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Mitochondrial mRNA fragments are circularized in a human HEK cell line

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ABSTRACT

The relatively recent focus on the widespread occurrence and abundance of circular RNAs (circRNA) in the human cell nucleus has sparked an intensive interest in their existence and possible roles in cell gene expression and physiology. The presence of circRNAs in mammalian mitochondria, however, has been under-explored. Mitochondrial mRNAs differ from those produced from nuclear genes because they lack introns and are transcribed as poly-cistronic transcripts that are endonucleolytically cleaved, leaving transcripts with very small 5′ and 3′ UTRs. Circular RNAs have been identified in the semi-autonomous organelles of single-celled organisms and plants but their purpose has not been clearly demonstrated. The goal of our project was to test the hypothesis, processed mRNAs are circularized in vertebrate mitochondria as a necessary RNA processing step prior to translation. Mitochondrial mRNAs were isolated from the human cell line HEK293 and evidence of circularization sought by treating RNA with RNAse-R and then amplifying putative 3′-5′ junction sites. Sequence results demonstrated the occurrence of mRNA circularization within each coding region of the mitochondrial genome. However, in most cases the circRNAs carried coding regions that had been truncated, suggesting they were not translatable. Quantification of the circularized versions of the mRNAs revealed they comprise a small portion (~10%) of the total mRNA. These findings demonstrate that mRNA circularization occurs in mammalian mitochondria but it does not appear to play a role in making translatable mRNAs.

1. Introduction

Mitochondria are an essential cukaryotic cell organelle responsible for energy production in the form of ATP. They possess their own genome independent of the nuclear genome encoding rRNAs, tRNAs, and genes ultimately leading to the expression of proteins necessary for oxidative phosphorylation. The modern mitochondrial genome is the product of an endosymbiosis of an α-protobacteria with an early eukaryotic ancestor (Small et al., 2013). Over time the mitochondrial genome has been simplified by drastic reduction to accommodate the energy demands of the modern eukaryotic cell (Rackham et al., 2012). With this reduction in size of the mitochondrial genome, gene expression has been modified and regulatory post transcriptional modifications arose.

Transcription of the mammalian mitochondrial genome is promoted bi-directionally from the D-loop region to create primary poly-cistronic transcripts (light and heavy) that extend nearly the entire length of the chromosome. The mRNAs flanked by tRNAs are released when the tRNAs are endonucleolytically cleaved by the enzymes RNaseP and RnaseZ (Holzmann et al., 2008; Sanchez et al., 2011), a process described by the punctuation model (Ojala et al., 1981). It is unknown how mRNAs unflanked by tRNAs are removed from the primary transcript. The individual mRNAs have very short or no 5′ and 3′ UTRs (Montoya et al., 1981; Temperley et al., 2010), and all but ND6 are polyadenylated by a mitochondrial specific polyA polymerase. These polyA additions stabilize transcripts and/or add the ultimate adenine necessary to complete a stop codon (Nagaike et al., 2005). In contrast, adenylation also occurs on truncated mRNAs where they appear to act as a degradation signal (Slomovic et al., 2005) and have also been located on small RNA fragments corresponding to mRNAs that are likely to be degradation products (Kuznetsova et al., 2017).

Circularized RNAs were discovered in viroids (Sänger et al., 1976) but it wasn't until the advent of high-throughput sequencing that their abundance in eukaryotic cells was appreciated (Salzman et al., 2012; reviewed in Wilusz, 2018). The majority of described eukaryotic circRNAs are generated from nuclear encoded genes where the spliceosome catalyzes the circularization of exons by way of a backsplicing mechanism using repeat sequences in flanking introns (Kramer et al., 2015). Their exact role and function is currently unknown however it is thought that they can alter microRNA levels, modify gene expression, act as a template for translation, or bind directly to proteins as repressors. They are also potential biomarkers for specific diseases (Qu

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et al., 2015; Wilusz, 2018). Circular RNAs are produced in organelles but these have primarily been described in yeast (Amberg et al., 1980), plants (Darbani et al., 2016; Sun et al., 2016), and algae (Cahoon and Qureshi, 2018). In the plant examples the circRNAs are fragments of coding regions whose role in gene expression is uknown (Darbani et al., 2016; Sun et al., 2016). In the case of the green alga *Chlamydomonas reinhardtii*, the circularization links the 3′ UTR to leaderless 5′ termini of full length mitochondrial mRNAs and may facilitate translation initiation (Cahoon and Qureshi, 2018). Since mammalian mitochondrial mRNAs have small to non-existent 5′ UTRs similar to *C. reinhardtii* we posed two questions. Do vertebrate mitochondria produce circular RNAs of their coding regions? Do these circular RNAs carry full length coding regions that could be translated? In this study we used RNA extracted from the human cell line HEK293 and a restrictive PCR assay to test the hypothesis: processed mRNAs are circularized in vertebrate mitochondria as a necessary RNA processing step prior to translation.

2. Materials and methods

HEK293 cells were obtained as a generous gift from Professor Walter Chazin, Vanderbilt University. Cells were cultured in 1x DMEM supplemented with 10% FBS under standard growth conditions (37 °C, 5% CO_2), and grown to confluence in 15 cm tissue culture dishes. Cells were washed three times using ice-cold 1x DPBS, harvested by scraping, and collected by centrifugation at 2000 × *g* for 2 min. Supernatant was removed and the harvested cells were immediately resuspended in the lysis buffer provided in Qiagen's RNeasy kit (Valencia, CA). The slurry was stored at −80 °C until RNA could be extracted using the manufacturer's instructions which included the optional DNase treatment.

Circularized mRNAs were PCR amplified using two sets of nested divergent primers (purchased from Integrated DNA Technologies, Coralville, IA) per coding region (Table S1) so only cDNAs made from circular mRNAs could be PCR amplified. This is similar to the methodology used in Cahoon and Qureshi (2018) and outlined in Fig. 1. Briefly, 2 μg of total RNA was treated with 20 U of RNase-R (Lucigen, Madison, WI) at 37 °C for 5 min to remove linear RNAs. After a heat inactivation step, the remaining circ-enriched RNA was converted to cDNA using MMLV reverse transcriptase (Promega, Madison, WI, Cat. # M1701) and gene specific primers (Table S1, primers ending in –PCR1_R). The 3′ and 5′ junctions of the circularized mRNAs were PCR amplified (Phusion polymerase, ThermoFisher, Waltham, MA) using the primers labelled 'PCR1-F' and 'PCR1-R' (Table S1) to produce primary products. To increase the specificity of the amplicons to be sequenced, each primary product was re-amplified using a second set of nested primers ('PCR2-F' and 'PCR2-R'). Amplicons were gel purified using the Zymoclean gel recovery kit (Zymoresearch Tustin, CA), and deep sequenced by Wright Labs (Huntingdon, PA). All sequence analysis was completed using Geneious Prime software v.11 (https://www.geneious.com). Unmerged sequences were trimmed to remove any bases with < 95% certainty and aligned to their coding regions. Sequences annealing to the 5′ or 3′ end of a coding region with 95% sequence identity were retained. Those sequences were sorted and like sequences binned as contigs using the *de novo* assembly option embedded in Geneious Prime. Contigs representing fewer than 50 sequences were discarded. For the remaining contigs, all were compared to the human mitochondrial genome using BLAST and 5′ and 3′ junctions indicative of circularized RNAs were identified. The production of amplicons was completed three or more times for each coding region. Sequencing was completed twice.

Linear and circular transcripts were quantified using Bio-Rad's (Hercules, CA) cfx Real-Time PCR system. Total mRNA was quantified for each transcript using a standard convergent primer pair (Table S1) that produced a single amplicon and were optimized per MIQE guidelines (Bustin et al., 2009). cDNA for total mRNA quantification was produced using 2 μg of total RNA as template, MMLV reverse transcriptase, and the gene specific primers designated 'PCR3-R' (Table S1).

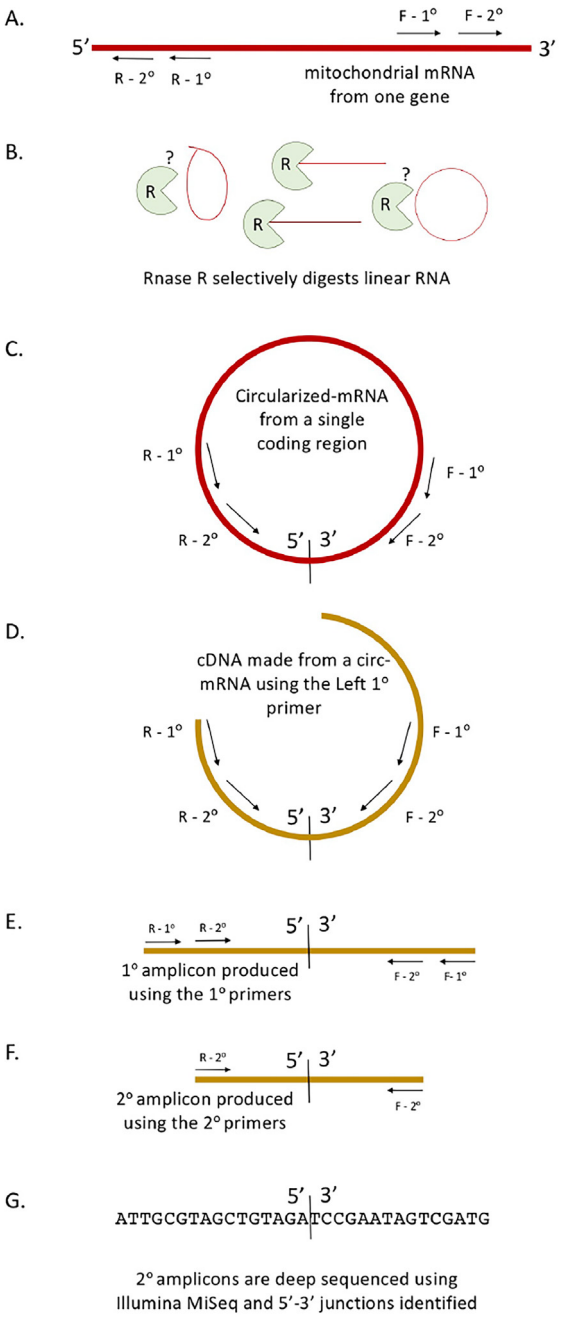

Fig. 1. Divergent primers were designed to produce cDNA and PCR amplicons from each mitochondrial mRNA (A). The exonuclease, RNaseR was used to remove linear RNAs (B). The divergent primers would be convergent and produce products only if the mRNAs occurred as circularized molecules (C). cDNAs were made from each mRNA using the 'R-1' primary primer (D). cDNAs were used as template in 'primary' PCR reactions that used the 'primary' primer pair (E) Products from the 'primary' PCR reaction were used as template to produce amplicons using the 'secondary' primer pair (F). Amplicons were deep sequenced (G).

qPCR reactions contained 1 μL of the cDNA reaction mix, 5 pmol of each primer ('PCR3-F' and 'PCR3-R'), 5 μL of qPCR master mix (Maxima SYBR Green/Fluorescein qPCR master mix, Thermo Scientific, cat. # K0241), and water for a final volume of 10 μL. DNA contamination controls were completed by removing the reverse transcriptase steps from the assay. A small amount of DNA was detected in all the biological replicates and was subtracted from the total cDNA estimate. circRNA was quantified using the same methodology but the 'PCR2-R'

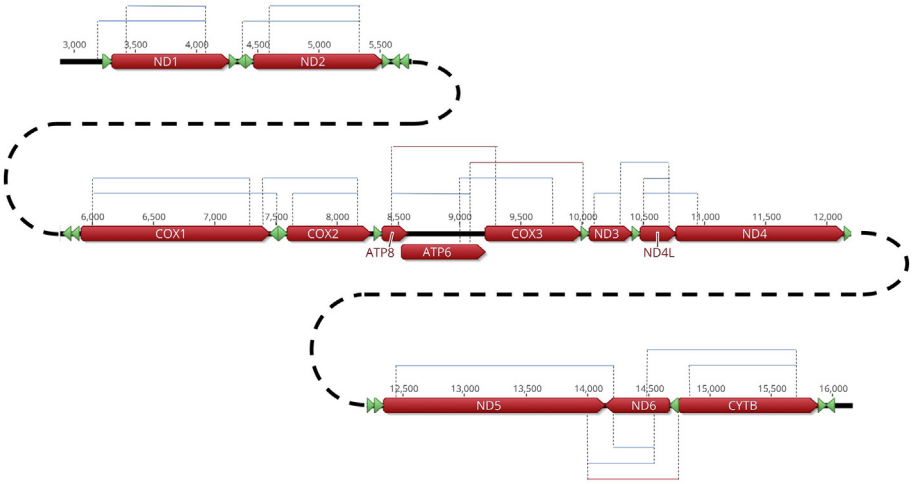

Fig. 2. The portion of the human mitochondrial genome with protein coding genes is represented by a dark line with maroon boxes (protein coding genes) and green arrowheads (tRNA coding genes). The fragments of the heavy strand primary transcript found to be circularized are represented as lines above the genome. Blue lines represent a fragmented coding region, red lines represent full length. Circularized fragments of the light strand primary transcript are below the genome. The genome diagram was initially generated using Geneious Prime. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gene specific primers were used for cDNA production and the 'PCR2-F' and 'PCR2-R' primers for qPCR. RNase treated templates were incubated with 20U of either RNase-R or RNase-A at 37 °C for 5 min prior to cDNA production. For each condition, RNA from three biological replicates plus three technical replicates of each of those was quantified (n = 9). Crossover threshold values were converted to an estimate of cDNA copy number using the methodology described in Grimes et al. (2014).

3. Results

Amplicons were produced from putative circularized RNAs derived from the 13 protein coding transcripts. Analysis of the amplicon sequences revealed 105 circular mRNA variants of 23 regions which are represented as linear fragments in Fig. 2. Nearly all of the circRNAs were made of fragments of coding regions and fell into three general categories. 1 - An internal fragment of the coding region that lacked both start and stop codons – e.g. *nd1*, *nd2*, *cox1*, *cox2*, *nd3*, *nd4L*, *nd6*, and *cytB*. 2 – A fragment of the coding region missing either the start or stop codon on one end while the opposite end extended into the coding region of an adjacent gene – e.g. *nd1*, *nd2*, *cox1*, *cox2*, *atp8*, *atp6*, *cox3*, *nd4L*, *nd5*, *nd6*, and *cytB*. 3 – The entire coding region was intact because the termini occured in the interiors of adjacent genes – e.g. *atp6*, *cox3*, and *nd6*. The circularized regions varied in length due to differences in the 3′ and/or 5′ termini that were ligated. Fig. 3 is a box plot demonstrating the variations in the sizes of each circularized RNA fragment. There was a wide variety of sizes but the variations followed no discernable pattern. For example, the internal cat. 1 circRNAs of nd2 were much more variable than the cat. 2 circRNAs of nd2 that included a portion of an adjacent tRNA. A similar comparison of the cat. 1 and cat. 2 circRNAs from cox1 reveal that both have narrow size ranges.

qPCR experiments were performed to compare steady state amounts of circRNA compared to total mRNA and to test whether the amplicons were produced from circRNAs rather than linear RNA or DNA (Fig. 4). circRNA steady state amounts of *cox1*, *nd2*, *nd5*, and *nd6* mRNAs as measured using divergent primers were found to exist as relatively small proportions of the total mRNAs detected using convergent primers (ranging from 8 to 12.6%). To test whether the amplicons analyzed by deep sequencing had been produced by circular RNAs, as hypothesized, or alternatively by linear RNAs produced by some form of splicing, RNA template was treated with RNase-R. This reduced the amount of template RNA in all four cases but did not eliminate it, suggesting the presence of circRNA. The non-selective RNase-A enzyme was used to test if the amplicons had been made from rearranged mitochondrial DNA that could occur in the nuclear genome rather than RNA. RNase-A removed all template, demonstrating that the amplicons had been produced from RNA.

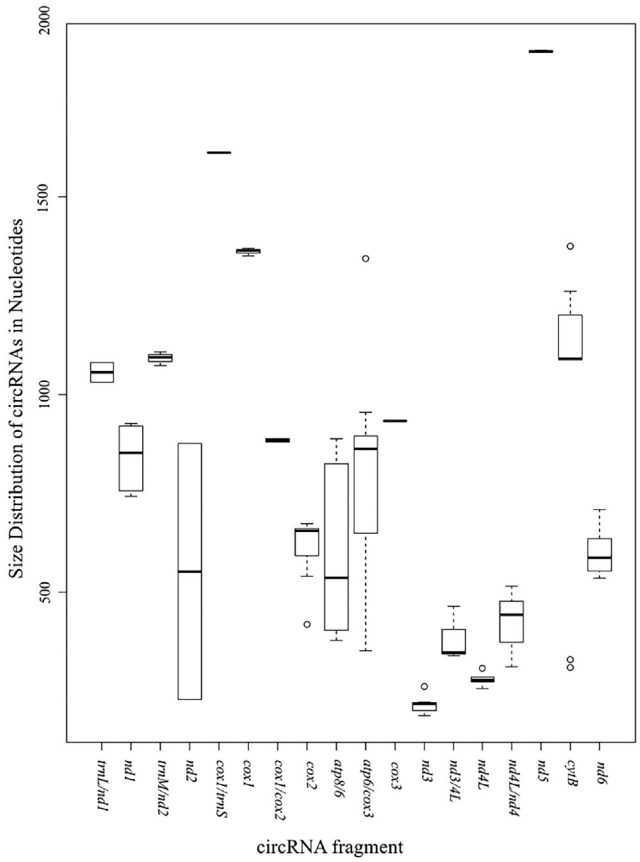

Fig. 3. The size distribution of each of the major circRNAs characterized in this study.

The 105, 3′-5′ junction sites were aligned to deduce the possibility of sequence conservation in and around the truncation and/or ligation sites. There was a nucleotide bias towards cytosine (followed by adenine) 7–8 nucleotides on either side of the 3′-5′ junction sites (Fig. 5). None of the circRNA fragments had 3′ polynucleotide additions (e.g. polyA) at the junction site.

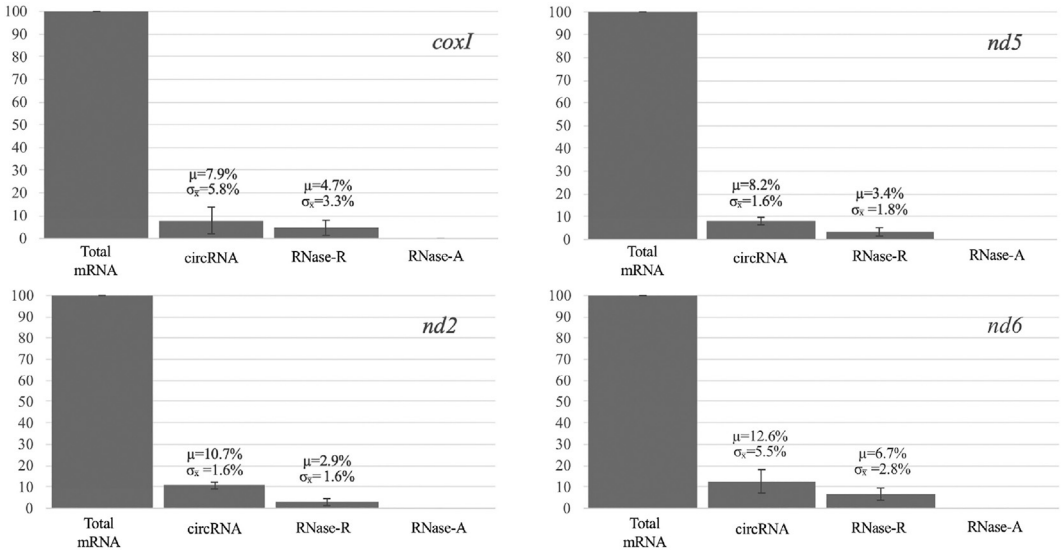

Fig. 4. qPCR quantification of the total mRNA (linear plus circular) from four mitochondrial genes was determined using standard convergent primers. The amount of total mRNA starting template was set at 100% for comparison. Total circRNA from fragments of each gene was determined using divergent primers. The circRNA assay was repeated using template RNA that had been treated with RNase-R or RNase-A. Each condition represents the results from three technical replicates completed from three biological replicates (n = 9).

4. Discussion

4.1. circRNAs occur in mammalian mitochondria

Circular RNAs have been characterized in human cells from a wide array of tissue and cell sources but with an exclusive focus on RNAs transcribed in the nucleus (reviewed in Wilusz, 2018). Naturally occurring circRNAs have been reported from mitochondria and chloroplasts of yeast, plants, and algae (Amberg et al., 1980; Darbani et al., 2016; Sun et al., 2016; Cahoon and Qureshi, 2018; Liu et al., 2019). Zhang et al. (2019) briefly reported 118 mitochondrial circRNAs in their sub-cellular survey of circRNAs from a human cell line HepG2, but chose to exclude them from further analyses due their low abundance compared to circRNAs of nuclear origin. In this paper we describe experiments intended to test the following hypothesis: Processed mRNAs are circularized in mammalian mitochondria as a necessary RNA processing step prior to translation. This was inspired by perceived similarities in gene density and transcript processing between mammalian mitochondria and those in the green alga, *C. reinhardtii* where leaderless transcripts are circularized as a possible strategy to provide a 5′ UTR and ribosome binding sites for translation. We report the presence and characterization of 105 circRNAs made from the protein coding regions of the human cell line HEK293. All but three were made from partially truncated coding regions so we reject our initial hypothesis since these circRNAs would be incapable of supporting translation. We also report that the circularized fragments occur as a small proportion of the total amount of transcript present for a particular gene. This small copy number is consistent with the observations of Zhang et al. (2019). A caveat regarding the quantification experiments is that the amplicons produced from the divergent primer sets were comprised of an admixture of fragments (see Fig. 3). This means they did not conform to MIQE guidelines (unlike the convergent primer sets) so we caution the reader to consider the circRNA quantifications to be rough estimates. Also, it is for that reason results from only four genes are presented. The amplicon melt curves for those four genes were consistent between the untreated circRNA and RNase-R treated samples so we were confident the same amplicons were being compared. This was not the case for the other mRNAs, so even though the quantifications were very similar to the four genes that are presented, we were uncertain the data were reliable. A second aspect of the quantification experiments that requires explanation is that RNase-R treatment reduced the amount of putative circularized RNAs. This reduction is consistent with the observations reported in Cahoon and Qureshi (2018) and we interpret them as indicating that some of the circRNAs are linearized by a natural process or by shearing during extraction.

The naturally occurring circRNAs we report from HEK293 cells

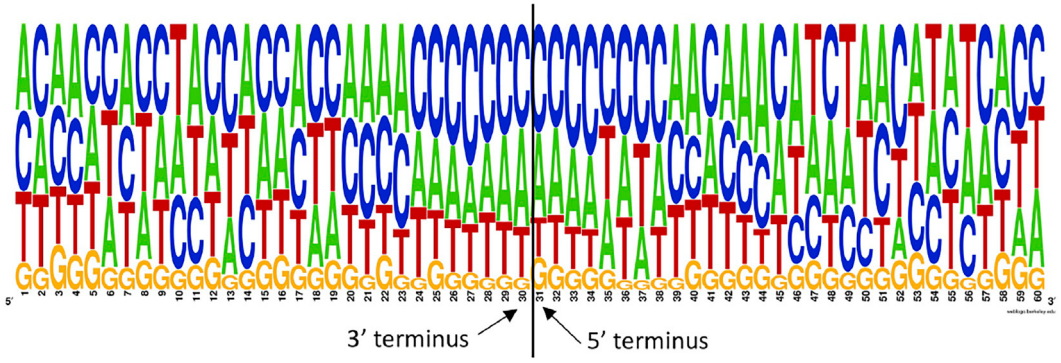

Fig. 5. The circRNA junction sites of the 105 circularized transcript fragments were aligned and a Logo plot generated to determine the most common nucleotide found at each position of the ligated 3′ and 5′ termini. The phosphodiester bond between the 3′ and 5′ termini is represented as a vertical black line. The plot was produced using WebLogo (Crooks et al., 2004).

made of truncated fragments of coding regions are consistent with some of the artificially circularized RNAs reported in mouse mitochondria by Kuznetsova et al. (2017). In that study, mitochondrial RNAs were artificially circularized and sequenced to map transcript processing intermediates. Naturally occurring circRNAs would have been inadvertently included in their analyses and interpreted as RNA processing intermediates. Interestingly, they reported that cells lacking a mitochondrial RNAseP endonuclease had a sharp increase in the circRNAs similar to the natural ones we report. This suggests that these natural circRNAs may be produced from specific processing intermediates awaiting digestion by endonucleases. The ligation process is considered to be inefficient (Lasda and Parker, 2014) and human mitochondrial circRNAs comprise a small portion of total organellar RNA, so if RNaseP is missing perhaps it increases the amount of available substrate for the ligation process.

The presence of naturally occurring circRNAs from predictable regions of primary mammalian mitochondrial transcripts insinuates a circularization mechanism and suggests they may play some functional role in mitochondrial maintenance. The most direct mechanism for the creation of circRNAs would hypothetically be a mitochondrial localized RNA ligase. Eukaryotic RNA ligation has been recognized for decades (Filipowicz and Gross, 1984), but to the best of our knowledge no RNA ligase has been found with a mitochondrial localization signal peptide. To speculate, a mitochondrial mechanism would hypothetically resemble a prokaryotic one. Recently, a RNA ligase was found to circularize a wide variety of RNAs (mRNAs, tRNAs, rRNAs, and non-annotated fragments) in the archaeon, *Pyrococcus abyssi* (Becker et al, 2017). Their work demonstrated a mechanism for the production of circRNAs in prokaryotes and may offer an avenue to identify similar enzymes encoded in eukaryotic nuclear genomes. Regarding the function of mitochondrial circRNAs, we can only offer conjecture based on the roles circRNAs play in other systems. For example, nuclear encoded circRNAs can act as miRNA “sponges” which would regulate RNA degradation (Zheng et al., 2016). They also directly interact with proteins to repress function (Du et al., 2016). Mitochondrial circRNAs in Barley have been shown to fluctuate in relation to micronutrient levels (Darbani et al., 2016) suggesting they play a role in cellular homeostasis by way of mitochondrial gene expression. With the randomness of the circularized fragments we observed and the relatively low amount of them it is also possible that these circRNAs may be part of a quality control mechanism to sequester incorrectly processed or sheared mRNAs.

4.2. Conserved repetitive sequences may be important for the mitochondrial ligation process

Mammalian nuclear circRNAs are produced by a backsplicing mechanism that requires a repetitive sequence such as ALU elements or other micro-repeats pairing (Jeck et al., 2013; Liang and Wilusz, 2014). Yeast does not appear to require complementary repeats and circularizes RNAs with lariat precursors (Barrett et al., 2015). No mechanism has been proposed for mitochondrial circRNA production so it is unknown if conserved or repeated sequences are necessary to complete the process. We aligned the 3′ and 5′ junction sites and found a nucleotide bias towards cytosine and adenine 7–8 nucleotides on either side of the junction site. In the green alga, C. reinhardtii mitochondria, circRNAs were found to contain a 3′ polyC nucleotide addition (Cahoon and Qureshi, 2018). The sequence bias in these human cells and the polyC addition in Chlamydomonas raises the possibility that there may be a nucleotide bias or conservation associated with the ligation process. This nucleotide bias suggests purposeful ligation of specific fragments which is inconsistent with the incorrect mRNA sequestration hypothesis offered in the previous section.

5. Conclusions

We provide the first characterization of circRNAs in human/mammalian mitochondria. The circRNAs we characterized were all derived from fragments of transcripts that were improperly processed for translation and comprised a small portion of the total RNA. It is possible that these circRNAs have a yet to be determined role in mitochondrial gene expression.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2019.11.002.

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