Mitochondrial DNA Priming by Mitochondrial RNA Polymerase

Mitochondria are DNA containing organelles and use their own RNA polymerases for both transcription and primer synthesis needed for replication of mitochondrial DNA (mtDNA). Human mitochondrial RNA (mtRNA) polymerase uses the same promoter, light strand promoter for both priming as well as polycistronic, genome length transcription. A similar mechanism of primer synthesis by RNA polymerase is found in replication of the E.coli ColE1 plasmid. In this system, however, transcription from an upstream promoter produces antisense RNA that inhibits downstream priming and favors transcription. Human mtDNA lacks that secondary promoter which could be used as a regulatory element. Therefore, human mtDNA is a unique system in which the molecular mechanisms governing the decision between primer formation and genomic length transcription are not completely understood.

Transitions from primer RNA to newly synthesized DNA have been mapped in vivo to sequences within or near to a region named the conserved sequence box II (CSBII). RNA processing enzymes were proposed to have a direct role in primer formation over genomic length transcription. Ribonuclease Mitochondrial RNA Processing complex (RNase MRP) has been shown to cleave transcripts at CSBII in a site-specific manner in vitro. This finding suggests that RNase MRP can produce free 3’ ends of RNA for priming, but whether RNase MRP is required for priming in vivo remains unknown. Alternatively, it is proposed that mtRNA polymerase may itself produce primers without need of extrinsic factors. In vitro transcription assays indicate that mtRNA polymerase terminates transcription with 60% efficiency at CSBII site. However, in this in vitro system, mtDNA replication machinery could not use those early terminated transcripts as primers, suggesting there is a clear need for additional factors even if early termination is important for primer formation.

Human mtRNA polymerase is a single subunit enzyme related to the T7 family of bacteriophage RNA polymerases. Interestingly, human and yeast mtRNA polymerases contain an additional a conserved N terminal domain, which is not found in T7 RNA polymerases. Partial deletion of this N terminal domain of yeast mtRNA polymerase has no effect on transcription but causes defects in mtDNA replication in vivo, suggesting a possible role for the N terminal domain of yeast mtRNA polymerase in priming. It is also reported that upon partial deletion of the N terminal domain, human mtRNA polymerase is still catalytically active in vitro, making it possible to question whether this domain plays a direct role in priming.

I hypothesize that extrinsic factors such as RNase MRP are required for priming and that the N terminal domain of human mtRNA polymerase has a direct role in primer formation.

Aim 1. Determine whether RNase MRP and/or other factors are required for priming.

The RNase MRP complex is found in the nucleus, where it is essential for processing of rRNAs, as well as in mitochondria. It consists of RNA which has the catalytic activity and several proteins. A specific region of the RNase MRP RNA has been identified which is essential for import of RNase MRP RNA into the mitochondria but at the same time is dispensable for transcription, nuclear localization and stability of the RNase MRP RNA. In order to test whether RNase MRP is required for priming of mtDNA in vivo, I aim to keep nucleus localized and cytoplasmic RNase MRP RNA functional but inhibit its import into mitochondria. I will delete the identified mitochondria targeting sequence to create a new RNase MRP RNA gene construct. By genomic targeting and homologous recombination in HEK293 cells, I aim to replace the endogenous RNase MRP RNA gene with my construct. I will confirm levels, localization and function of new RNase MRP RNA by Northern Blotting against RNase MRP RNA and rRNAs. I will isolate mtDNA and perform qPCR analysis to quantify mtDNA levels. Also, I will perform PCR analysis to map the 5’ end of newly synthesized mtDNA. Additionally, I will treat purified mtDNA with RNase H which cleaves RNA only if it is hybridized to DNA and perform PCR analysis to detect new 5’ ends after RNA degradation.
is required for primer formation in mitochondria, I expect only WT cells should produce new 5’ ends after RNase H treatment. If, however, in the absence of RNase MRP RNA in mitochondria, mtDNA levels are not decreased or mtDNA still produces new 5’ ends after RNase H treatment, then it is likely that RNase MRP is not required for primer formation. There might be yet to be identified redundant enzymes to process the primer or processing RNA might not be crucial for primer formation. Some other factors mediating primer hand off to mtDNA polymerase may be sufficient to use early terminated transcripts as primers. To address that, I will use different mitochondria fractions and incubate them with previously identified transcription and replication players\[^{8}\]. I will look for which fractions are capable of coupling priming to DNA synthesis in vitro.  

On the other hand, if RNase MRP is required for primer formation in vivo, the next step would be to determine the mechanism in vitro. Previously, it was shown that transcripts at CSBII site produce G quadruplex RNA structure\[^{14}\] and that RNase MRP complex cleaves transcripts in vitro at the CSBII site. However, it is still not clear whether RNase MRP recognizes DNA, RNA sequence or the secondary G quadruplex structure of RNA. I will address this question by altering G quadruplex structure of RNA in different ionic conditions\[^{14}\]. Additionally, I will test whether purified RNase MRP complex is sufficient to couple priming to DNA synthesis by mtDNA polymerase in vitro. Thus, these findings would be important to provide in vivo analysis of whether RNase MRP is required for priming and identification of other factors.

**Aim2: Determine the mechanism of how the N terminal domain of human mtRNA polymerase is involved in primer formation.**

Complete deletion of the N terminal domain of mtRNA polymerase creates defects in vitro in promoter melting, both in human and yeast\[^{8,16}\]. However, partial (half) deletion of the N terminal domain of human mtRNA polymerase has no effect on transcription in vitro\[^{8}\], similar to what has been observed for yeast in vivo\[^{10}\]. To test the effect of this deletion on priming in vivo, first I aim to down regulate endogenous mtRNA polymerase by siRNAs against 5’ UTR region of gene. I will confirm down regulation in HEK293 cells by RT-PCR analysis and western blotting. I will transfect and express my construct which lacks canonical UTR regions and has a partial deletion at the N terminal domain. I will check by Northern Blot analysis whether this deletion causes any defects in transcription of mtDNA in vivo. Similar to what has been observed for yeast, I expect this deletion to have no major effect on transcription. I will then test whether this deletion affects mtDNA replication in vivo. I will isolate mtDNA and perform qPCR analysis to compare levels of mtDNA with respect to WT HEK293 cells. Secondly, I will analyze and map the 5’ ends of newly synthesized mtDNA with the same techniques described earlier\[^{8}\]. If the N terminal domain is required for priming, I predict partial deletion of N terminal domain would create problems in mtDNA replication. If it does disrupt mtDNA replication in vivo, the next step would be to identify the mechanism of how the N terminal domain is involved in priming in vitro. If the N terminal domain is important for early termination, I expect upon partial deletion of the N terminal domain, mtRNA polymerase would not be able to terminate transcription at CSBII in vitro. Alternatively, the N terminal domain might be important for RNA-DNA hybrid formation, which is crucial for priming and longer than the canonical hybrid seen during transcription. I will test this model by RNase H and RNase A treatments following in vitro transcription assays on biotinylated mtDNA. It was previously shown that RNA-DNA hybrid is resistant to RNase A treatment, yet prone to RNase H degradation\[^{16}\]. If the N terminal domain is crucial for RNA-DNA hybrid formation, upon its partial deletion, I predict that in vitro transcripts would be washed away from DNA and no longer degraded by RNase H. To sum up, these experiments would be important to test whether the N terminal domain of human mtRNA polymerase has a direct role in priming and for identification of the mechanism.

This proposal aims to test the possible models of mtDNA priming by mtRNA polymerase. These findings would be helpful to understand the molecular mechanisms regulating the decision between genomic length transcription and primer formation on mtDNA.
Three possible models of priming

References: