Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone

Mark Bowmaker,¹ Ming Yao Yang,¹ Takehiro Yasukawa,¹ Aurelio Reyes,¹,² Howard T. Jacobs,³ Joel A. Huberman⁴ & Ian J. Holt.¹*

1. Dunn Human Nutrition Unit, Wellcome Trust-MRC Building, Hills Road Cambridge, CB2 2XY, UK.
2. Sezione di Bioinformatica e Genomica di Bari. Istituto Tecnologie Biomediche. CNR. Via Amendola 168/5, 70126 Bari, Italy.
3. Institute of Medical Technology and Tampere University Hospital, FIN-33014, University of Tampere, Finland, and Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK.
4. Department of Cancer Genetics, Roswell Park Cancer Institute, Elm & Carlton Streets, Buffalo, NY 14263-0001, USA.

* address for correspondence
email holt@mrc-dunn.cam.ac.uk
tel. + 44 (0) 12 23 25 28 40/2
fax. + 44 (0) 12 23 25 28 45

Running title: mitochondrial DNA origin of replication

The first two authors made an equal contribution to the study.
ABSTRACT

Previous data from our laboratory suggested that replication of mammalian mitochondrial DNA initiates exclusively at or near to the formerly designated origin of heavy-strand replication $O_{H}$, and proceeds unidirectionally from that locus. New results obtained using two-dimensional agarose gel electrophoresis of replication intermediates demonstrate that replication of mitochondrial DNA initiates from multiple origins across a broad zone. After fork arrest near $O_{H}$, replication is restricted to one direction only. The initiation zone of bidirectional replication includes the genes for cytochrome $b$ and NADH dehydrogenase subunits 5 and 6. The products of bi-directional theta replication include previously characterized ribonucleotide-rich replication intermediates, suggesting that bi-directional theta replication is the major mechanism for copying mammalian mitochondrial DNA in solid tissues, irrespective of the extent of ribonucleotide incorporation.
INTRODUCTION

Neutral/neutral two-dimensional agarose gel electrophoresis (N/N 2D-AGE) has been widely used to define a variety of replication intermediates (RIs) (1-4). Initiation of replication within a DNA restriction fragment gives rise to a characteristic initiation or “bubble” arc, whereas origin-less fragments that are passively replicated by forks moving from one end to the other generate characteristic “Y” arcs (5). The technique of N/N 2D-AGE has proved applicable to mapping replication origins in the whole range of living organisms, from simple plasmids (5,6) to complex eukaryotic genomes (7,8). The method can identify other features of the replication process such as fork arrest. Replication fork barriers (RFB) produce spots on Y arcs (2). The higher the frequency of fork arrest the stronger the spot and the weaker the arc downstream of the RFB.

Mitochondrial DNA (mtDNA) of mammals is a closed circular molecule of approximately 16.5 kb; it encodes 13 proteins that contribute to oxidative phosphorylation (Fig. 1A). Replication of mtDNA has been studied in a number of organisms using N/N 2D-AGE (9-12). The patterns of RIs obtained were consistent with conventional coupled leading- and lagging-strand (or strand-coupled) replication. However, for many years it was believed, based initially on electron microscopic images of mtDNA of cultured cells, that mammalian mtDNA was replicated primarily via a unique, strand-asymmetric, unidirectional mechanism (13) (Fig. 1B). More recently we used N/N 2D-AGE to demonstrate that human, mouse and rat mtDNA replication involves conventional, double-stranded replication intermediates (RIs) with coupled leading- and lagging-strand synthesis in addition to partially single-stranded molecules that appeared consistent with a strand-asymmetric mechanism (14). In a later study, the isolation procedure was improved and the more highly purified mitochondria yielded only replication intermediates that were substantially duplex; moreover, the partially single-stranded forms seen in the previous study could be generated by RNase H treatment. Hence, it was concluded mammalian mtDNA replication intermediates contain extensive ribonucleotide patches, and apparent strand-asymmetric replication intermediates result from RNA degradation during isolation (15). Two major objections to the conclusions of this study were raised recently; first that partially single-stranded molecules (including products of strand-asymmetric replication) would not be detected by N/N 2D-AGE due to branch-migration and second that blocked restriction sites could arise from transcription intermediates (16). However, intact partially single-stranded replication intermediates have been analysed successfully using N/N 2D-AGE (17), and in any case branch migration of nascent DNA strands would produce a linear single-strand arc on N/N 2D agarose gels (18).
Transcription intermediates would inevitably be partially single-stranded whether or not replication was occurring on the same molecule, whereas the novel slow-moving Y-like arcs we described were substantially duplex (15). Although extensive ribonucleotide incorporation created duplex RIs akin to strand-coupled RIs (15), it was not known where or by what mechanism these intermediates originated.

When we earlier screened the mammalian mitochondrial genome for origins of replication, we detected initiation arcs exclusively in restriction fragments that included the previously defined, strand-asymmetric origin of replication, \(O_H\) (14). Based on this observation, we proposed that strand-coupled replication was unidirectional, with an origin close to or coincident with \(O_H\) (Fig. 1C). \(O_H\) has been mapped precisely in humans (19) to nucleotide position (np) 191 of the 16,569 bp mtDNA molecule (20). It was mapped at np 16,008 of the (differently numbered) 16,298 bp rat mitochondrial genome (21) and in the mitochondrial genome of mouse to np 16,065 (22). However, our previous study (14) did not exclude an alternative model, namely bi-directional, strand-coupled replication, initiating at one or more sites downstream of \(O_H\), followed by fork arrest at \(O_H\) (Fig. 1D and Fig. 2 scenarios II and III). Our original interpretation depended on the assumption that molecules derived from \(O_H\)-containing fragments coincident with a simple Y arc represented broken bubbles (14). The assumption was tenuous, given that it had earlier been shown that broken bubbles form arcs distinct from a simple Y arc (23).

Here we report that more detailed mapping studies refute our previous model. Instead they support the alternative proposition, in which initiation of strand-coupled replication occurs downstream of \(O_H\). Initiation of strand-coupled mtDNA replication is not restricted to the large non-coding region, which contains the major promoters for the two strands of mammalian mtDNA. Moreover, strand-coupled replication is the major mechanism of replication of mammalian mtDNA irrespective of ribonucleotide incorporation.
EXPERIMENTAL PROCEDURES

Mitochondrial DNA was extracted from highly purified mitochondria of human placenta and mouse or rat liver as described previously (15) with one modification. Whereas previously mitochondria were lysed immediately after pelleting following isolation from a density sucrose-gradient, in this study purified mitochondria were resuspended in 75mM NaCl, 50mM EDTA (pH 7.6) with 500 micrograms/ml Proteinase K without detergent. After incubation at 50 °C for 30 minutes, mitochondria were lysed by the addition of SDS to a final concentration of 0.5% and the incubation extended for a further 120 minutes. DNA was precipitated from solution after successive phenol and chloroform extractions, and resuspended in 10 mM Tris/0.1mM EDTA (pH 7.6). Mitochondrial DNA (0.1-1 mg) was digested with restriction endonucleases under conditions recommended by the manufacturer (New England Biolabs). Where indicated, single-strand nuclease treatment was with one unit of S1 nuclease (Promega) for 120 seconds at 37 °C after restriction digestion.

Two-dimensional agarose gel electrophoresis and hybridization. For fragments of two to five kilobase pairs (kbp) in size neutral/neutral two-dimensional agarose gel electrophoresis was performed by the standard method (3). Briefly, first dimension electrophoresis was 0.7V/cm for 20 hours at room temperature in a 0.4% agarose gel, without ethidium bromide (EB); the second dimension gel was 1% agarose with 300 ng/ml EB, electrophoresis was 6V/cm for four hours at 4°C. In the case of fragments >5 kb first dimension electrophoresis was in a 0.35% agarose gel at 1.5V/cm for 20 hours, and second dimension electrophoresis was 3V/cm for 18 hours in a 0.875% agarose gel. After Southern blotting, specific regions of human, mouse or rat mtDNA were amplified by the polymerase chain reaction and then radioactively labeled using random primers. Five microlitres of [α-32P] dCTP (3000 Ci/mmol, Amersham) was incubated with three units of Klenow DNA polymerase and 50 ng of DNA that had been annealed with 50 ng of hexadeoxyribonucleotides, for 15-30 minutes at 37°C. Oligonucleotide primers and the region of mtDNA each pair amplified are listed below.

Human mtDNA

From (20): probe h1 5'-TAACCACTCACGGGAGCTCT-3' and 5'-AAGGCTAGGACCAAACCTAT-3', np 23-668, probe h2 5'-CATGTGCCTAGACCAAGAAG-3' and 5'-TAGAATCCGAGTATGTTGGAG-3', np 12,499-13,922; probe h3 5'-GAATCATCCGCTACCTTCAC-3' and 5'-GTTTACAAGACTGGTGTATTAG-3', np 14,977-15,926; probe h4 5'-CTGTTCGCTTCATTCATTGC-3' and 5'-GTCATTATGTGTTGTCGTGC-3', np 8,540-9,212; and probe h5 5'-AACGAATGATTTCGACTCAT-3' and 5'-TCTGTGGTTGTCGTAGGCAGAT-3' np 10,425-11,602.
Mouse mtDNA:
From (22); probe m1 5'-CAAAGGTTGTCCCTGCGCT-3' and 5'-TGTAGCCCATTCTCTCTCCA-3', np 69-790; probe m2 5'-CGCCTAATCAACAACCGTCT-3' and 5'-TGGTAGCTTTGTTGGCTGA-3', np 8,032-8,497; probe m3 5'-CATAGGCTGGCAGACGAACA-3' 5'-GAGGGGTGGATTGGATGGGT-3', np 12,777-13,435; and probe m4 5'-CACAACCAACATCCCCCTCA-3' and 5'-GCTGTGGCTATACTGACTG-3', np 13,867-14,518.

Rat mtDNA:
From (21); probe r1 5'-GGCATCTGGTTCTTACTTCAGGG-3' and 5'-TGTTTTTGGGGTTTGGCATTG-3', np 15,765-16,120; probe r2 5'-ACCTACTAGGAGACCCAGACA-3' and 5'-CCTGAGAAGACTGACTCTTCA-3', np 14,866-15,331; and probe r3 5'-GCCTACCCATTCATCATCCTCT-3' and 5'-GTGTGGGAAGGTTGGAGGTTT-3', np 10,916-11,436. Southern hybridization was carried out in 0.25 M sodium phosphate pH 7.2, 7% SDS buffer overnight at 65 °C. Post-hybridization washes were 1 x SSC followed by 0.1 x SSC, 0.1% SDS both for 30 minutes at 65 °C. Filters were exposed to X-ray film and developed after 0.5-10 days.
RESULTS

Initiation arc intensity increases with increasing distance from O₄r

DNA fragments containing a unique unidirectional origin located towards one end, such that the incipient replication fork passes through much of the fragment, will not produce a simple Y arc. Rather the RIs will initially contribute only a bubble arc (illustrated in Fig. 2A). However, Accl and Dral digested fragments of human and mouse mtDNA containing O₄r lacked an appreciable bubble arc (Fig. 3A and D). Although bubble arcs arising from a unidirectional origin located close to the center of a fragment can be difficult to distinguish from simple Y arcs, a bubble arc was detected previously in the same fragment of mtDNA of a tumor cell line (14). Thus, the Accl result cannot be reconciled with unidirectional replication from O₄r. Furthermore, our previous suggestion that the Y-like species resulted from bubbles broken during processing (14) is undermined by other observations. First, Hamlin and co-workers have shown that broken replication bubbles form arcs distinct from a simple Y arc (23). Second, intact bubble arcs were seen with other enzymes such as HincII and Clal (e.g. Fig 3B and 3E). Third, both Dral and Accl were subsequently found to yield intact bubble arcs from human or rat mtDNA when probed for fragments lacking O₄r (see below). Moreover, as detailed in Fig. 2, aspects of the results from the O₄r-containing HincII fragment of human placental mtDNA, in particular the simple Y arc arising from the unit length fragment (1n), can only be reconciled with initiation of replication at one or more sites outside the fragment in some molecules. On the other hand, the weak bubble arc indicates that replication does initiate within the fragment, at least in a minority of molecules, though not necessarily at O₄r. A slightly larger O₄r-containing Clal fragment of mouse mtDNA gave a similar result, i.e. a bubble arc accompanied by a slightly more pronounced Y arc (Fig 3E). The relative abundance of bubble and Y molecular species altered markedly when still larger fragments of human or mouse mtDNA were studied; the bubble arc signal increased considerably, with a concomitant decrease in signal from the simple Y arc (Fig. 3C and F). Moreover, the absence of a detectable ascending Y arc close to 1n in the O₄r-containing 6.6 kbp AflIII fragment of mouse mtDNA indicates that replication initiates rarely outside the fragment. Thus, both human and mouse mtDNA fragments containing O₄r exhibited a trend according to size: bubble arcs were barely detectable in small fragments (1.5 and 2 kbp downstream of O₄r), moderate in intermediate sized fragments (3-4 kbp downstream of O₄r), and prominent in large fragments (≥ 6 kbp downstream of O₄r). The quantitative differences described above were reproducible both between and within different samples; the gels shown have been selected on the basis of
image quality. Therefore, these data suggest that replication initiates frequently at multiple sites downstream of $O_h$ and only rarely at or near $O_h$.

Two straightforward and readily testable predictions stem from these conclusions, first, initiation arcs should be detectable in fragments of mammalian mtDNA lacking $O_h$; second, the initiation arcs should not map to a single site.

**Strand-coupled replication initiates in fragments of human and mouse mtDNA lacking $O_h$**

Analysis of a number of mtDNA fragments lacking $O_h$, derived from purified human placenta and mouse liver mitochondria, revealed bubble arcs on N/N 2D agarose gels (Figures 4 and 5). For example, human mtDNA fragments spanning, np 13,366-16,456 ($NciI$) and np 13,366-16,390 ($AvaII$) both lack $O_h$ (located at np 191) therefore, the bubble arcs associated with them (Fig 4A-C) must have arisen from initiation at sites other than $O_h$. In the case of the np 13,366-16,456 and np 13,366-16,390 human mtDNA fragments (Fig. 4A-C) a complete Y arc was also detected, indicating that strand-coupled replication frequently initiates at sites outside the fragment. This conclusion was confirmed when the filter shown in Fig. 4A was reprobed for the $NciI$ fragment spanning np 8,152-12,125, revealing a weak bubble arc (Fig. 4D). The disparity in signal between the bubble arcs of the two $NciI$ fragments (np 13,366-16,456 and np 8,152-12,125) indicates that more initiation events occur in the fragment adjacent to (but not containing) $O_h$. Additional prominent non-standard arcs (slow-moving Y-like arcs) were evident in many cases (e.g. Fig. 4A'-D'); these are the result of blocked restriction enzyme sites attributable to extensive ribonucleotide incorporation on the newly synthesised mitochondrial L-strand (for details see (15)).

A number of fragments of mouse mtDNA lacking $O_h$ analysed by N/N 2D-AGE also revealed a complete bubble arc, and a complete Y arc, such as an $NsiI$ fragment spanning np 10,758-15,903 (Fig. 5A), a $BglII$ fragment spanning np 12,664-15,949 (Fig. 5B). Bubble arcs were also detected in a $XhoI$ fragment (np 10,907-15,973), a $PsisI$ fragment (np 10,971-15,590) and a $BanII$ fragment (np 12,959-15,742), which similarly lack $O_h$ (data not shown). A unidirectional origin located close to the common end of these various fragments, around np 15,500, with synthesis away from $O_h$, could explain these data. However, a complete bubble arc was also detected associated with an $AccI$ fragment of mouse mtDNA spanning np 9,599-14,647 (Fig 5C) and a $PflMI$ fragment np 10,836-14,604 (Fig. 5D). Thus, if unidirectional replication is to account for these findings there must be a third
origin close to the ends of these fragments around np 14,500, with the direction of synthesis again away from $O_{ht}$. Alternatively, bi-directional replication initiates at multiple sites downstream of $O_{ht}$, with one advancing fork undergoing arrest upon reaching $O_{ht}$. Consistent with the latter interpretation is the pronounced thickening of the descending portion of the Y arc in several fragments, most notably as seen in Fig. 5D. Moreover, multiple unidirectional origins of similar strength cannot account for the abrupt termination of the Y arc in $O_{ht}$-containing fragments, which indicates that many replication intermediates share a common end mapping at or near $O_{ht}$.

Although $O_{ht}$ acting as the major unidirectional origin would be compatible with this observation, the absence of bubble arcs in some $O_{ht}$-containing fragments (Fig 3A and D) and their weakness in others (Fig. 3B and E) categorically contradict this idea. Therefore, only bidirectional replication from multiple origins with subsequent arrest of one advancing fork at $O_{ht}$, is compatible with all the data (Fig. 2 panel C, diagrams II and III).

Analysis of larger fragments of mouse mtDNA such as a 6.65 kbp $Avr$II fragment (np 8,178-14,825) (Fig. 5E) and a 7.15 kbp $Bsa$I fragment (np 7,735-14,884) (Fig. 5F) yielded a similar ratio of bubble to simple Y molecules as smaller fragments such as the $Acc$I fragment spanning np 9,599-14,647 (Fig. 5C). This suggests that the first fork to exit the fragment does so at the $O_{ht}$ proximal end (14,825, 14,884 or 14,647). Only large fragments containing $O_{ht}$ produced a prominent bubble arc, therefore the majority of bubbles of 5 kb or more include a fork that has reached, and stalled at, $O_{ht}$. As mentioned above, $O_{ht}$-containing $Afl$III fragment of mouse mtDNA lacked a portion of the ascending Y arc close to ln suggesting that few origins are located in the 2 kb of the fragment furthest from $O_{ht}$ (circa np 10,118-12,000). Further evidence that origins are not scattered throughout the mouse mitochondrial genome came from a 6.9 kb $Bam$HI fragment (np 4,275-11,167) and a 7.6kb $Xba$I fragment (np 938-8,529), each of which lacked a bubble arc and was associated with a simple Y arc (Fig 5G and 5H, respectively). Other fragments of mammalian mtDNA that lacked an initiation arc are described below.

**Replication origins of rat mtDNA map to a broad region downstream of $O_{ht}$**

Analysis of mtDNA replication intermediates in rat liver lends support to the idea that bidirectional initiation in a zone downstream of $O_{ht}$ is general among mammalian mtDNAs. As with human and mouse mtDNA, bubble arc versus Y arc intensity was greater in large fragments containing $O_{ht}$ than small $O_{ht}$-containing fragments (Fig. 6A versus 6B and 6C). The presence of a prominent simple Y arc arising from ln in the small fragments (Fig. 6B and C) again suggests that initiation events occur not only downstream of $O_{ht}$ but also outside these fragments,
i.e. at nucleotide numbers below 13,811. Moreover, given that the bubble arc was more truncated than the Y arc in such fragments, unidirectional theta-replication initiating at O_h is excluded as a major mode of replication for rat liver mitochondria. Unidirectional replication from O_h would produce a bubble arc that extended to a point in line with the terminus of the bubble arc (marked with a vertical broken line in Fig. 6C). The smear on the descending Y arc that ends abruptly in a pronounced spot (Fig. 6D) suggests that replication fork arrest occurs at multiple loci. Therefore, the data in rat are consistent with initiation of bidirectional replication in the O_h-distal portion of the restriction fragment (np ~14,500-15,000), followed by stalling of the rightward-moving replication fork within a ~500-bp region terminating at O_h. Alternative initiation events further from O_h within this fragment, or outside the fragment altogether, could account for the prominent Y arc signal. The replication forks coming from these more O_h-distal origins would also stall near O_h and would contribute to the strength of the pause region signal on the Y arc.

To test for the possibility of alternative initiation events O_h-distal to 14,500, we examined other restriction fragments of rat mtDNA. Analysis of the 4.6 kb AccI fragment (np 9,236-13,841), MfeI fragment, np 10.815-14,701, and BamHI fragment, np 9,361-14,436 all yielded bubble arcs (Fig 6E-J). BglI and BclI fragments of rat mtDNA adjacent to, yet lacking, O_h and mapping between np 12,643 and np 15,927 were also associated with bubble arcs (Fig. 6K and L). These data support the view that rat mtDNA replication initiates by a strand-coupled theta-mechanism, at multiple sites downstream of O_h.

**Fine-mapping of the origin of strand-coupled replication in rat mtDNA**

Analysis of overlapping fragments of DNA has been used previously to map origins of replication, (e.g. (24). A complete bubble arc is consistent with bidirectional replication from an origin located at the centre of a fragment; in practise an origin anywhere within the central third of a fragment (sometimes called the “detection zone”) gives rise to a clear bubble arc, whereas origins closer to the ends of fragments are difficult, if not impossible, to detect by N/N 2D-AGE (25). Where replication is unidirectional, only origins at the extreme end of a fragment will produce a complete bubble arc. Accordingly, overlapping fragments on either side of a fragment in which such an origin is located will, in one case lack the origin whilst in the other case the pattern formed will be a truncated bubble arc accompanied by a double Y arc. None of these scenarios is consistent with the results. In the case of rat mtDNA, the AccI (np 9,237-13,842), BamHI (np 9,361-14,436), BglI (np 12,643-15,927) and BclI (np 12,012 -15,861) fragments cover a region that overlaps the extreme fragments (AccI and BglI) by less than
half their length, yet all were associated with essentially complete bubble arcs (Fig. 6D-J). A discrete origin, whether bidirectional or unidirectional, would give alterations in the patterns of bubble-, Y- and X-shaped replication intermediates when overlapping fragments were compared.

The only explanation consistent with the data is that bidirectional theta-replication initiates at different positions in different molecules, and that these origins are scattered throughout an extensive zone stretching approximately from 500 base pairs downstream of O₁ (in the rat) to the ND4 gene. Based on this analysis, theta replication initiates in a zone that spans approximately np 11,500–15,500 of rat mtDNA (Fig. 6M). The data sets for mouse and human indicate mtDNA initiation zones of np 11,300–15,500 and np 10,500–16,000, respectively. Discrete origins, such as the SV40 origin, give rise to bubble arcs that are particularly prominent near their apex (26). The prominent apex of the bubble arc of mouse mtDNA (Fig. 3F) contrasts with that of human (Fig. 3C) and is consistent with a narrower initiation zone in mouse than human mtDNA. In any event, the exact boundaries of the initiation zone may be slightly outside of the defined limits, since origins of theta replication located near one end of a fragment are difficult to detect (25). Conversely, the breadth of the zone may be exaggerated by the absence of informative sites. Where two forks of a bidirectional origin travel at different speeds the size of the zone would be exaggerated in the direction of the slower moving fork. Origins need not be evenly dispersed across an initiation zone, nor need all origins be activated at equal frequency. In human mtDNA theta replication appears to be more frequent in the O₁ proximal part of the initiation zone. There are a number of reports of initiation zones, including several in mammalian nuclear DNA, for example dihydrofolate reductase (27), β-globin (28) and immunoglobulin H loci (29).

**Absence of coupled replication origins in other regions of mammalian mtDNA**

The above data indicate that mammalian mtDNA origins are dispersed across a wide zone in three different species: rat, mouse and human. However, this zone does not encompass the entire genome, much of which is free of origins that give rise to initiation arcs on N/N 2D-AGE. The fragments that define a contiguous origin-free zone covering the rest of the mitochondrial genome are as follows: in rat, np 15,861-2,340, 7,654-12,012, 2,340-7,654, 3,066-6,204, and 16,183-3,064; in human, 3,659-7,658 and 1,505-6,259; and in mouse, 16,174-2,349, 934-8,529, 3,102-7,084, 4,275-11,167, 5,276-9,817, 7,084-11,322, and 14,647-5,710 (see supplementary data fig. 4).
DISCUSSION

N/N 2D-AGE has been employed widely since 1987, and is the pre-eminent method for analysing replication intermediates, including origins of replication (1,30,31); to date it has proved totally reliable. In the present study, extensive analysis of mitochondrial DNA of three mammals revealed a set of over-lapping fragments associated with initiation arcs (Figs. 3-6). These data are indicative of multiple origins of replication distributed across a region of four or more kilobases, located downstream of the 3’ end of the short displacement loop.

Can the initiation arcs account for all the observed mtDNA replication intermediates?

Many of the initiation arcs derived from mammalian mtDNA were relatively weak, compared with the accompanying standard Y arcs (Figs. 4-6). The question therefore arises as to whether replication might sometimes initiate elsewhere, but by another mechanism that does not generate a standard bubble arc on 2D gels. Where bidirectional replication initiates at multiple sites, most initiation events will occur towards one or other end of a restriction fragment, hence one replication fork will exit the fragment before the other; the bubble structure will be cleaved by the restriction enzyme and a partial Y arc created (Supplementary data fig. 5). Any initiation event outside the fragment will contribute a complete Y arc. Thus, a zone of initiation predicts a prominent standard replication fork (Y) arc to accompany the initiation arc. This is exactly what was seen for the well-characterized DHFR locus of nuclear DNA (24,27). Replication fork arrest will of course prevent one fork from exiting a fragment, thus the increased prominence of initiation arcs in large O_H containing fragments is entirely consistent with our model for mtDNA that proposes a bi-directional initiation zone downstream of a powerful RFB (O_H). As the majority of RIs of human, mouse and rat mtDNA form classical bubble arcs in large O_H-containing fragments (Fig. 3C and F, and Fig. 6A) we conclude that strand-coupled theta-replication arising from multiple origins is the major mechanism of replication for mammalian mtDNA, at least for the tissues examined. Furthermore, because the large O_H-containing fragments studied here include sites that are known to be ribonucleotide blocked, e.g. at nps 13,842 and 14,632 of rat mtDNA (15), the simple and slow-moving Y arcs described previously (15) must be generated by a common (theta) mechanism. Otherwise molecules comprising the initiation arc would form a minority of replication intermediates associated with large O_H-containing fragments, which was not the case (Fig 3C, 3F, and Fig 6A).
Unidirectional versus bidirectional replication

*E. coli* plasmids such as Col E1 were long-believed to replicate in a unidirectional manner and this mechanism appeared to be confirmed by N/N 2D-AGE analysis (6). However, a later more detailed study of a closely related plasmid (p15A) indicated that replication is initially bidirectional (32), as proposed here for mammalian mitochondrial DNA. Fork-direction analysis of mtDNA fragments would add support to the model as it predicts that for fragments outside the initiation zone, replication forks will enter at the end proximal to the 3’ end of the D-loop.

Role of O_h and the D-loop in mtDNA replication

In our revised model, O_h acts not as the origin of strand-coupled replication but as a terminus for mtDNA replication. O_h is always preserved in partially deleted mtDNAs, whereas most if not the entire initiation zone of human mtDNA, as defined in this report, is redundant (33). Similarly, in the most extensively studied replication initiation zone, the human nuclear DHFR locus, all sequence elements are known to be dispensable (34). The most obvious candidate for an essential *cis*-element for mtDNA replication outside the initiation zone is the triple stranded D-loop region, located in the major non-coding region (35,36). Short regions of triplex DNA are known to inhibit transcription e.g. (37); hence the function of the D-loop may be to mediate replication fork arrest by forming a structural barrier. An attractive feature of this hypothesis is that it can account for the observed stalling not at a single site, but across a region of several hundred base pairs (e.g. Fig. 3A and D, and Fig. 6D). The fact that replication almost never extends upstream of O_h towards the rDNA genes suggests that any fork reaching O_h stalls at that point. By analogy with *E. coli* Col E1 (38) this could be due to the inability of the replisome helicase to separate regions of RNA:DNA hybrid formed by the short D-strand primer, and which persist in mtDNA after replication. If D-loops do indeed mediate fork arrest of O_h-proximal replication forks (Fig. 7), then the vast majority of replicating molecules must have arisen from molecules containing a D-loop, as almost all appear to undergo fork arrest in the D-loop region. Therefore, either D-loop synthesis is so frequent that there is generally a D-loop present in each molecule that initiates replication or, more likely, the D-loop itself provides the conditions under which replication can initiate in the zone downstream of it, via topological alteration of mtDNA structure. In this regard, it is noteworthy that regions of triplex DNA can promote homologous recombination at sites up to four kilobases distant (39). In our revised model, O_h serves as a bidirectional replication terminus. It should be noted that the predicted ‘double-Y’ arcs associated with replication termination are often very faint or not discernible above the background of the strong stall at O_h.
Double Y-arcs in \( \text{OH} \)-containing fragments are more clearly detectable in mtDNA from cultured cells recovering from induced mtDNA depletion, although they are not seen under standard growth conditions (see Fig. 7 of ref. 14). This suggests that the final stages of mtDNA replication are completed rapidly, under normal conditions.

**Mitochondrial DNA replication and pathological rearrangements**

Pathological rearrangements of human mtDNA invariably spare \( \text{OH} \) and the D-loop, whereas the region which contains the initiation zone is frequently deleted (33,40,41). As well as indicating the functional importance of \( \text{OH} \), this also suggests that the zone itself does not supply the signals essential for initiation of replication. Perhaps any DNA located downstream of the D-loop could function as an origin zone due to its proximity to the D-loop, and membrane components (42). Indeed this may be a general feature of initiation zones; as mentioned above the signals essential for nuclear DHFR gene replication have recently been shown to lie outside the well-characterized initiation zone (34).

Another feature of pathological rearrangements of human mtDNA is that they commonly occur in the major arc between \( \text{OH} \) and \( \text{OL} \) (40,41). Replication fork arrest is a well-recognized prelude to genome rearrangement (43-45). A replication fork barrier is located close to \( \text{OL} \) (14), and the data reported here suggest that \( \text{OH} \) acts as a prominent replication fork barrier. Taken together these observations offer a partial explanation for the location of pathological rearrangements; that is, molecules in which replication has stalled at the fork barriers near \( \text{OH} \) and \( \text{OL} \) may be favoured substrates for illicit recombination.

**ACKNOWLEDGEMENTS**

We thank Jen Chi-Min and Joanna Poulton, University of Oxford for helpful discussions on the possible role of the D-loop in mitochondrial DNA replication; Lodovica Vergani, University of Padova for help in preparing human mitochondrial DNA samples. TY holds an overseas fellowship from the Japanese Society for the Promotion of Science, and AR is the recipient of a Royal Society Visiting Fellowship. The UK Medical Research Council provided financial support for this work. HTJ was supported by the Academy of Finland and Tampere University Hospital Medical Research Fund and JAH was supported by grants from the National Institutes of Health, USA (GM49294, CA95908 and CA84302). The collaboration between IJH and HTJ is supported by the European Union project MitEURO (QLG1-CT-2001-00966).
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**FIGURE LEGENDS**

**Figure 1.** Panel A The mammalian mitochondrial genome encodes 13 polypeptides and the RNA elements necessary for their translation. ND – NADH dehydrogenase, Cyt b - cytochrome b, COX – cytochrome c oxidase, A – ATP synthase, rRNA – ribosomal RNA genes. The 22 transfer RNAs are denoted according to the single letter code. tRNA^Pro^ gene marks the end of the large non-coding region and the end of the D-loop. Panel B, the strand asymmetric model of mammalian mtDNA replication in which both strands are synthesised continuously from physically and temporally distinct sites termed O_H and O_L, the diagram depicts a replication intermediate where the leading strand has traversed two-thirds of the genome, exposing O_L, thereby enabling second-strand synthesis to begin in the opposite direction (for details see, (13)). Panel C, strand-coupled unidirectional replication from a discrete origin (O_H) (14). O_H defines both the start site for replication and the terminus; the broken line with an arrowhead indicates the direction of replication away from O_H. Panel D the new model supported by data presented in this report: bidirectional q-replication originating downstream of O_H.
with subsequent fork arrest (fork a) at $O_h$. Note that after arrest of fork a, the replication intermediates associated with the different models depicted in panels C and D are indistinguishable.

**Figure 2. Mechanisms of initiation of mammalian mtDNA replication.** Panel A, diagramatic illustration of the previously published 2D gel-blot of the $O_{hr}$-containing HincII fragment of human mtDNA (14) and figure 3B. Panel B illustration of the predicted pattern of replication intermediates (RIs) for a discrete unidirectional origin located at $O_{hr}$. Beneath in panel C are 3 possible interpretations. Horizontal lines represent linear duplex DNA molecules spanning, from left to right, np 13,637-1,008. Vertical bars indicate the position of $O_{hr}$, arrows the direction(s) of replication. The previously published interpretation (14), scenario I, assumed unidirectional fork movement leftwards from $O_{hr}$, creating a truncated bubble arc, which converts to a prominent burst bubble structure lying on the standard Y arc, as the fork exits the fragment. Bidirectional initiation from one or more positions less than halfway from $O_{hr}$ to the left-hand end of the fragment, with fork arrest at $O_{hr}$ (scenario II), would generate exactly the same intermediates. Bidirectional initiation from one or more points further from $O_{hr}$ with fork arrest at $O_{hr}$ (scenario III), would generate a more truncated bubble arc, plus an enhanced portion of the standard Y arc, since the leftward-moving fork would exit the fragment before the rightward-moving fork had reached $O_{hr}$. The combination, in the published gel, of an enhanced portion of the standard Y arc, plus an extensive bubble arc, suggests that scenarios II and III, or even all three scenarios, may operate in different molecules. Scenario I cannot alone account for the pattern of RIs detected.

**Figure 3. Bubble arc intensity increases with increasing distance from $O_{hr}$.** Restriction digested samples of human placenta mtDNA (A-C) and mouse liver mtDNA (D-F) separated on Brewer-Fangman gels and probed for $O_{hr}$-containing fragments of mtDNA. The restriction enzyme used appears in the bottom left corner of each image. Gel blots were hybridized with probe h1 (panels A and B), h2 (panel C), m1 (panel D), or m2 (panels E and F). Panels A, B and D are reproduced from (14). Panel G shows the approximate lengths of each of the fragments analysed aligned with respect to $O_{hr}$ (filled circle). The size indicated in kilobases (kb) is the portion of the fragment downstream of $O_{hr}$ (in the direction of the cytochrome b gene). A 7.3 kb, $BtgI$ fragment of mouse mtDNA spanning np 9,215-224 also yielded a prominent initiation arc similar to that associated with the 6.6 kbp $Afl$III fragment in panel F (Supplementary data fig. 1). [For an interpretation of the image in panel F see supplementary data fig.2. Another $O_{hr}$-containing fragment lacking an appreciable bubble arc is shown in
supplementary data fig. 3, together with longer exposures of panels A and D]. Schematic maps of human and mouse mtDNA marked with restriction sites defining the probed fragments appear at the head of the figure.

**Figure 4. Initiation arcs associated with fragments of human mtDNA lacking O₄.** Mitochondrial DNA from highly purified human placental mitochondria was digested with *NciI* (panels A, B and D) or *AvaII* (panel C). The products were separated by two-dimensional gel electrophoresis. In all cases, the detected fragment lacks O₄. Hence, where a bubble arc is associated with a fragment (arrowed) it cannot be the result of unidirectional replication initiating at O₄ (scenario I in Fig. 2). Panels A’-D’ are shorter exposures of panels A-D; these shorter exposures show that the most prominent replication intermediates form standard and slow-moving Y-like arcs. Each prominent spot on the duplex linear arc greater in mass than the unit length linear fragment, and the accompanying slow-moving Y-like arc can be explained by ribonucleotide incorporation at the restriction site (see (21). In addition, there appeared to be a major pause site coinciding with a point on the ascending slow-moving Y-like arc (panels A’ and C’), which was single-strand nuclease sensitive (panel B’). This species was common to all fragments of mammalian mtDNA where one end mapped to the D-loop region (see also for example (21)fig. 3C, 3D, 3K). Thus, we attribute the apparent pause to D-loop containing molecules that were not cleaved by the restriction enzyme on the displaced strand; note however that this does not establish a direct relationship between D-loop containing molecules and molecular species forming the slow-moving Y-like arc. Gel blots were hybridized with probe h3 (panels A-C); h4 and h5 (panel D). A schematic map at the top of the figure shows the restriction sites for human mtDNA that define the fragments analysed in panels A-D.

**Figure 5. Initiation arcs are associated with overlapping fragments of mouse mtDNA lacking O₄.** Purified mouse liver mtDNA samples were digested with *NsiI* (panel A), *BglI* (panel B), *AccI* (Panel C), *PflMI* (Panel D), *AvrII* (Panel E), *BsaI* (Panel F), *BamHI* (Panel G) or *XbaI* (Panel H). Gel blots were hybridized with probe m2 (panels A and B); m3 (panels C-F) or m4 (panel G). Each detected fragment lacks O₄. A schematic map at the top of the figure shows the restriction sites for mouse mtDNA that define the fragments analysed in panels A-G.

**Figure 6. A series of overlapping fragments of rat mtDNA is associated with initiation arcs.** A schematic map at top of figure indicates the restriction sites defining the fragments of rat mtDNA that were associated with an initiation arc. Rat liver mtDNA was digested with *BtgI* (panel A), *BsaWI* (panel B), *BsrFI* (panels C, D), *AccI* (panels E, F), *MfeI* (panels G, H), *BamHI* (panel I, J), *BglI* (panel K) or *BcII* (panel L), then
treated (panels F, H and J) or not treated (other panels) with S1 nuclease prior to 2D electrophoresis. S1 resistant molecules cannot be the products of either strand-asymmetric replication or transcription that entails extensive RNA-DNA hybrid formation, as both these processes would generate partially single-stranded molecular forms. Gel blots were hybridized with probe r1 (panels C and D), r2 (panels A, B, K and L) or r3 (panels E-J). The initiation arcs are more truncated than the Y arcs in panels B and C, and are marked by a broken vertical line. Panel D is a shorter exposure of panel C, the arrow in panel D indicates a portion of the Y arc that forms a prominent smear, consistent with replication pausing in a region that terminates at or near O1. Note that the BclI site at 13,389 (21) was absent in all rat mtDNA samples analysed. Two other fragments of rat mtDNA, a Dral fragment (np 13,177-15,516) and an NdeI fragment (np 12,741-15,633) yielded complete or nearly complete bubble arcs, whilst an HgaI fragment (np 12,217-211) revealed a truncated bubble arc (data not shown).

Prominent X-like spikes were evident in some digests (6F and 6H), typically these are characteristic of recombination intermediates. Such forms appear at high abundance in all fragments of human heart mtDNA (46), although their role in mtDNA homeostasis is an enigma. **Panel M. The initiation zone of bidirectional strand-coupled replication of rat mtDNA.** Fragments of rat mtDNA that produced a bubble arc are aligned with a portion of the rat mitochondrial genome: a set of overlapping restriction fragments were each associated with a complete bubble arc, yet the fragments do not share a common end or mid-point. The only model compatible with the data is one in which bidirectional replication initiates heterogeneously, within the zone indicated, in different molecules.

**Figure 7. Proposed model of bi-directional initiation of replication of mammalian mitochondrial DNA.** Replication initiation is depicted originating at multiple sites (ori) that lie in a defined region (see text for details), replication is bi-directional, however, a directional replication fork barrier (RFB) maps to a position in the major non-coding region close to the locus previously designated as the origin of heavy (leading) strand synthesis (Ohi). The Ohi proximal fork stalls at or within ~500 bp of Ohi. The model is based on the observations in this report. **Viz.** origins of replication map to the centre of different partially overlapping fragments of mammalian mtDNA indicating that initiation occurs at multiple sites; origins are dispersed over a region of 4-6 kilobase pairs; one end of the initiation zone maps to within a kilobase of the 3’ end of the D-loop. Replication forks rarely pass through the entire fragment where it contains Ohi, which suggests that forks frequently stall in this region. Stalling may be mediated by short D-loops, whose 5’ ends map to Ohi. Replication fork arrest predominates at a locus, which maps within 200 bp of Ohi, nevertheless, some forks arrest downstream of Ohi.
within the region defined by the short D-loop, suggesting that triple stranded (or triplex) DNA may precipitate replication fork arrest, albeit at relatively low frequency compared to $O_h$. 
16S rRNA

12S rRNA

cyt b

ND5

ND1

ND6

ND2

ND4/4L

ND3

COX3

COX1

COX2

16.5 kb

Figure 1, Bowmaker et al
Figure 3, Bowmaker et al
Figure 4, Bowmaker et al.
Figure 5, Bowmaker *et al.*
Figure 6A-L.
Figure 6M Bowmaker et al.
Figure 7, Bowmaker et al
Supplementary Data, Fig.1 Mouse liver mtDNA digested with BtgI and separated by 2D-AGE. The membrane was probed for the 7.3 kb fragment of mouse mtDNA spanning np 9,215-226 that includes O$_{H}$. The fragment extends 907 np further downstream of O$_{H}$ than the similar AflIII fragment (Fig. 3F).
Supplementary Data, Fig.2  Panel A, the AflIII fragment of mouse liver mtDNA spanning np 10,118-360 reproduced from Fig. 3F, with interpretations alongside (Panels B and C). Arc a represents molecules where strand-coupled replication has initiated within the fragment. b comprises molecules where one replication fork has arrested at or near \( O_R \) and the other has exited the fragment at np 10,118. c is a slow-moving Y-like arc; based on our previous study (Yang et al. 2002) we attribute this to ribonucleotide occupation of the restriction site at 10,118. Failure to cleave at np 10,118 predicts fragments spanning np 6,092-360, and indeed a probe to the region 6,092-10,118 also hybridized with the slow-moving Y-like arc (data not shown). d are molecules where the replication fork has exited the fragment at np 6,092 yet ribonucleotides persist at np 10,118 preventing cleavage at the latter site. e are presumed broken bubbles and/or RNase degraded replication intermediates. Spot f also hybridized to a probe that detected the adjacent fragment np 360-1,771, whereas it failed to hybridize to a probe detecting the other adjacent fragment (np 6,092-10,118) (data not shown). These results combined with its position on the gel lead us to propose it as the terminal double-Y species, where both replication forks have reached \( O_R \); extension of at least one branch of the replication intermediate (owing to ribonucleotide blockage at np 360) cause it to be more retarded than a standard replication intermediate. Note that a species consistent with a standard terminal double Y fragment was detected routinely in smaller fragments containing \( O_H \) (e.g. Fig. 3A, 3D and supplemental Fig. 3A), and yet is absent in this fragment. In panel B the numbers indicate the predicted size in kilobases of linear duplex DNA fragments, these are consistent with size markers analysed on other equivalent gels (data not shown). In panel C, fine lines represent duplex DNA and a ribonucleotide patch that prevents cleavage appears as a broader line than duplex DNA. Arrows indicate the inferred direction of replication.
Supplementary Data, Figure 3. O₄ containing fragments. Incomplete Y arcs were invariably associated with O₄ containing fragments. In every case terminus of the Y arc mapped to O₄ within the limits of the technique (+/- 200bp) (Fig. 3A, B, D-F, Fig. 4A-C and panel A above). In the manuscript, the presence of a (truncated) Y arc and the absence of an appreciable bubble arc in small O₄-containing fragments is attributed to initiation events occurring downstream of O₄, however an alternative explanation warrants consideration. Bidirectional origins outside the central third of a fragment can be difficult to detect (Linskens & Huberman 1990), implying that the position of a bubble within a fragment affects its mobility. This problem will be exacerbated in the case of unidirectional replication. Therefore, the failure to detect bubble arcs in small fragments (<3kbp) where O₄ is located near the centre of the fragment might reflect a limitation of the N/N 2D-AGE technique. However, a “unidirectional” origin at the centre of a 4 kb fragment of Col E1 DNA did yield a (truncated) bubble arc as expected (Martin-Parras et al 1991). Hence we could reasonably have expected a similar prominent arc in association with a 5 kb fragment of human placental mtDNA spanning np 14,258-2,650 digested with PvuII and BamHI (Panel A), if replication initiation occurred predominantly at or very near O₄, yet none was detected after standard exposure times. Moreover, a partial bubble arc was detected in the human AccI O₄-containing fragment after prolonged exposure to X-ray film (panel B, cf Fig. 3A of the manuscript), and we previously detected a faint (truncated) bubble arc in the same human AccI fragment derived from cultured cells amplifying mtDNA (Holt et al., 2000 Fig. 7C), suggesting that the technique is capable of resolving intact bubbles associated with a 2.8 kb restriction fragment. No bubble arc was detectable in the mouse Dral fragment spanning np 14,171-1,478, treated without (panel C) or with (panel D) single-strand nuclease, even after prolonged exposure (a shorter exposure of the single-strand nuclease treated sample (panel D) appears in Fig. 3D of the manuscript).
Supplementary Data, Fig. 4. Fragments of mammalian mtDNA lacking an initiation arc. The limits of the initiation zone of bidirectional replication of mammalian mtDNA are defined not only by those fragments that yielded a bubble arc, but equally by the fragments that did not reveal a bubble arc. Other fragments of mammalian mtDNA that lacked an appreciable bubble arc included the following. Fragments of mouse mtDNA spanning np 3,102-7,084 (BclI) and 5,276-9,817 (DraI) (Panels A-D). An MfeI and a BclI fragment of rat mtDNA spanning np 15,792-3,444 and np 7,065-12,012 respectively (Panels E-G); and an Accl fragment of human mtDNA (np 1,504-6,238) (Panel H). Note that all of these restriction enzymes yielded intact bubble arcs with other fragments of mammalian mtDNA, indeed in three cases a bubble arc was detected on the same membrane when other probes were applied.
Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone
Mark Bowmaker, Ming Yao Yang, Takehiro Yasukawa, Aurelio Reyes, Howard T. Jacobs, Joel A. Huberman and Ian J. Holt

J. Biol. Chem. published online September 23, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308028200

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