seconds to break open the mitochondria.

The skeletal muscle mitochondria isolation procedure was the same as above except that it included a more vigorous homogenization and a different isolation buffer consisting of 0.1M KCl, 50 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$, and 1 mM EGTA.

After isolation, mitochondrial and cytosolic protein concentrations were estimated by the Bradford method [Bradford, 1974].

RNR Assay

The RNR activity assays performed were in reference to Slabaugh *et al.* (1984).

After the mitochondria were isolated, 20µL of enzyme preparation is mixed with 10µL water and 10µL of 4X reaction mixture. The 4X reaction mixture consists of 1.0 M HEPES (pH 8.0), 0.5M dithiothreitol, 100mM AMP-PNP, 1mM ferric chloride, 1mM CDP (cold), 8mM magnesium acetate, and 100cpm of ^{3}H CDP per pmol of cold CDP.

After the reaction mixture had been incubated for 60 minutes in a 37ºC water bath, the reaction was stopped by the addition of 4.4µL of 10N perchloric acid.

The stopped reaction has chilled on ice for 15 minutes, it was transferred to a clean microfuge tube to be suspended in boiling water for 20 minutes. The boiling hydrolyzed all of the nucleotides to their corresponding monophosphates. After boiling, 4 µL of 10mM dCDP, dUDP and CDP are added as TLC markers. 12 µL of 5M KOH is then added to precipitate out any remaining salt in the reaction mixture. Once the salts have ceased to precipitate out of solution, the reaction mixture is centrifuged and then transferred to a clean microfuge tube, at which time the reaction mixture is ready for TLC separation.

RNR Analysis

After the RNR reaction has completed, the CMP (substrate) and the dCMP (product) were separated via chromatography using cellulose TLC plates. The resolving solvent was made via mixing: 220mL of 90% ethanol, 80mL of saturated sodium borate (pH 7.8), 20mL of saturated ammonium acetate (pH 9.8), and 1mL 0.25 M disodium EDTA (pH 8.0).

The separation process typically takes 15 hours, after which, the products and substrates can be seen to have fully separated under a short wave UV lamp.
Finally, the separated products and substrates are cut out of the TLC plate, placed in a scintillation vial and eluted using 0.5N HCl. Quantification of products and substrates is performed by liquid scintillation counting using a Beckman LS6500 scintillation counter.

RNR Gene PCR Amplification

The following protocol for PCR amplification was designed by Song *et al.* with modifications courtesy of Dr. Indira Rajagopal.

Amplification and sequencing of the putative mtRNR gene was performed using a human skeletal muscle cDNA library. PCR amplification of the presumed mtRNR gene from the cDNA library was performed using primers designed from conserved ends of the Vaccinia virus R1 subunit sequence. Amplification conditions were as follows:

dH_2O	38 µl
PCR buffer (10x)	5 µl
dNTP mix (10 mM each)	1 µl
F-primer (4/12/05) 1 µM	1 µl
R-primer (4/12/05) 1 µM	1 µl
Taq polymerase (Invitrogen)	1 µl
template (cDNA)	1 µl
$MgCl_2$ (50 mM)	2 µl

The PCR cycles were as follows:

96° C 1 min			1 cycle
96° C 15 sec	60° C 30 sec	72° C 1 min.	30 cycles
72° C 1 min			1 cycle

DATA ANALYSIS

RNR Activity Studies

In this study, our investigation of mtRNR began with yeast. Since mtRNR activities in unicellular eukaryotic organisms have never been studied before, we were uncertain if there would even be any measurable activity in yeast. To our surprise, there was mtRNR activity and its specific activity was higher than cytosolic RNR activity (Fig. 8). This relatively higher mtRNR activity was consistent with what Song *et al.* from our laboratory saw in rat hepatocytes [Song *et al.*, 2005].

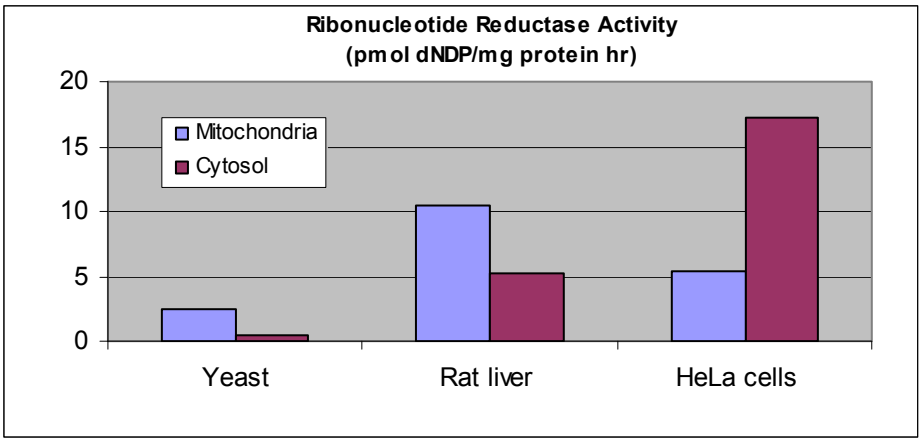

Figure 8. A comparison of mtRNR activity to cytosolic RNR activity in three different cell types. The rat liver and HeLa cell RNR activities are in reference to Song *et al.* 2005.

To investigate the activity of mtRNR in yeast, we quantified the amount of dCDP produced in yeast with respect to time (Fig. 8 and 9). Through quantifying yeast mtRNR activity in substrate saturated environments, we learned mtRNR in yeast has relatively slow rates of catalysis when compared to mammalian tissues.

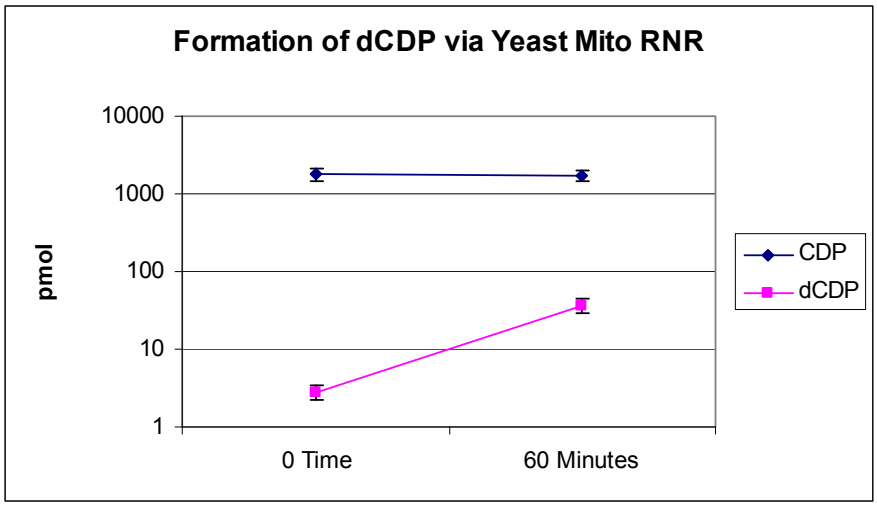

Figure 9. Yeast mtRNR converting CDP to dCDP. The error bars indicate the maximum deviation between 3 reaction runs. To standardize the amount of yeast mtRNR used without purification, each reaction contained 25µg of total yeast mitochondrial protein. Note: the amount of product formed is minute compared to the amount of substrate.

In comparing mtRNR activity between five different tissues, we found the specific activities to be very different. The largest difference in mtRNR activity was between heart and skeletal muscle mitochondria. We will discuss later some of the physiological reasons for why this 30 fold difference in activity occurs.

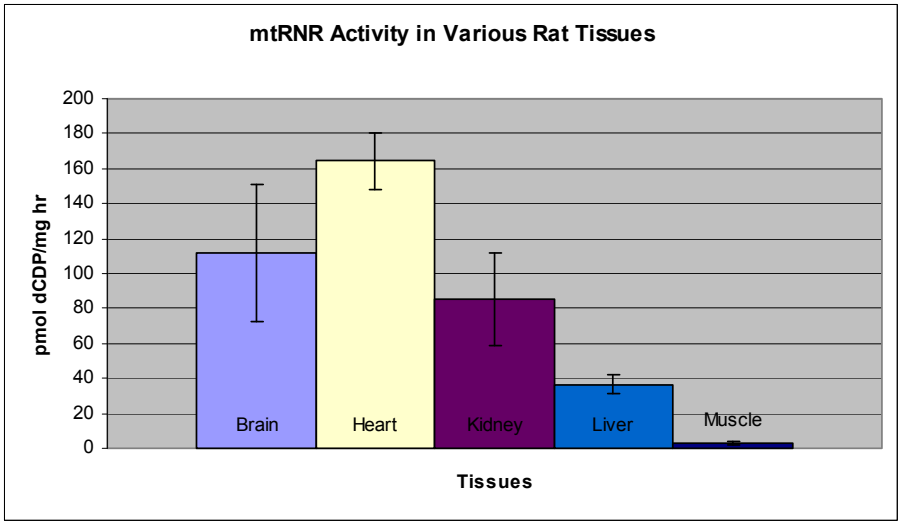

Figure 10. Comparison of mtRNR activity between five different rat tissues. With the exception of brain, all error bars indicate the maximum deviation in mtRNR activity between two rats. Because brain mtRNR activity was only

measured in one rat, its error bar represents the deviation between two experimental runs.

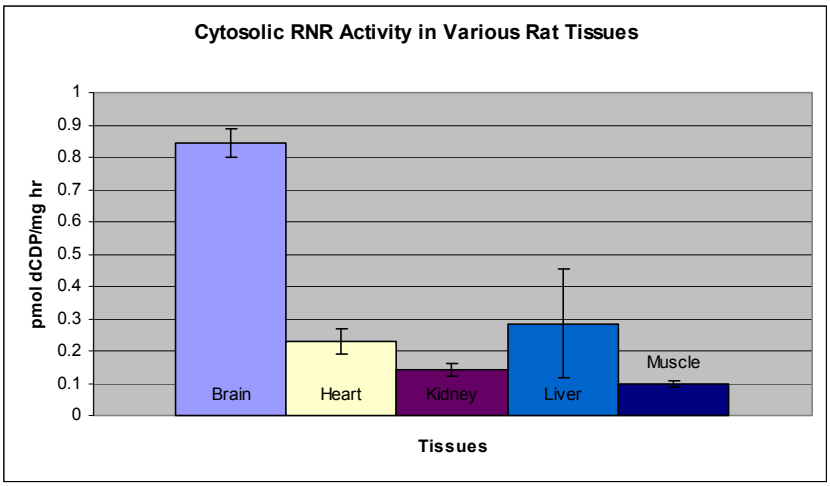

Figure 11. A comparison of cytosolic RNR activity between 5 different rat tissues. The error bars represent the range between two experiments. Because RNR activities were quantified based on the amount of total cellular protein used, the brain activity was relatively high due to its low protein content.

To test the distinctiveness of mtRNR, we studied rat liver mtRNR activity in the presence of various dATP inhibitor concentrations (Fig. 12). Contrary to what Young *et al.* reported in 1994, we did not see a stimulation of mtRNR activity at low dATP concentrations. However, we did observe a difference in response between mtRNR and cytosolic RNR to increasing concentrations of dATP (Fig. 13). Our finding not only demonstrates that there is indeed a distinct form of mtRNR, but suggests that mtRNR is regulated differently than cytosolic RNR in vivo.

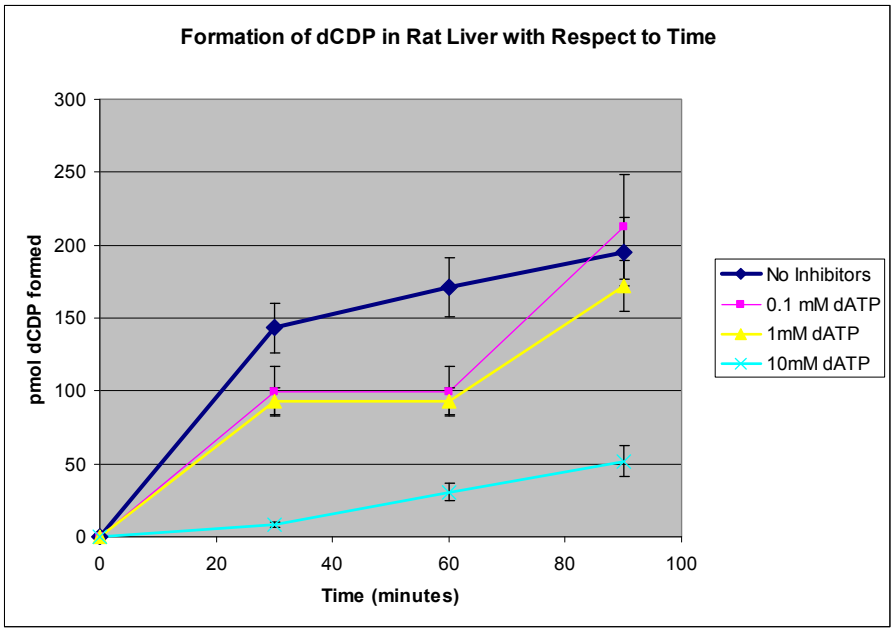

Figure 12. A figure showing the synthesis of dCDP by rat liver mtRNR with respect to time. Each line represents a different concentration of dATP inhibitor used. The error bars represent the range between two experiments.

Figure 12 illustrates how increasing concentrations of dATP affect mitochondrial dCDP formation with respect to time. In Figure 13, however, we are illustrating that mtRNR activity actually varies nonlinearly with respect to increasing dATP concentration. This contrast in mtRNR and cytosolic RNR response to dATP suggests that mtRNR is indeed a unique enzyme.

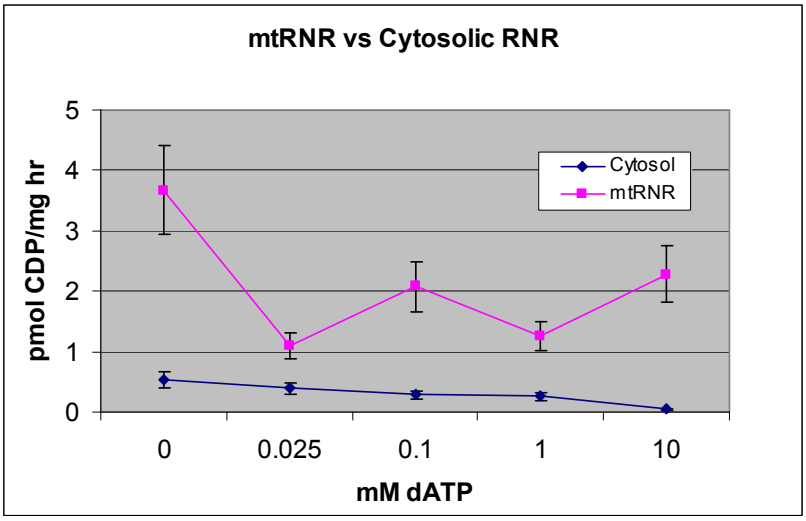

Figure 13. A comparison of rat liver mtRNR activity and rat liver cytosolic RNR activity in the presence of dATP inhibitor. The error bars represent the range between a two measurement of the same rat liver. Note how rat liver mtRNR and cytosolic RNR respond differently to increasing dATP inhibitor.

One experiment that we would like to repeat is our comparison of cytosolic and mitochondrial RNR activities in the presence of hydroxyurea (Fig. 14). This experiment produced interesting results in that mtRNR appeared to be insensitive to high concentrations of hydroxyurea, while cytosolic RNR was very sensitive. Given that hydroxyurea is known to readily neutralize the amino acid radicals essential for RNR activity, our data suggests that mtRNR may have a very different activity compared to the major cytosolic forms of RNR.

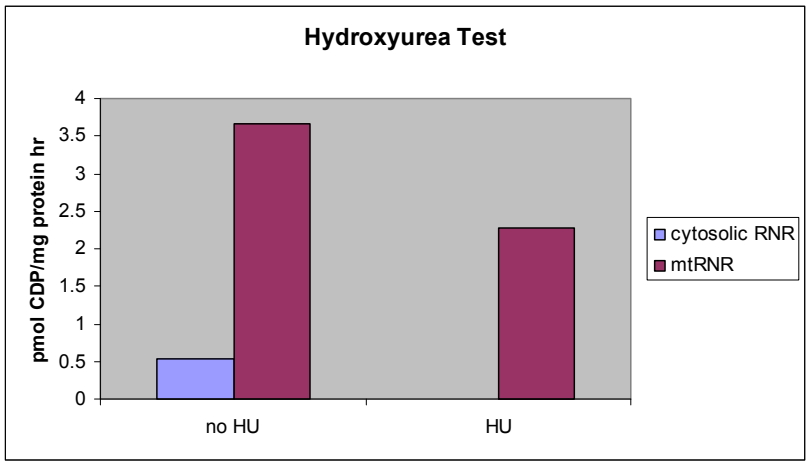

Figure 14. A comparison of rat liver mtRNR activity and rat cytosolic RNR activity in the presence of hydroxyurea. Note that the mtRNR activity was less sensitive to hydroxyurea than cytosolic RNR.

Amplification and Sequencing of the Putative mtRNR Gene

Using primers designed from the conserved ends of the vaccinia virus RNR R1 subunit gene, which we have presumed to be homologous with the mtRNR R1, we have amplified via PCR a potential mtRNR gene out of a skeletal muscle cDNA library (Fig. 15). This putative gene, at approximately 650 base pairs, seems to be of the appropriate size for a ribonucleotide reductase R1 subunit. Unfortunately, our attempts at sequencing the mtRNR gene have been unsuccessful. Feedback from our on campus DNA sequencing facility (Center for Genome Research and Biocomputing) suggests that our amplified product is not pure enough for sequencing. To alleviate this problem, we are currently trying to purify our PCR product via using a series of size-filtering DNA columns.

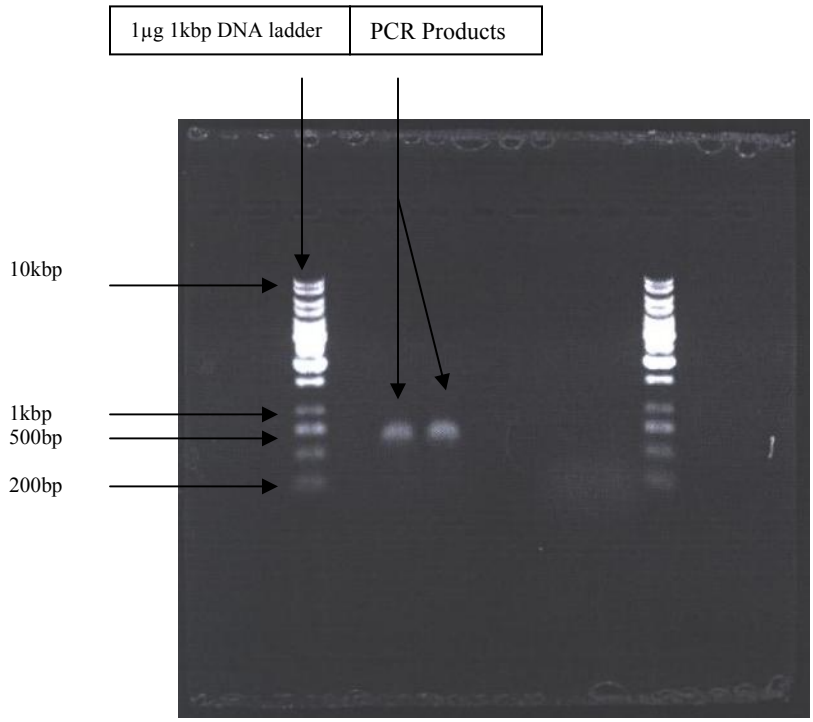

Figure 15. Amplification of the predicted mtRNR gene from human skeletal muscle cDNA using conserved vaccinia virus R1 sequence primers (0.8% Agarose gel).

DISCUSSION AND CONCLUSIONS

RNR Activity Assays

Our investigation of yeast and different rat-tissue mitochondria produced several important observations.

First, we demonstrated that ribonucleotide reductase activity is in fact present in a variety of mitochondria. This important finding suggests that all mitochondria have the capacity for de novo dNTP synthesis. In mammalian mitochondria, we found the mtRNR activity to be several-fold higher than in the mitochondria of yeast. We also found that mitochondrial RNR activities can vary significantly between different tissues. Although cytosolic RNR activities were relatively similar amongst different tissues, we observed large variations in mtRNR activities. For example, we found that mtRNR activity in cardiac muscle was over 50 times higher than in skeletal muscle. In cells with high energy requirements, such as the heart and brain, we saw relatively high mtRNR activities. The author speculates that since these cells have a steady demand for high energy molecules, it is possible that their mtDNA will undergo more damaging events. In order to repair these mtDNA damages in a timely manner, it likely that their mtRNRs will be more active in producing dNDPs. Conversely, in tissues such as white muscle fiber, which have metabolically less active mitochondria [Martini, 2006], it is possible that their mtRNRs are also less active due to low demands for dNDPs.

When mtRNR activities were compared to cytosolic RNR activities in rats, we found that the mtRNR activities were several-fold higher in all tissues. This was not a surprising observation since it is known that mitochondrial DNA synthesis continues throughout the cell cycle, and that mitochondrial regeneration is active even in

nonproliferating tissues [Karol and Simpson, 1968]. This means that mitochondria would require more active RNRs to supply sufficient dNTPs for DNA repair and replication.

We speculate that because mtDNA are subjected to more oxidative DNA damaging events than nuclear DNA, mtRNRs would need to be more active in producing dNTPs for repair in mitochondria. However, in physiologically irregular cells such as HeLa cells, evidence seems to show a reversal in the ratio of mtRNR activity to cytosolic RNR activity [Song *et al.*, 2005]. Since not much is known about mitochondrial dNTP metabolism to date, we can only speculate on how these differences in ratios arise. Also, because mtRNR activity appears to vary with respect to tissue type (Fig. 10), it is possible that different mitochondria are using different pathways for dNTP accumulation. Thus, mitochondria with high dNTP requirements may have low mtRNR activities due their utilization of salvage pathways. In addition, the lower activity in both yeast mtRNR and cytosolic RNR when compared to rat liver and HeLa cells may use the same rationale. Because yeast cells proliferate relatively quickly, it is possible for them to prefer salvage pathways for their method of cytosolic and mitochondrial dNTP accumulation.

In mammals, neurons, podocytes (kidney cells), hepatocytes, and muscle fibers rarely divide after maturity [Martini, 2006]. Since their nuclear demands for dNTPs are based primarily on DNA repair needs, it makes good metabolic sense for cytosolic RNRs to less active than mtRNRs (Fig. 10 and 11).

Although brain cytosolic RNR activity appears to be the highest out of all of the tissues we measured (Fig. 11), the author feels that this observation is not a good representation of reality. By tissue mass, the amount of dCDP produced by cytosolic

RNR in each tissue was similar. Since brain cells are relatively high in fat by weight, our RNR activity calculation using total cellular protein was probably misleading. For future reference, a better method for comparing RNR activities between tissues would be to compare with reference to the number of cells used, or the activity of a reference enzyme.

In comparing mtRNR and cytosolic RNR activities in the presence of inhibitors, we found that the two enzyme forms responded very differently to hydroxyurea and dATP. While rat liver cytosolic RNR activity was almost completely inhibited by 1mM hydroxyurea, we found that rat liver mitochondrial RNR activity was relatively unaffected. Since hydroxyurea is a radical scavenger, and readily neutralizes catalytic radicals on RNRs [Jordan et al, 1998], our observation raises two questions. If mtRNR is resistant to hydroxyurea, does that mean that it is more effective in protecting its catalytically essential radical? Perhaps it is possible that mtRNR uses an entirely different mechanism for catalysis, one which does not use a radical amino acid. To answer this question, we would need to isolate mtRNR and conduct EPR (electron paramagnetic resonance) experiments to search for such a catalytic radical amino acid.

Even though both rat liver mtRNR and cytosolic RNR were inhibited by dATP, we found that they responded very differently (see Fig. 12). This suggests that the two enzyme forms have distinct pathways for in vivo regulation.

RNR Sequencing

The PCR amplification of what appears to be a putative mtRNR gene of the appropriate molecular size from skeletal muscle cDNA using conserved RNR sequence primers suggests that there is in fact a distinct mitochondrial RNR. Although the PCR amplification was productive, our sequencing attempts have been unsuccessful. At present, it appears that we have not been able to amplify the mtRNR gene with enough purity for sequencing. Through future purification workups, we hope to successfully clone, sequence, and overexpress the mtRNR protein for further analysis.

BIBILIOGRAPHY

Arnér, E. S. J., Eriksson, S. E. (1995) Mammalian deoxyribonucleoside kinases. *Pharmacol. Ther.* **67**,155-186

Baruffini, E., Lodi, T., Dallabona, C., Puglisi, A., Zeviani, M., Ferrero, I. (2006) Genetic and chemical rescue of the Saccharomyces cerevisiae phenotype induced by mitochondrial DNA polymerase mutations associated with progressive external ophthalmoplegia in humans. *Human Mol. Genet*. **15**, 2846-2855

Bogenhagen, D., Clayton, D. A. (1976) Thymidylate nucleotide supply for mitochondrial DNA synthesis in mouse L-cells. *J. Biol. Chem*. ***251***, 2938-2944

Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantization of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem*. **72**:248-254.

Bridges, E. G., Jiang, Z., Cheng, Y-c. (1999) Characterization of a dCTP transport activity reconstituted from human mitochondria. *J. Biol. Chem.* **274**, 4620-4625

Bourdon, A., Minai L., Serre, V., (2007) p53R2: DNA repair or mitochondrial DNA synthesis? *Nat. Genet*. **39**, 776–780.

Chinnery, P. Samuels, D. Elson, D. Turnbull, D. (2008) Accumulation of mitochondrial DNA mutations in ageing, cancer, and mitochondrial disease: is there a common mechanism? *The Lancet* , **360**, 9342, 1323 - 1325

Desler, C., Munch-Petersen, A., Rasmussen, L. J. (2006) The role of mitochondrial dNTP levels in cells with reduced TK2 activity. *Nucleosides Nucleotides Nucleic Acids* **25**, 1171-1175

Dolce, V., Fiermonte, G., Runswick, M. J., Palmieri, F., Walker, J. E. (2001) The human mitochondrial deoxynucleotide carrier and its role in the toxicity of nucleoside antivirals. *Proc. Natl. Acad. Sci. U. S. A*. **98**, 2284-2288

Enríquez, J. A., Ramos, J., Pérez-Martos, , López-Pérez, J., Montoya, J. (1994) Highly efficient DNA synthesis in isolated mitochondria from rat liver. *Nucl. Acids Res.* **22,** 1861-1865

Ferraro, P., Nicolosi, L., Bernardi, P., Reichard, P., Bianchi, V. (2006) Mitochondrial deoxynucleotide pool sizes in mouse liver and evidence for a transport mechanism for thymidine monophosphate. *Proc. Natl. Aad. Sci. U. S. A.* **103** ,18586-18591

Ferraro, P., Pontarin, G., Crocco, L., Fabris, S., Reichard, P., Bianchi, V. (2005) Mitochondrial deoxynucleotide pools in quiescent fibroblasts**.** *J. Biol. Chem.* **280**, 24472-24480

Hirano, M., Marti, R., Ferreiro-Barros, C., Vila, M. R., Tadesse, S., Nishigaki, Y., Nishino, I., Vu, T. H. (2001) Effects of intergenomic communication: Autosomal disorders that cause multiple deletions and depletion of mitochondrial DNA. *Sem. Cell. Dev. Biol.* **12**, 417-427

Holt, I. J., Lorimer, H. E., and Jacobs, H. T. (2000) Human mtDNA sublimons resemble rearranged mitochondrial genomes found in pathological states. *Cell* **100**, 515-524

Johnson, A. A., Ray, A. S., Hanes, J., Suo, Z., Colacino, J. M., Anderson, K. S., Johnson, K. A. (2001) Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. *J. Biol. Chem.* **276**, 40847-40857

Jordan, A., and Reichard, P. (1998) Ribonucleotide reductases. *Annu. Rev.Biochem* **67**, 71-88

Karol, M. H., Simpson, M. V. (1968) DNA biosynthesis by isolated mitochondria: A replicative rather than a repair process. *Science* **162**, 470-473

Kaukonen, J., Juselius, J. K., Tiranti, V., Kyttälä, A., Zeviani, M., Comi, G. P., Keränen, S., Peltonen, L., Suomaleinin, A. (2000) Role of adenine nucleotide translocator 1 in mtDNA maintenance. *Science* **289**, 782-785

Lacombe, M. L., Milton, L., Munier, A., Mehus, J. G., Lambeth, D. O. (2000) The human Nm23/nucleoside diphosphate kinases. *J. Bioenerg. Biomembr*. **32**,247-258

Lara, M. C., Weiss, B., Illa, I., Madoz, P., Massuet, L., Andreu, A. L., Valentino, M. L., Anikster, Y., Hirano, M., Martí, R. (2006) Infusion of platelets transiently reduces nucleoside overload in MNGIE. *Neurology* **67**, 1461-1463

Lee, H., Hanes, J., Johnson, K. A. (2003) Toxicity of nucleoside analogues used to treat AIDS and the selectivity of the mitochondrial DNA polymerase. *Biochemistry* **42**,14711-14719

Leeds, J. M., Mathews, C. K. (1987) Cell cycle-dependent effects on deoxyribonucleotide and DNA labeling by nucleoside precursors in mammalian cells. *Mol. Cell Biol*. **7**, 532-534

Lewis, W., Haase, C. P., Miller, Y. K., Ferguson, B., Stuart, T., Ludaway, T., McNaught, J., Russ, R., Steltzer, J., Santoianni, R., et al (2005) Transgenic expression of the deoxynucleotide carrier causes mitochondrial damage that is enhanced by NRTIs for AIDS. *Lab. Invest*. **85**, 972-981

Lindhurst, M. J., Fiermonte, G., Song, S., Struys, E., De Leonardis, F., Schwartzberg, P. L., Chen, A., Castegna, A., Verhoeven, N., Mathews, C. K., et al (2006) Knockout of

Slc25a19 causes mitochondrial thiamine pyrophosphate depletion, embryonic lethality, CNS malformations, and anemia. *Proc. Natl. Acad. Sci. U. S. A*. **103**, 15927-15932

Martini, Frederic (2006). Human development. *Anatomy and Physiology*. San Francisco California: Pearson. 503-514.

Mandel, H., Szargel, R., Labay, V., Elpeleg, O., Saada, A., Shalata, A., Anbinder, Y., Berkowitz, D., Hartman, C., Barak, M., et al (2001) The deoxyguanosine kinase gene is mutated in individuals with depleted hepatocerebral mitochondrial DNA. *Nat. Genet.* **29**, 337-341

Marcelino, L. A., Thilly, W. G. (1999) Mitochondrial mutagenesis in human cells and tissues. *Mutat. Res.* **434**, 177-203

Mathews, C.K., Song S. (2007) Maintaining precursor pools for mitochondrial DNA replication. *FASEB J.* **21** 2294-2303

Mathews, C. K. (2006) DNA precursor metabolism and genomic stability. *FASEB J.* **20**,1300-1314

Mathews, C. K., Slabaugh, M. B. (1986) Eukaryotic DNA metabolism: are deoxyribonucleotides channeled to replication sites?. *Exp. Cell. Res*. **162**, 285-295

McKee, E. E., Bentley, A. T., Hatch, M., Gingerich, J., Susan-Resiga, D. (2004) Phosphorylation of thymidine and AZT in heart mitochondria. *Cardiovasc. Toxicol*. **4**, 155-167

Muscular Dystrophy Association (MDA) . Mitochondrial disorder*s*. Retrieved May 27, 2008, from Neuromuscular Web site: http://neuromuscular.wustl.edu/mitosyn.html

Mootha, V. K., Bunkenborg, J., Olsen, J., Hjerrild, M., Wisniewski, J. R., Stahl, E., Bolouri, M., Ray, H., Sihag, S., Kamal, M., et al (2003) Integrated analysis of protein expression, tissue diversity, and gene regulation in mouse mitochondria. *Cell* **115**, 629-640

Nakano, K., Bálint, É., Ashcroft, M. & Vousden, K.H. (2000). A ribonucleotide reductase gene is a transcriptional target of p53 and p73. *Oncogene* **19**, 4283–4289

Nishigaki, Y., Marti, R., Copeland, W. C., Hirano, M. (2003) Site-specific somatic mitochondrial DNA point mutations in patients with thymidine phosphorylase deficiency. *J. Clin. Invest*. **111**, 1913-1921

Nishino, I., Spinazzola, A., Hirano, M. (1999) Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder. Science 283,689-692
Parker, W. B., Cheng, Y-c. (1994) Mitochondrial toxicity of antiviral nucleoside analogs. J. *NIH Res*. **6**, 57-61

Ponamarev, M. V., Longley, M. J., Nguyen, D., Kunkel, T. A., Copeland, W. C. (2002) Active site mutation in DNA polymerase associated with progressive external ophthalmoplegia causes error-prone DNA synthesis. *J. Biol. Chem*. **277**, 15225-15228

Pontarin, G., Ferraro, P., Valentino, M. L., Hirano, M., Reichard, P., Bianchi, V. (2006) Mitochondrial DNA depletion and thymidine phosphate pool dynamics in a cellular model of mitochondrial neurogastrointestinal encephalomyopathy. *J. Biol. Chem.* **281**, 22720-22728

Pontarin, G., Gallinaro, L., Ferraro, P., Reichard, P., Bianchi, V. (2003) Origins of mitochondrial thymidine triphosphate: dynamic relations to cytosolic pools. *Proc. Natl. Acad. Sci. U. S. A*. **100**, 12159-12164

Powell, D.R., Desai, U., Sparks, M.J., Hansen, G., Gay, J., Schrick, J., Shi, Z., Hicks, J. (2005) Rapid development of glomerular injury and renal failure in mice lacking p53R2. *Pediatr. Nephrol*. **20**, 432–440.

Rampazzo, C., Ferraro, P., Pontarin, G., Fabris, S., Reichard, P., Bianchi, V. (2004) Mitochondrial deoxyribonucleotides, pool sizes, synthesis, and regulation. *J. Biol. Chem.* **279**, 17019-17026

Rampazzo, C., Gallinaro, L., Milanesi, E., Frigimelica, E., Reichard, P., Bianchi, V. (2000) A deoxyribonucleotidase in mitochondria: involvement in regulation of dNTP pools and possible link to genetic disease. *Proc. Natl. Acad. Sci. U. S. A*. **97**, 8239-8244

Saada, A., Ben-Shalom, E., Zyslin, R., Miller, C., Mandel, H., Elpeleg, O. (2003) Mitochondrial deoxyribonucleoside triphosphate pools in thymidine kinase 2 deficiency. *Biochem. Biophys. Res. Commun.* **310**, 963-966

Saada, A., Shaag, A., Mandel, H., Nevo, Y., Eriksson, S., Elpeleg, O. (2001) Mutant mitochondrial thymidine kinase in mitochondrial DNA depletion myopathy. *Nat. Genet*. **29**, 342-344

SaveJude.com (2008). Mitojourny. Retrieved May 27, 2008, from Jude's Mitochondrial Disease Web site: http://www.judesmitojourney.com/index.htm

Scheffler, I.E. (2000) A century of mitochondrial research: achievements and perspectives. *Mitochondrion* **1**, 3-31

Schapira, A. H. V. (2006) Mitochondrial disease. *Lancet* **368**, 70-82

Song, S., Pursell, Z. F., Copeland, W. C., Longley, M. J., Kunkel, T. A., Mathews, C. K. (2005) DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity. *Proc. Natl. Acad. Sci. U. S. A*. **102**, 4990-4995

Song, S., Wheeler, L. J., Mathews, C. K. (2003) Deoxyribonucleotide pool imbalance stimulates deletions in HeLa cell mitochondrial DNA. *J. Biol. Chem.* **278**, 43893-43896

Spinazzola, A., Marti, R., Nishino, I., Andreu, A. L., Naini, A., Tadesse, S., Pela, I., Zammarchi, E., Donati, M. A., Oliver, J. A., Hirano, M. (2002) Altered thymidine metabolism due to defects of thymidine phosphorylase. *J. Biol. Chem.* **277**, 4128-4133

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraisi, K., Fukuda, S., Matsui, K., Takei, Y., Nakamura, Y. (2000) A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* **404**, 42-49

Taylor, R. W., Turnbull, D. M. (2005) Mitochondrial DNA mutations in human disease. *Nat. Rev. Genet.* **6**, 389-402

Thelander, L. Reichard P. Ribonucleotide Reductases *Annu. Rev.Biochem.* **48**: 133-158 (1979)

United Mitochondrial Disease Foundation (UMDF). Retrieved May 27, 2008, from Mitochondrial Diseases Web site: http://www.umdf.org/site/c.dnJEKLNqFoG/b.3042073/

Vigilant, L., Stoneking, M., Harpending, H., Hawkes, K. & Wilson, A.C. (1991) African populations and the evolution of human mitochondrial DNA. *Science* **253**, 1503−1507.

Wallace, D. C. (1999) Mitochondrial diseases in man and mouse. *Science.* **283**, 1482-1488

Wallace, D. C. (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Ann. Rev. Genet.* **39**, 359-407

Wang, L., Eriksson, S. (2003) Mitochondrial deoxyguanosine kinase mutations and mitochondrial DNA depletion syndrome. *FEBS Lett*. **554**, 319-322

Wang, L., Saada, A., Eriksson, S. (2003) Kinetic properties of mutant human thymidine kinase 2 suggest a mechanism for mitochondrial DNA depletion myopathy. *J. Biol. Chem.* **278**, 6963-6968

Young, J. P., Leeds, J. M., Slabaugh, M. B., Mathews, C. K. (1994) Ribonucleotide reductase: evidence for specific association with HeLa cell mitochondria. *Biochem. Biophys. Res. Commun.* **203**, 46-52