EMBO reports

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Exploring our origins—the importance of OriL in mtDNA maintenance and replication

Robert Neil Lightowlers & Zofia Maria Alexandra Chrzanowska-Lightowlers

In this issue of EMBO *reports*, an article by Falkenberg *et al* [1] reports *in vitro* and *in vivo* experiments to help resolve the controversy regarding the mechanism of mammalian mitochondrial DNA (mtDNA) replication. After a series of impressive experiments by Clayton and others in the 1970s and 1980s a model of mammalian mtDNA replication was proposed and generally accepted [2]. This model was simple but was also unusual, lacking a strong precedence in nature. It posited that replication was initiated by priming at a promoter element in the non-coding region, known as the light (L)-strand promoter (LSP)—as the two strands had previously been shown to have slightly different buoyant densities on caesium chloride gradients—with DNA polymerase γ extending the primer just downstream from the origin of heavy-strand replication (OriH). Replication of the L-strand initiated once the origin OriL became exposed, which occurred only after approximately two-thirds of the H-strand had been synthesized, folding into a stem–loop structure to facilitate priming. Subsequently, synthesis was primed at this structure and the entire L-strand was copied (Fig 1A; [3]). This is described as the 'strand asynchronous' or 'strand-displacement' model and requires neither coupling of strands during the replication process, nor Okazaki fragment production by classic lagging-strand synthesis. This mechanism had been accepted, but an additional model was raised in 2000 [4]. Holt and colleagues employed the classical method of neutral–neutral two-dimensional gel electrophoresis for the first time to investigate replication intermediates of mtDNA. Single-stranded nuclease-insensitive intermediates were discovered that were consistent with strand synchronous and unidirectional replication (Fig 1B). This more classical strand synchronous mode was most apparent in cultured cells that had been treated to promote an increase in mtDNA replication, prompting the authors to conclude that these intermediates were found in addition to those that would be predicted from the Clayton model. In a following report [5], little evidence of single-stranded replication intermediates could be found in highly purified mitochondria from animal tissue. Observing that ribonucleotides were present in major parts of the newly synthesized L-strand, it was suggested that in the earlier studies regions of nascent L-strand might potentially have been degraded by RNase H activity before isolation of mitochondria. This has led to intense debate between protagonists. There has also subsequently been a series of refinements to the strand-coupled method and a model whereby RNA intermediates are laid down as lagging-strand intermediates, known as RNA incorporation throughout the lagging strand (RITOLS; [6]). Further, Clayton and colleagues reported putative origins additional to OriL for L-strand replication [7]. The result has been that few in the mitochondrial research community feel they can judge sufficiently how mammalian mtDNA replicates under physiological or even non-physiological conditions.

One prediction that separates the two original models is the role of what was initially described as the origin of L-strand replication. The strand asynchronous model describes an essential role for this *cis*-acting element, whereas the strand-coupled model designates no specific function. The mammalian mitochondrial genome is found in many copies per cell and it has been described for more than 20 years that subsets of molecules can survive and propagate with large-scale deletions. This is often the case in patients with particular syndromes associated with mitochondrial dysfunction. Strikingly, most of these, often large, deletions spare OriL. In fact, one early claim that a pathogenic deletion had been mapped to show loss of OriL proved on further evidence to have been a partly duplicated mtDNA molecule containing more than one origin [8]. This *in vivo* data would strongly support the importance of OriL in human mtDNA maintenance. Surely it must be simple to show the crucial nature of this element by generating well-chosen mutations in this sequence, transfecting mitochondria and observing replication of the mutated genomes? Unfortunately, such an experiment is impossible, as there is no established, robust and accepted method for transfecting mitochondria in intact mammalian cells [9].

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Into this rather opaque arena step Falkenberg and colleagues. The authors had previously shown that human mitochondrial RNA polymerase can prime DNA synthesis from the stem–loop structure found *in vitro* at OriL (10). To determine the importance of this structure *in vivo*, they have now turned to the ploymerase gamma mutator mouse. This mouse carries a mutation in the proofreading subunit of DNA polymerase gamma, which effectively

performs saturation mutagenesis of mtDNA *in vivo*. Consequently, propagation is allowed of only mutated mtDNA that can still be efficiently replicated and maintained. Falkenberg *et al* argue that if this OriL domain was indeed essential for replication it should be relatively spared from the accumulation of mutations compared with other regions of mtDNA. To test their hypothesis they analysed an approximately 1 kb region of mtDNA spanning the designated OriL region and compared this with regions encoding tRNAs, open-reading frames and non-coding sequence. Their data support their case, as the Wanrooij et al paper published in this issue of EMBO *reports* shows that the point mutation frequency is reduced over OriL. Mutation analysis revealed an interesting bias as to which regions could more easily tolerate changes. The non-template side of the stem and the loop were found to be more tolerant to mutation with a surprisingly high variation of insertion and deletion permitted within the loop (figure 1C in [1]). This result was recapitulated by data from their impressive reconstituted *in vitro* replication system that used synthetic versions of the wild-type or mutated human OriL orthologue as template. Thus, *in vivo* and *in vitro* approaches presented here allow the authors to delineate precisely the structural requirements of human OriL that were needed to prime lagging-strand synthesis of these synthetic DNA substrates. Finally, bioinformatics programmes were used to identify putative OriL sequences in an extensive number of vertebrate mitochondrial genomes—although an OriL-like sequence has yet to be identified in the chicken genome [11]. Further data mining of other species suggests that this feature evolved early in vertebrates and seems highly conserved.

There is no doubt that this elegant body of work highlights the importance of a region in the genome referred to as OriL. However, how can we reconcile the claim that mtDNA replication intermediates are essentially duplex at all times, irrespective of whether they are DNA:DNA or RNA:DNA [12]? Holt and colleagues have also reported something of note at or around the OriL when investigating replication intermediates. Indeed, in the RITOLS paper, Yasukawa *et al* suggested the region around OriL might be where the lagging-strand RNA begins to be replaced by DNA in the process of strand maturation [6].

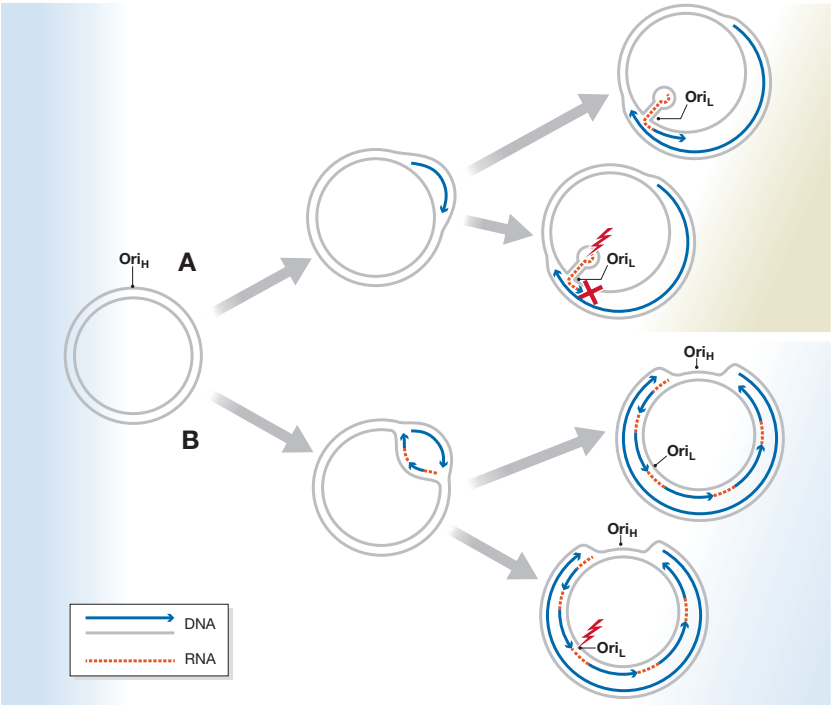

Fig 1 | Models of mammalian mtDNA replication. (**A**) Strand displacement or asynchronous mode of replication. DNA synthesis initiates at OriH. L-strand synthesis occurs only after approximately two-thirds of nascent H-strand has been made. The stem–loop structure at OriL is formed and DNA synthesis is primed by mitochondrial RNA polymerase. L-strand synthesis is continuous. Mutations in the region of OriL would be predicted to affect mtDNA replication. Wanrooij *et al* have mapped mutations around this site *in vivo* and *in vitro*, concluding that this region is indeed essential for mtDNA synthesis [1]. (**B**) Strand-coupled mode of replication. DNA synthesis initiates in a unidirectional mechanism from both strands within the non-coding region. The lagging-strand synthesis is discontinuous. In this model, it is not predicted that mutations in the region originally referred to as OriL would affect mtDNA replication. OriH, origin of heavy-strand replication; OriL, origin of light-strand replication; mtDNA, mitochondrial DNA.

The beauty of this report by Falkenberg and colleagues is that it gathers *in vivo* genetic, *in vitro* reconstitution and bioinformatic data together for the first time to provide evidence for the strand asynchronous model of mammalian mtDNA replication. The balance of evidence would seem to support such a replicative mode. The question remains whether mtDNA always and exclusively replicates in this manner *in vivo*, or whether other mechanisms are used under certain physiological conditions, as originally suggested by Holt *et al*? Until a simple and reliable method for mitochondrial transformation is found, the question of whether mammalian mtDNA can ever be replicated in a strand-coupled mode, or indeed in the absence of OriL, might be extremely difficult to answer unequivocally.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Robert Neil Lightowlers and Zofia Maria Alexandra Chrzanowska-Lightowlers are at the Wellcome Trust Centre for Mitochondrial Research at Newcastle University, UK.
E-mail: r.n.lightowlers@ncl.ac.uk

EMBO *reports* (2012) **13,** 1038–1039; published online 13 November 2012; doi:10.1038/embor.2012.175