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Coupled Leading- and Lagging-Strand Synthesis of Mammalian Mitochondrial DNA

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Summary

Analysis of mammalian mtDNA by two-dimensional agarose gel electrophoresis revealed two classes of replication intermediate. One was resistant to single-strand nuclease digestion and displayed the mobility properties of coupled leading- and lagging- strand replication products. Intermediates of coupled, unidirectional mtDNA replication were found in mouse liver and human placenta and were the predominant species in cultured cells recovering from transient mtDNA replication. Replication intermediates sensitive to single-strand nuclease were most abundant in untreated cultured cells. These are presumed to derive from the orthodox, strand-asynchronous mode of mtDNA replication. These findings indicate that two modes of mtDNA replication operate in mammalian cells and that changes in mtDNA copy number involve an alteration in the mode of mtDNA replication.

Introduction

Mammalian mtDNA is a covalently closed circular molecule of approximately 16 kb. After denaturation, the two strands, termed heavy (H) and light (L) on the basis of their nucleotide composition, can be separated by cesium chloride density-gradient centrifugation. For over 25 years, mammalian mtDNA replication has been believed to proceed solely via a strand-asynchronous, asymmetric mechanism (Clayton, 1982). This mode of mammalian mtDNA replication has some similarities with that of ColE1 and some other plasmids, but differs from that of chromosomal DNA of both eukaryotes and eubacteria. Replication of the H strand on this model is proposed to initiate at a single site (O_H), and to proceed unidirectionally until two-thirds of the way around the genome, where a second "origin" (O_L) is exposed on the displaced heavy strand. DNA synthesis from the light (lagging) strand "origin" then initiates in the opposite direction. In this strand-asymmetric model of replication, DNA synthesis is continuous on both strands. Nevertheless, the possibility that coupled leading- and lagging-strand synthesis of mammalian mtDNA could occur was suggested when duplex replication intermediates of rat hepatocyte (Koike and Wolstenholme, 1974; Wolstenholme et al., 1974) and of HeLa mtDNA (Crews et al., 1979) were detected by electron microscopy.

The preferred analytical method for studying DNA replication has become neutral/neutral two-dimensional agarose gel electrophoresis, as pioneered by Brewer and Fangman in the late 1980s (Brewer and Fangman, 1987; 1988). This technique separates DNA molecules on the basis of size and shape. Thus, particular types of replication intermediate (RI) resolve in specific and predictable ways. The simplest type of replication intermediate is the standard replication fork, where replication occurs simultaneously on both strands, albeit discontinuously on one strand. Restriction fragments containing such replication forks are essentially duplex throughout and produce on 2D gels a characteristic, so-called "Y arc," representing the passage of the replication fork along the fragment. Brewer-Fangman gels can also give information on origins of replication and replication pause sites (RPS) (Friedman and Brewer, 1995).

Mitochondrial DNA (mtDNA) replication has been studied using this technique in yeast (Han and Stachow, 1994; Lockshon et al., 1995), sea urchin (Mayhook et al., 1992), and the malaria parasite *Plasmodium* (Preiser et al., 1996). In all these organisms, standard replication fork arcs were detected. In order to determine whether such RIs could also be detected in mammalian mtDNA, we analyzed DNA from purified mitochondria of human placenta and mouse liver. The data unambiguously support the existence of RIs that derive from coupled leading- and lagging-strand DNA synthesis, initiated unidirectionally at or near O_H. Other RIs were also detected that contain substantial regions of single-strandedness, and which could represent intermediates of the previously described strand-asymmetric mode of mtDNA replication. We conclude that there are two alternative mechanisms or modes of mtDNA replication operating in mammals.

The occurrence of two different classes of mtDNA replication intermediate raises many questions regarding the mechanisms by which mtDNA replication is regulated in different physiological and developmental contexts. As a first step toward understanding how mtDNA synthesis might be regulated in situations where copy number is actively modified, we additionally investigated the representation of mtDNA replication intermediates in cultured human cells in which mtDNA amplification was induced by prior depletion via drug treatment. These replication intermediates were compared with

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those present in growing cells where only maintenance synthesis of mtDNA was taking place. Replication intermediates that were detected during reamplification of mtDNA were almost exclusively of the double-stranded class. These are inferred to be the products of the conventional coupled leading- and lagging-strand replication, initiating unidirectionally from the same origin detected in placenta. By contrast, in growing cells undergoing mtDNA synthesis merely to maintain copy number from one cell generation to the next, the predominant mtDNA replication intermediates were partially single-stranded, as predicted by the orthodox strand-asymmetric model. These findings indicate that different modes of mtDNA synthesis operate in human cells under different conditions, depending on whether copy number is being modulated or merely maintained.

Results

Two-dimensional, neutral-neutral agarose gel electrophoresis is a powerful technique for analyzing the relative amounts and fine structure of replication intermediates. We reasoned that applying it to mammalian mtDNA would also enable us to investigate such questions as the topology of mtDNA during replication, the possible presence of RPS in regions of protein-binding, as well as defects in mtDNA replication associated with pathological states.

The probes employed and the fragments they were predicted to detect are shown for mouse and human mitochondrial DNA in Figure 1.

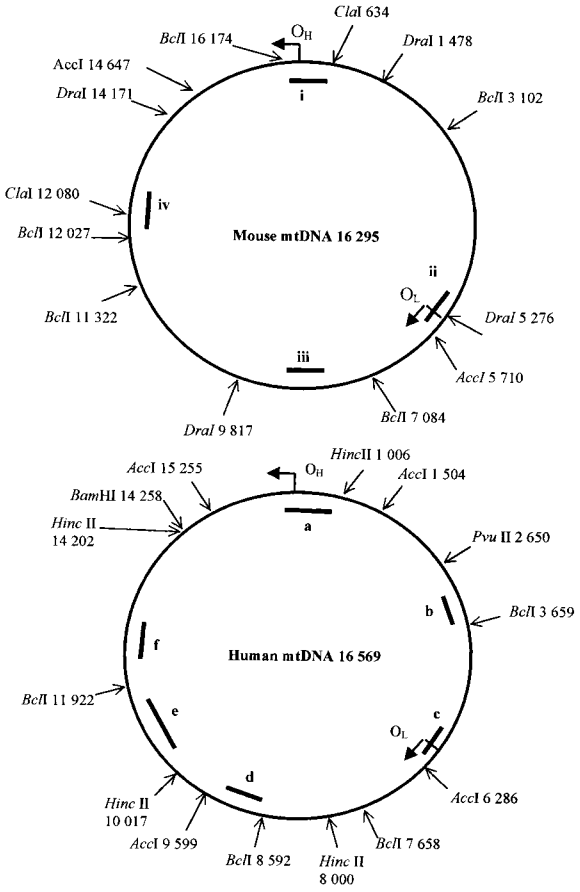

Figure 1. Cartoons of the Mouse and Human Mitochondrial Genomes

The cartoons show the restriction enzyme sites that define the fragments analyzed in the study. The PCR amplified probes are indicated as broad lines on the inside of the circles; (i)–(iv) for mouse mtDNA, (a)–(f) for human mtDNA.

Analysis of Mouse Mitochondrial DNA Replication Intermediates

We initially applied Brewer-Fangman gel technology to the analysis of mouse liver mtDNA digested with a restriction enzyme demonstrated not to cut ssDNA, DraI. Two-dimensional gel blots were first probed for a region of the mouse mitochondrial genome contained within a 4.5 kb DraI fragment that excludes both of the previously mapped origins, O_H and O_L. The orthodox model would predict that whereas the probe should detect the 4.5 kb unit fragment of dsDNA from nonreplicating molecules, the digestion products generated by replicating molecules would have a strand length in the first dimension of at least the next largest partial digestion product created by digestion behind the origin, namely of 12.5 kb. Furthermore, being highly nonlinear, they would lie far from the gel diagonal of duplex DNA fragments.

Mouse mtDNA Replication Intermediates Form Standard Replication Arcs

Surprisingly, however, the experiment visualized a strong Y arc proceeding from the unit-length fragment (Figure 2B). Such Y arcs represent standard products of conventional DNA replication of the synchronous and symmetrical type (Brewer and Fangman, 1987; Brewer and Fangman, 1988; Brun et al., 1995), resulting from the passage of a duplex replication fork through a nonorigin-containing restriction fragment. The Y arc was partially obscured by a complex, convoluted arc lying far from the linear duplex arc (Figure 2A) that was presumed to represent the partially single-stranded RIs predicted by the orthodox model. This conclusion is supported by the fact that they were completely eliminated when a portion of the DraI digest was treated with single-strand-specific nuclease (Figure 2B), leaving the standard Y arc as the only major nonlinear species. The fact that the molecular species comprising the Y arc were resistant to single-strand nuclease digestion further confirms that they were completely double-stranded. Fragments of most other regions of mouse mtDNA also gave rise to standard replication-fork arcs that were more readily apparent when single-strand nuclease was employed after restriction digestion (e.g., Figure 2D). Standard replication fork arcs were not observed when fragments including the presumed heavy-strand replication origin (O_H) were analyzed. Instead, partial or discontinuous arcs were obtained, consistent with unidirectional but still strand-symmetrical replication initiating at O_H.

Mouse O_H was assigned previously to the region around nucleotide 16,080 that lies within the 3.6 kb DraI fragment spanning nucleotide pairs (np) 14,171–1,478 (Bibb et al., 1981). The filter shown in Figure 2B was stripped and hybridized with probe (i) that detected the 3.6 kb DraI fragment containing O_H. The autoradiograph

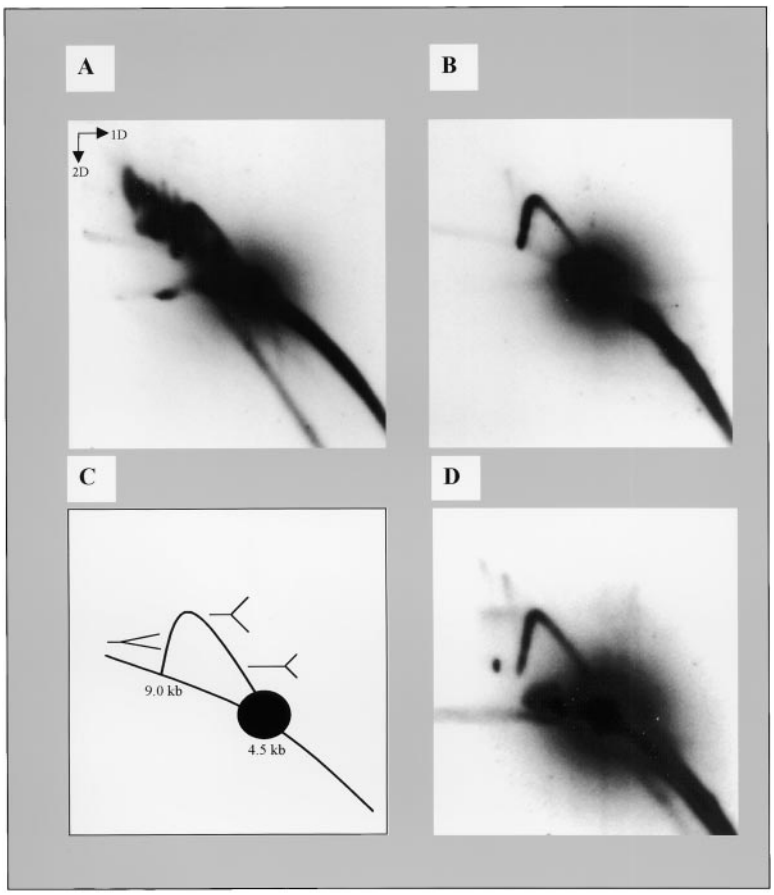

Figure 2. Standard Duplex Replication Fork Arcs Derived from Mouse Liver Mitochondrial DNA

Purified mouse mitochondrial DNA was digested with DraI or AccI and separated on a Brewer-Fangman gel as described in the experimental procedures.

(A and B) After blotting the DraI filter was hybridized with probe (iii) that detected a 4.5 kb fragment of mouse mtDNA, np 5,276–9,817. (A) and (B) represent a single digest of mouse liver DNA that was divided in two; half was incubated in S1 nuclease buffer without S1 nuclease (A) and the remainder was incubated with S1 nuclease (B). On this and subsequent figures, the arrows marked 1D and 2D at the top left-hand corner of (A) indicate the direction of first and second dimension electrophoresis (− to +), respectively.

(C) shows in cartoon forms the duplex (single-strand nuclease- resistant) RIs that make up the Y arc, the extent of replication fork expansion is shown illustratively at three points along the Y arc.

(D) Brewer-Fangman gel of AccI digested mouse liver mtDNA probed for the 5 kb fragment spanning np 9,599-14,647 after S1 nuclease treatment. AccI cleaves single as well as double-stranded DNA.

revealed an arc similar to the ascending portion of a Y arc, with a prominent spot at the apex of the arc, but the Y-like arc lacked a descending arm (Figure 3A). The prominent spot and the absence of a descending Y arc are both consistent with unidirectional replication from an origin within the fragment. Notable by its absence was a standard replication bubble arc. However, it has been shown previously that nicked replication bubbles form an arc similar to a Y arc (Kalejta and Hamlin, 1996).

A similar result was obtained when a ClaI digest of mouse mtDNA, that yields a fragment of 4.8 kb (12,080–634) in which O_H is located 850 nucleotides from one end, was analyzed. Replication from O_H to 634 via 12,080 would predict a prominent spot on the descending arm of a Y arc. This was indeed the case (Figure 3B). Moreover, in some preparations of mouse mtDNA, ClaI digests revealed a faint intact replication (bubble) arc (data not shown). The intact replication bubble arc was much clearer when the products of BclI digestion were probed for the fragment 16,174– 12,027, which also contains O_H (Figure 3C). The dearth of intact bubbles in (A) and (B) can therefore be attributed in part to the presence of nonspecific endonucleases contaminating the commercial preparations of DraI and ClaI. In conclusion, all regions of mouse mtDNA yielded standard duplex replication intermediates, consistent with a single unidirectional origin at O_H, as well as other material that was single-strand nuclease sensitive.

Human Mitochondrial DNA Duplex RIs Are Essentially the Same as Those of Mouse

Brewer-Fangman gels of human placental mtDNA revealed prominent, standard replication fork arcs that, as in the case of mouse liver, were resistant to digestion by single-strand-specific nuclease (Figures 4A and 4B). Additional molecular species were detected that were, once again, like mouse liver mtDNA, digestible by single-strand nuclease (data not shown). As with mouse mtDNA, analysis of fragments that included the previously localized O_H (np 191; Crews et al., 1979) revealed duplex RIs consistent with the presence of an origin of replication (Figures 4C and 4D). AccI digestion yields a fragment of 2.8 kb spanning np 15,255–1,504, in which O_H is centrally located. The single-strand nuclease-treated human AccI O_H-containing fragment revealed a pattern (Figure 4C) strikingly similar to the equivalent mouse DraI fragment (Figure 3A). A faint Y arc was apparent on 2D blots of AccI-digested placental mtDNA probed for the O_H region, but much more prominent was a single spot that was coincident with the top of the ascending Y arc (Figure 4C). This spot comprises cleaved replication bubbles initiated unidirectionally at O_H and exiting the AccI fragment at np 15,255.

HincII digestion of human mtDNA yields a 3.4 kb fragment spanning O_H (np 14,201–1,006). Using the same O_H region probe as earlier, a partial Y-like arc was detected, terminating in a prominent single-strand nuclease-resistant spot above the arc of duplex linear DNA molecules

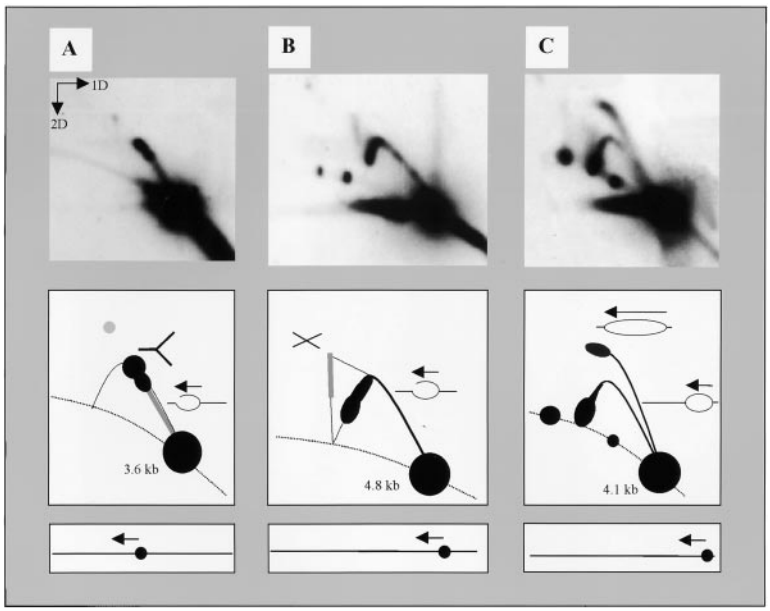

Figure 3. Coupled Leading- and Lagging-Strand DNA Synthesis Initiates at, or near, O_H

(A) The membrane shown in Figure 2B of DraI-digested and single-strand nuclease-treated mouse mtDNA was stripped and reprobed (probe [i]) to detect the fragment np 14,171-1,478 that contains O_H (np 16,070) near its center. The single-strand nuclease-resistant molecular species formed an arc that appears to be coincident with the ascending portion of a standard Y arc and terminate in a prominent spot. The prominent spot and absence of a descending Y arc are both consistent with unidirectional replication. Furthermore, the location of the prominent spot places the origin of replication close to the center of the fragment. Nicking by endonuclease is the proposed explanation for the absence of a replication bubble arc, as per the accompanying cartoon. S1 nuclease–treated ClaI (B) and BclI (C) mouse liver mtDNA digests were separated on Brewer-Fangman gels, as before and probed with PCR product (iv). ClaI digestion of mouse mtDNA yields a 4.85 kb fragment spanning np 634–12,080. Unidirectional replication from np 16,070 to 12,080 would result in almost 80% of the fragment being copied before the replication fork exited the fragment. Thus the prominent "cleaved replication bubble" spot would be expected to appear on the descending portion of a standard Y arc, as is the case (B). The abundant species that make up the truncated Y-like arc that terminates at the prominent spot are again presumed to be nicked replication bubbles. The BclI-digested sample, probed for the region np 16,174–12,027, showed a clearer bubble arc of a type that is synonymous with the presence of an origin of replication within a fragment (C). O_H is predicted to lie within 100 bases of one end of this fragment (Bibb et al., 1981). Thus, the result is in complete agreement with the hypothesis that coupled leading- and lagging-strand replication initiates at the previously defined O_H (np 16,080) and proceeds unidirectionally, exiting the fragment at np 12,027. The presumed nicked-bubble arc appears to meet the linear duplex arc both because the origin is extremely close to the "beginning" of the fragment and because cleaved replication bubbles accumulate forming a prominent spot (see text for details). Cartoons in (A)–(C) depict the observed arcs and the structures they represent: nicked bubble arc terminating in a prominent spot above the (dotted) linear duplex arc (A and B), an expanding bubble arc (C), and unresolved four-way junctions (B). The boxed line, at the base of each cartoon denotes the linear (1n) fragment with the position of a unidirectional origin of replication marked as a filled circle; an arrow indicates the direction of replication. No other mode of replication is consistent with the observed RIs. Note that the predicted location of the origin in all three cases is at or near the previously defined O_H.

(Figure 4D). Significantly, in this digest, the prominent spot was located on the *descending* portion of the Y arc. Moreover, a standard replication bubble arc was visible (Figure 4D). These observations substantiate the earlier conclusion that unidirectional replication initiates at O_H and proceeds in the direction np 191 to 15,255.

Recovery from Induced Mitochondrial DNA Depletion in Human Cultured Cells Is Predominantly via the Coupled Leading- and Lagging-Strand Replication Mechanism

Two different drug treatments were employed for inducing transient mtDNA depletion: ethidium bromide (EB) and dideoxycytidine (ddC). These drugs act by completely different mechanisms. The former intercalates into DNA, drastically altering its topology, although the precise reason why this leads to loss of mtDNA is not clear. Yeast, avian, and mammalian cell lines have all been entirely depleted of their endogenous mtDNA using this drug (Goldring et al., 1970; Desjardins et al., 1985; King and Attardi, 1988). When EB is used transiently, removal of the drug is followed by reamplification of mtDNA to restore normal copy number (Wiseman and Attardi, 1978; King and Attardi 1988). ddC acts as a chain terminator for mtDNA synthesis after its intracellular conversion to ddCTP. The mitochondrial DNA polymerase is hypersensitive to the drug (Simpson et al., 1989), which may therefore be used at appropriate concentrations to inhibit mtDNA replication selectively. Human 143B osteosarcoma cells were treated with 50 ng/ml EB, or 50 μM ddC. After 72 hr of treatment, removal of either drug led to progressive restoration of normal mtDNA copy number (data not shown). Mitochondrial RIs were analyzed in DNA samples from cells grown for 72 hr in the presence of one or other of the drugs, followed by 24 hr in the absence of drug. The generation time of the osteosarcoma cells was unaffected by drug treatment up to 120 hr, as determined by MTT assays (Mosmann, 1983) of cell number and viability (data not shown).

AccI digests of 143B cell DNA, after 72 hr drug treatment (EB or ddC) and 24 hr recovery, were fractionated by neutral/neutral two-dimensional agarose electrophoresis and probed with radiolabeled mtDNA fragments corresponding to the region np 3,122–3,558. As anticipated from the Cambridge mtDNA reference sequence (Anderson et al., 1981), the probe detected a fragment of 4.8 kb (np 1,504–6,286). Arising from the 4.8 kb unit length fragment was an arc of the type generated by a standard replication fork (Y) arc (Figure 5).

We considered the possibility that the high abundance of species observed after drug treatment represent intermediates of mtDNA destruction, rather than DNA synthesis. This was excluded when cells treated continuously for 72 or 96 hr with ddC with no recovery period

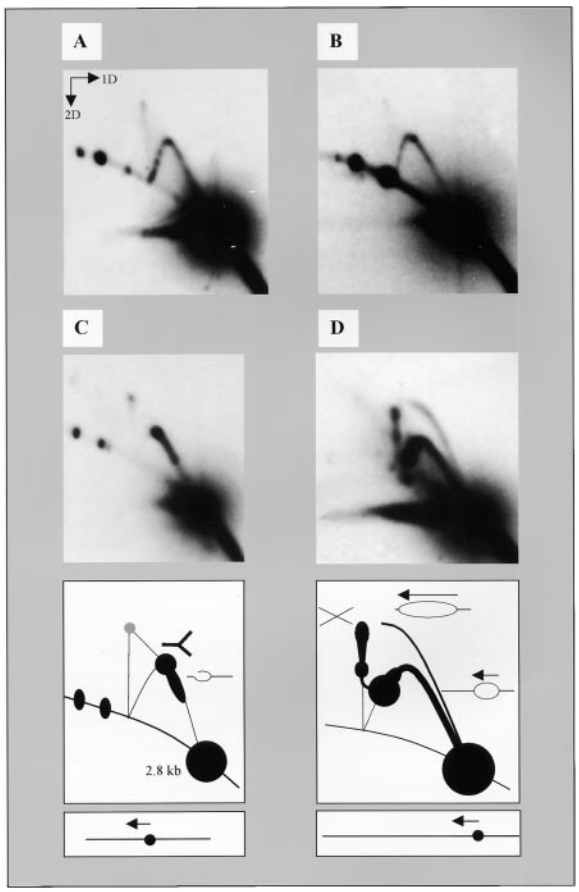

Figure 4. Standard Replication Arcs Are Found in Human Placental Mitochondrial DNA

After restriction enzyme digestion, DNA samples were separated on Brewer-Fangman gels and in some cases treated additionally with single-strand-specific nuclease.

(A) BclI-digested and S1 nuclease–treated human placental mtDNA hybridized to probe (e) detecting a 3.3 kb fragment, np 8,592–11,922. The signal along the Y arc is not of even intensity: several spots (RPS) are apparent indicating that the rate of DNA synthesis is not uniform.

(B) The filter in (A) was stripped and rehybridized with probe (c), to detect the fragment spanning np 3,659–7,658 that contains O_L near its center. As with the equivalent region of mouse mtDNA (data not shown), an RPS is located near the apex of the arc, which maps at or close to O_L.

(C) AccI and S1 nuclease digested human placental mtDNA hybridized to probe (a) to detect the 2.8 kb fragment, np 15,255– 1,504. O_H is located close to the center of the fragment. The single-strand nuclease-resistant RIs are similar to those observed for the equivalent region of the mouse mitochondrial genome (Figure 3A). Thus, the inference is the same as for mouse mtDNA that coupled leading- and lagging-strand replication initiates at or near O_H and proceeds unidirectionally. Once the replication fork is beyond np 15,255, cleaved replication bubbles accumulate giving rise to the prominent spot near the apex of a standard Y arc.

(D) A classical intact replication bubble arc was detected in a HincII fragment of human mitochondrial DNA. S1 nuclease, HincII digested human placental mtDNA hybridized with probe (a) that detects the fragment spanning np 14,202–1,006. The location of human O_H within the HincII fragment is similar to that of mouse O_H in the 4.8 kb ClaI fragment described above (Figure 3B). HincII digested human mtDNA hybridized to probe (a) gave substantially the same result as the earlier described ClaI analysis of mouse mtDNA on short autoradiographic exposure. In the longer exposure shown, an intact replication bubble arc is clearly visible. The duplex-nicked and intact replication bubble arcs shown were obscured in samples that were not treated with single-strand nuclease (data not shown). Cartoons accompanying (C) and (D) depict the major single-strand nuclease-resistant species. The box at the base of the 2D gel cartoons represents the linear fragment with the location of the origin marked (filled circle); an arrow indicates the direction of replication.

were analyzed and found to lack such intermediates (e.g., Figure 5D). The putative mtDNA RIs were not detected in DNA preparations from either of two different ρ^0 cell lines (i.e., cells that lack mtDNA), derived respectively, from 143B osteosarcoma and A549 lung carcinoma cells (data not shown). They cannot, therefore, be derived from nuclear pseudogenes of mtDNA.

In order to compare the mtDNA RIs present in cells recovering from transient depletion with those present in cells maintaining a constant mtDNA copy number, AccI digests of DNA from cells treated as above and from parallel cultures of cells not treated with any drug were analyzed by 2D gel electrophoresis. To distinguish the two classes of RI, aliquots of each digest were further treated with a single-strand-specific nuclease. When probed for regions of the mitochondrial genome distant from the replication origin region O_H, mtDNA RIs from cells recovering from transient mtDNA depletion formed a standard Y arc resistant to single-strand- specific nuclease (Figures 6A and 6B). In contrast, RIs from untreated cells did not form a complete Y arc (Figure 6C). Furthermore, the species detected were dramatically modified by single-strand-specific nuclease treatment (Figure 6D). Samples from untreated cells did contain a small amount of material that was single-strand nuclease-resistant (Figure 6D), forming a faint Y arc seen most clearly near to its apex where RIs are sometimes compressed.

As with solid tissues, a standard Y arc was weak or undetectable when fragments containing O_H were analyzed. Since the RIs detected in untreated cells were mainly single-strand nuclease sensitive, we used a restriction enzyme (AccI) capable of cutting both single- and double-stranded DNA to compare the RIs from the O_H region in treated and untreated cells. O_H is located at np 191 near the center of an AccI fragment that spans np 15,255–1,504. Analysis of RIs deriving from this fragment in untreated cells (Figure 7) revealed a major spot (denoted [i] in Figure 7A), migrating close to the unit length linear 2.8 kb fragment, plus a minor spot (denoted [ii]), much further from the arc of linear duplex molecules. In samples from cells recovering from drug induced mtDNA depletion, spot (ii) was the major nonlinear species seen (Figure 7C), although spot (i) was still weakly detected, and was also single-strand nuclease sensitive (Figure 7D). In DNA samples from drug-treated cells left to recover an additional 24 hr (i.e., 48 hr of recovery as opposed to the usual 24), the pattern observed was intermediate, with spot (i) still prominent, but spot (ii) still much more prominent than in untreated cells (Figure 7B).

We infer that spot (ii) corresponds with cleaved, double-stranded replication bubbles, as seen previously in placental DNA samples treated with single-strand-specific nuclease and probed for the region of O_H (Figure

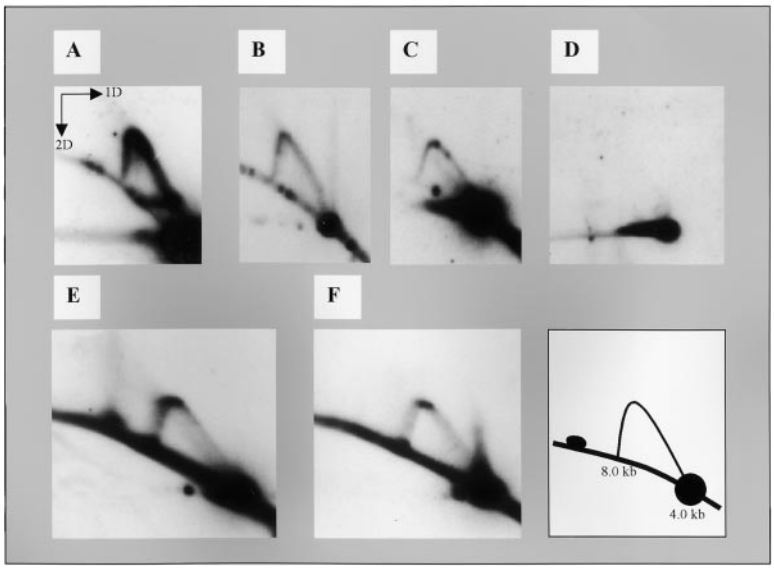

Figure 5. Mitochondrial DNA Replication Intermediates Derived from Human Cultured Cells Recovering from Transient mtDNA Depletion Form Standard Replication Fork Arcs

Osteosarcoma cells were treated with drug for 72 hr and DNA harvested after a further 24 hr without drug (+72, −24). DNA was digested with AccI, an enzyme that cleaves single-stranded DNA, separated by two-dimensional agarose gel electrophoresis and hybridized with probe (b) that detects the fragment np 1,504–6,286. The major (1n) spot was estimated to be of the order of 4.8 kb and the point of return to the double-stranded linear arc as 9.6 kb, based on the migration of DNA size markers (Life Technologies).

(A) DNA from cells treated with 50 ng/ml EB (+72, −24).

(B) DNA from cells treated with 50 mM ddC (+72, −24).

(C) A similar blot of human placental mtDNA digested with AccI and S1 nuclease, and hybridized to probe (b). Cultured cells harvested directly after ddC treatment of 72 or 96 hr contained no detectable mitochondrial RIs whatsoever (e.g., D). Note that the DNA samples in (A), (B), and (D) were not treated with single-strand-specific nuclease. Likewise, standard replication fork arcs of mtDNA were obtained with restriction enzymes that cut double-, but not single-stranded DNA, such as BclI (E and F). DNA from human tumor cell lines recovering from drug-induced transient mtDNA depletion was digested and separated as before and hybridized with probe (b), which in this case detected the 4 kb mtDNA fragment (np 3,568–7,657). O_L is centrally positioned in the 4 kb BclI fragment. (E) BclI-digested 143B DNA (EB +72, −24) without MBN; (F) as (E) but with MBN. The cartoon depicts the standard Y arc of (E) and shows the single-strand nuclease-sensitive spot lying close to the arc of linear duplex molecules; this species may represent a strand-asymmetric product where leading-strand synthesis has stalled at O_L.

7F and 4C). The position of this prominent spot, near to the apex of a presumptive standard Y arc, coincides exactly with that predicted for unidirectional, strand-synchronous replication initiating at O_H near the center of the fragment, and exiting the fragment at np 15,255. Conversely, the partially single-stranded species comprising spot (i) is the prominent replication intermediate expected from the orthodox, strand-asynchronous mode of mtDNA replication. In the orthodox mode, DNA synthesis also initiates unidirectionally at O_H and proceeds by strand displacement, again exiting the fragment at np 15,255. AccI is able to digest both the duplex replicated arm and the displaced parental strand at this position, generating a product sensitive to single-strand nuclease migrating on 2D gels with the mobility properties of spot (i) (Figure 7A). In the orthodox replication mode, a large fraction of replicating mtDNA molecules remains partially single-stranded at this AccI site until light strand synthesis, initiated distantly at O_L, reaches this position, eventually converting the partially single-stranded Y intermediate to duplex. In support of this, a faint arc connects spots (i) and (ii) (Figure 7A) that would represent extension of the light strand from the end of the fragment back to O_H.

The intermediate or composite pattern of RIs observed after 48 hr of recovery (Figure 7B) suggests that by this time, mtDNA synthesis is reverting to the mode that predominates in untreated cells. AccI-digested DNA from placenta also gave an intermediate or composite pattern of RIs from the origin region (Figure 7E), indicating, as inferred previously, that solid tissues contain both types of mtDNA replication intermediate. Moreover, comparison of placental mtDNA treated with and without single-strand nuclease showed clearly that spot (i) was sensitive to the enzyme, whereas spot (ii) was resistant to single-strand nuclease digestion (Figures 7E and 7F).

Other single-strand nuclease-resistant intermediates from the origin region were also evident, most clearly in the DNA sample from cells recovering from mtDNA depletion for 48 hr (Figure 7B). These too represent predicted products of unidirectional but strand-synchronous replication. They include a partial, intact double-stranded replication bubble arc (iv) and a double (Y) replication fork arc (iii), extending up from spot (ii). The latter can be interpreted as resulting from the eventual passage of the replication fork into the other end of the fragment at np 1,504, with eventual termination at the origin.

Discussion

The analysis of human and mouse mtDNA replication intermediates reported here leads us to propose a novel mechanism of mtDNA replication in mammals. Our data imply that in addition to the previously described, “orthodox”, strand-asymmetric mechanism (Clayton, 1982), synchronous leading- and lagging-strand replication, beginning at (or near) the previously assigned heavy strand origin of replication, proceeds unidirectionally around the entire circular molecule. The data obtained from mouse liver and human placenta were essentially the same. Thus, it seems likely that unidirectional, coupled leading- and lagging-strand replication is ubiquitous in mammalian mitochondria.

Neutral/neutral two-dimensional agarose gel electrophoresis has been validated as a means of identifying

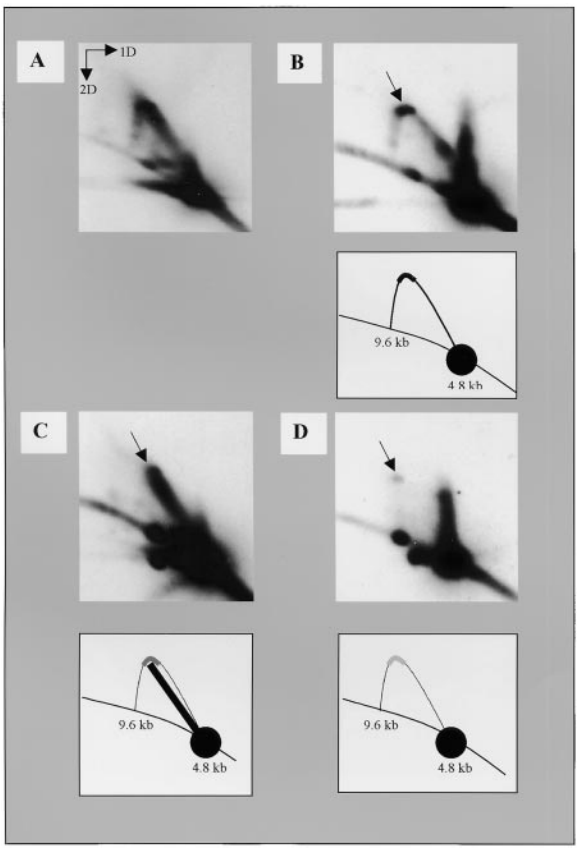

Figure 6. Comparison of Mitochondrial RIs of Cells Amplifying mtDNA, with Those of Cells Maintaining a Constant mtDNA Copy Number

Cells amplifying mtDNA were those recovering from transient drug-induced mtDNA depletion (A and B), whereas cells not treated with drugs were merely maintaining a constant number of mtDNA molecules (C and D). The major molecular species comprising the apparent standard replication fork arc detectable in human cultured cells recovering from transient depletion, are single-strand nuclease-resistant, whereas those from untreated cells are single-strand nuclease-sensitive. The restriction enzyme, two-dimensional agarose gels and probe were as per Figures 5A–5D.

(A) 143B cell DNA, EB (+72, −24) without mung bean nuclease (MBN).

(B) 143B cell DNA, EB (+72, −24) with MBN.

(C) 143B cell DNA, without drug and without MBN.

(D) 143B cell DNA without drug, with MBN. An arrow marks the apex of the standard Y arc in (B)–(D). Comparison of the three panels shows that the molecular species comprising a Y arc were far more abundant in cells reamplifying mtDNA than in untreated cells, but that nevertheless there was some material coincident with a Y arc in untreated cells, and that this was more resistant to single-strand nuclease than the bulk of the nonlinear species in untreated cells. Vertical and horizontal "trails" from the linear (1n) fragment were common, and were most common when restriction- digested samples were further treated with single-strand nuclease. This was not, however, universal; we therefore infer that the trails are artifacts that do not represent the products of restriction enzyme or single-strand nuclease digestion. Cartoons accompany (B)–(D), illustrating the major molecular species.

replication intermediates of various types in *Saccharomyces cerevisiae* (Nawotka and Huberman, 1988; Brewer and Fangman, 1991). Its more general applicability has also been demonstrated (Brun et al., 1995). Therefore, it can be confidently stated that the standard replication fork arcs observed in mouse and human tissues and human cultured cells represent fully double-stranded molecules of a structure identical to the products of synchronous and symmetrical mtDNA replication. The restriction enzyme-digested RIs predicted by the orthodox model of mammalian mtDNA replication are myriad and complex. Nevertheless, it is intuitively obvious that continuous DNA synthesis of two strands from physically and temporally different origins will yield RIs, almost all of which contain regions of single-strandedness. Han and Stachow (1994) have previously presented a detailed theoretical analysis of the types of RI predicted by an asynchronous, asymmetrical mechanism of replication of this type. They detected standard replication fork arcs in mtDNA of *Schizosaccharomyces pombe* akin to the single-strand nuclease-resistant molecules found in mouse and human samples presented here. They, like us, concluded that such RIs could not be the products of asynchronous, asymmetrical replication, nor of DNA synthesis occurring synchronously and in opposite directions from two closely positioned origins.

It is important to stress that we did not find RIs deriving from conventional DNA replication *instead* of those predicted by the orthodox model. We found evidence for both types of RI in the same DNA preparations, from two very different tissue types, mouse liver and human placenta. Preliminary data from other tissues (I. J. H., unpublished data) suggest that this is a general finding. Further studies will be required to establish whether this is true for all tissues at all stages of development. It may be, for example, that one mode is typical of growing cells and the other of quiescent cells, and that the two tissues we have studied in detail contain cells of both types.

Nevertheless, finding both types of RIs in the same DNA preparation must mean either that two completely different mechanisms of mtDNA replication are operating simultaneously in the same cells or at least in the same tissue, or that a single mechanism of mtDNA replication generates RIs of both types. The two types of mtDNA replication may represent extremes of a spectrum where the frequency of lagging-strand initiation is variable. In this scheme, the orthodox model represents one extreme in which lagging-strand synthesis occurs once and only once at O_L, whereas promiscuous and efficient lagging-strand initiation is synonymous with coupled leading- and lagging-strand DNA synthesis. Bridging these extremes, there may be instances where lagging-strand initiation occurs randomly but infrequently, generating intermediates of both type.

The analysis of mtDNA RIs in cultured cells indicates that products from both modes of mtDNA replication can also be detected in tumor cell lines. In growing cells undergoing mtDNA replication that merely maintains copy number from one cell generation to the next, partially single-stranded intermediates predominate, whereas in cells allowed to recover for 24 hr from transient, drug-induced mtDNA depletion, the replication intermediates detected are almost exclusively of the

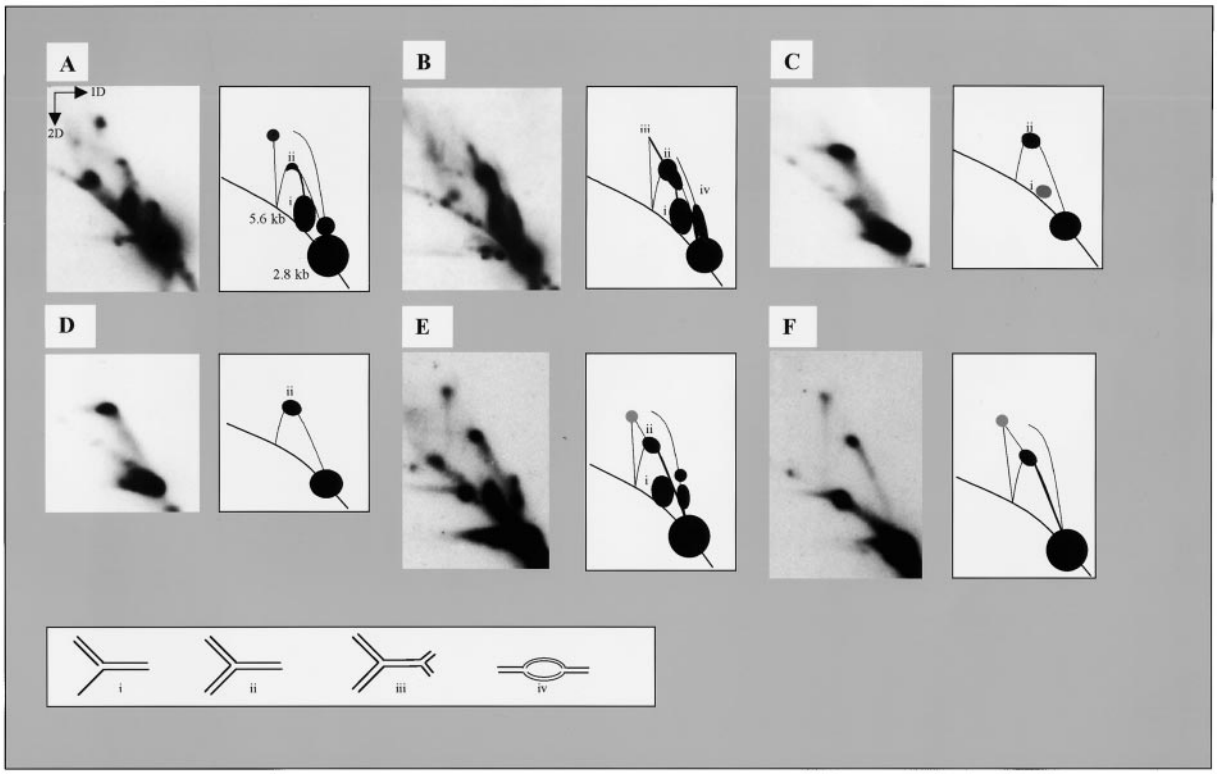

Figure 7. Duplex Mitochondrial DNA Replication Intermediates of a Type Associated with a Standard Origin of Replication Predominate in Fragments that Contain O_H in DNA Samples Derived from Cells Recovering from Transient mtDNA Depletion.

All the DNA samples shown were digested with AccI, and after separation probed for sequences within the 2.8 kb fragment spanning np 15,255–1,504, that includes O_H.

(A) 143B osteosarcoma DNA derived from cells grown without drug.

(B) 143B DNA, EB (+72, −48).

(C) 143B DNA, EB (+72, −24).

(D) 143B DNA, EB (+72 −48), with MBN.

(E) Human placental mtDNA.

(F) Human placental mtDNA after MBN treatment.

EB, ethidium bromide; MBN, mung bean nuclease. Cartoons of the major molecular species detected accompany the blots shown in (A)–(F) and are represented illustratively at the base of the figure: (i) presumed cleaved partially single-stranded replication bubbles, based on their mobility and their sensitivity to single-strand nucleases; (ii), presumed cleaved duplex replication bubbles; (iii), double Y arc; and (iv), intact bubble arc.

fully double-stranded class. Two drugs that induce mtDNA depletion by entirely different mechanisms gave essentially identical results. In cells allowed to recover from mtDNA depletion for an additional 24 hr, both classes of replication intermediate were of similar abundance, indicating that mtDNA replication in the cells was returning to "maintenance mode" as opposed to "amplification mode."

If it is assumed that they represent two alternative mechanisms of mtDNA replication, then the clear implication is that one mechanism is used principally for maintenance synthesis in growing cells, the other mainly for amplification. The alternative view, outlined above, postulates that both classes of intermediate are generated by a single replication mechanism, involving infrequent but random lagging-strand initiation. In this scenario, the difference between amplification/recovery and maintenance synthesis would be the frequency of lagging-strand initiation in relation to the processivity of leading-strand synthesis.

Leading-strand processivity could be influenced by any of several factors. One possibility is the presence of fork-impeding ("antihelicase") proteins bound to the template. Virtually any DNA-binding protein could serve such a role, although to produce the apparently random pattern of lagging-strand initiation, such a protein or proteins must have little sequence specificity for binding. During mtDNA depletion, continued synthesis of such DNA-binding proteins would radically shift the ratio of DNA to bound protein. This hypothesis is attractive, since there is a well-characterized candidate for such a factor, namely the so-called mitochondrial transcription factor, mtTFA. This DNA binding-protein of the HMG family has weak sequence specificity (Laudet et al., 1993; Ikeda et al., 1994) and is generally believed to be present in mitochondria in growing cells in substoichiometric amounts, occupying only the highest affinity binding sites in the D loop region (Ghivizzani et al., 1994), i.e., with most of the mtDNA molecules not coated with the protein. Alternatively, the composition of the replication complex itself may be different in the two cases. The so-called β subunit of the mitochondrial (gamma)

DNA polymerase stimulates the processivity of the (*Xenopus*) enzyme in vitro (Carrodeguas et al., 1999), and this protein, or its activity, might be absent in cells recovering from mtDNA depletion. Other components of the replication machinery may be responsive to mtDNA depletion. It is not excluded, for example, that an entirely different DNA polymerase may be induced to enter mitochondria under such circumstances, to boost the overall rate of DNA replication. This alone may increase the frequency of lagging-strand initiation without any effect on leading-strand processivity. A third possibility would be fluctuations in the intramitochondrial pools of dNTPs.

Many predictions of these models are testable, and useful information may also come about via proteome or cDNA expression analysis of cells grown under conditions of mtDNA depletion, reamplification, and maintenance. Experiments to test the above ideas will reveal the extent to which the phenomena documented here are best regarded as a cellular response to an externally applied stress or as a model for developmentally programmed changes in mtDNA copy number. They may also give valuable insights into the nature of the machinery of mtDNA synthesis more generally, about which remarkably little is known.

Our findings demonstrate clearly why the orthodox, strand-asynchronous model of mtDNA synthesis has gained such general acceptance, despite the fact that solid tissues contain prominent replication intermediates whose fully double-stranded structure is incompatible with it. Replication intermediates of the type predicted by the orthodox model greatly predominate in growing, cultured cells that undergo a daily doubling of biomass. However, the degree to which this is a valid model for any cell type in vivo is debatable. It will be interesting to revisit this issue in contexts where cells are rapidly proliferating (e.g., early development, gut epithelia, and blood).

In conclusion, our findings prompt many questions regarding the enzymes and other factors involved in mammalian mtDNA replication. A full analysis of their properties will allow us to resolve these questions and establish the precise mechanism of mammalian mtDNA replication.

Experimental Procedures

Purification of Mammalian Mitochondria

Mitochondria were isolated from human placenta or mouse livers after cell breakage and differential centrifugation. Homogenization buffer (HB) comprised 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8), and 0.1% BSA (fatty acid free). All operations were performed at 4°C or on ice. The ratio of buffer to tissue was 10 ml per gram wet weight. Human placenta (200 g) was homogenized for 4 S in a Waring type blender, whereas mouse livers (5 g) were subjected to six strokes with a Dounce homogenizer. Homogenized tissue was centrifuged at 1,000 g for 5 min, and the resultant supernatant recentrifuged at 9,000 g for 10 min. The crude mitochondrial pellet was resuspended in HB and the centrifugation steps repeated. The final pellet was resuspended in HB lacking BSA and loaded on a 20%–60% Percoll gradient (Pharmacia). After centrifugation at 60,000 g, the mitochondrial layer was removed, diluted 3-fold with HB lacking BSA and recentrifuged at 10,000 g for 15 min. Nucleic acid was phenol-chloroform extracted from the mitochondrial pellet after overnight incubation in 75 mM NaCl, 50 mM EDTA (pH 8), 1% SDS, 0.5 mg/ml proteinase K at 37°C. DNA was suspended in 10 mM Tris, 1 mM EDTA (pH 8.0) and stored at −20°C.

DNA Isolation from Tumor Cell Lines

DNA was extracted from human 143B osteosarcoma and A549 lung carcinoma cells by standard methods (Sambrook et al., 1990). Partial mtDNA depletion was effected using either 50 μM ddC or 50 ng/ml ethidium bromide. On removal of the drug, to enable reamplification of mtDNA to normal copy number, cells were washed and incubated with fresh medium without drug. The medium was replaced again after 1, 4, and 8 hr, to ensure better removal of drug.

DNA Modification

Whole cell or isolated mtDNA was digested with AccI, BclI, ClaI, DraI, or HincII under conditions recommended by the manufacturers (Promega and New England Biolabs). Of these enzymes only AccI cleaved M13 single-stranded DNA (data not shown). Where indicated, restriction-digested mtDNA was ethanol precipitated, washed, and resuspended in single-strand nuclease buffer (Promega). After dividing the solution equally between two tubes, one unit of single-strand-specific, S1 or mung bean nuclease was added to one tube and both tubes were incubated at 37°C for 1 or 2 min, respectively. These treatments were capable of substantially degrading 2 μg of M13 single-stranded DNA incubated separately or together with digested mtDNA samples (data not shown). In our hands, MBN and S1 nuclease modified putative mtDNA RIs, M13 single-stranded DNA and supercoiled plasmid DNA in identical fashion, except that S1 nuclease was more processive.

Neutral/neutral two-dimensional agarose electrophoresis was performed as described previously (Brewer and Fangman, 1987; Friedman and Brewer, 1995). Briefly, first dimension electrophoresis was at 0.7 V/cm for 20 hr in a 0.4% agarose gel, without ethidium bromide (EB). Each lane was excised and rotated through 90 degrees, a second gel of 1% agarose with 300 ng/ml EB was cast around the lane and once set the second gel was electrophoresed at 6 V/cm for 4 hr at 4°C. After Southern blotting, specific portions of the mitochondrial genome were probed for using random-primed PCR-amplified fragments. Five microliters of 3000 Ci/mmol ^{32}P-dCTP (NEN) was incubated with three units of Klenow DNA polymerase and 50 ng of DNA that had been annealed with 50 ng of hexadeoxyribonucleotides for 90 min at 37°C. The regions of mouse and human mtDNA amplified and oligonucleotide primers (5′-3′) are listed below.

Mouse mtDNA

From Bibb et al., 1981: (i) CAAAGGTTTGGTCCTGGCCT and TGTAG CCCATTTCTTCCCA, np 69–790; (ii) CACCTTCGAATTTGCATTCG and CTGTTCATCCTGTTCCTGCT, np 5,215–5 709; (iii) CGCCTAAT CAACAACCGTCT and TGGTAGCTGTTGGTGGGCTA, np 8,032–8,497; (iv) CATAGCCTGGCAGACGAACA and GAGGTGGATTTTGG GATGGT, np 12,777–13,435.

Human mtDNA

From Anderson et al., 1981: (a) TAACCACTCACGGGAGCTCT and AAGGCTAGGACCAAACCTAT, np 23–668; (b) TGTACGAAAGGA CAAGAGAA and TAGAAGAGCGATGGTGAGAG, np 3,122–3,558; (c) ACACTCATCGCCCTTACCA and GTAGAGAATAGTCAACGG, np 5,451–5,932; (d) CTGTTCGCTTCATTCATTGC and GTCATTATGTGT TGTCGTGC, np 8,539–9,192; (e) AACGAATGATTTCGACTCAT and TCTGTTTGTCGTAGGCAGAT np 10,424–11,601; (f) ATCAGTCTCTT CCCCACAAC and TTTCTGCTAGGGGGTGGAAG, np 12,472–13,141.

The map position of each PCR product is shown on the cartoons of the mouse and human mitochondrial genomes (Figure 1). Southern hybridization was carried out in 0.25 M sodium phosphate (pH 7.2), 7% SDS buffer overnight at 65°C. Posthybridization washes were 1× SSC followed by 0.1× SSC 0.1% SDS both for 30 min at 65°C. Filters were exposed to X-ray film and developed after 0.5–7 days.

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