Bidirectional Replication Initiates at Sites Throughout the Mitochondrial Genome of Birds*§

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Analysis of mitochondrial replication intermediates of Gallus gallus on fork-direction gels indicates that replication occurs in both directions around circular mitochondrial DNA. This finding was corroborated by a study of chick mitochondrial DNA on standard neutral two-dimensional agarose gels, which yielded archetypal initiation arcs in fragments covering the entire genome. There was, however, considerable variation in initiation arc intensity. The majority of initiation events map to regions flanking the major non-coding region, in particular the NADH dehydrogenase subunit 6 (ND6) gene. Initiation point mapping of the ND6 gene identified prominent free 5′ ends of DNA, which are candidate start sites for DNA synthesis. Therefore we propose that the initiation zone of G. gallus mitochondrial DNA encompasses most, if not all, of the genome, with preferred initiation sites in regions flanking the major non-coding region. Comparison with mammals suggests a common mechanism of initiation of mitochondrial DNA replication in higher vertebrates.

The study of initiation of θ replication in prokaryotes, viruses, and plasmids led to the discovery of a single discrete origin for each genome, which was essential for replication (1). The large chromosome size of nuclear DNA necessitates multiple origins of replication (2); nevertheless, it was widely assumed that nuclear DNA would initiate replication at a discrete site for a given region of DNA. The identification of autonomously replicating sequence elements seemed to support this idea (3). However, nuclear DNA replication often initiates at a multiplicity of sites across a broad zone (4–7). Even the EBV genome, which contains a site that behaves like a classic discrete origin when cut out of the genome and placed in a plasmid, is replicated from numerous origins distributed over a broad zone when it is intact (8). Nor is the initiation zone size-fixed; in flies, the initiation zone size is dependent on developmental stage (9). Recently, we reported that the 16.5-kb circles of mammalian mitochondrial DNA (mtDNA) initiate replication from multiple sites across a zone of ~4 kilobases (10).

Although there is great diversity in the size and organization of mitochondrial genomes of plants, fungi, and animals (11), within the animal kingdom they are very similar (12), particularly so among vertebrates (13). One might therefore anticipate a conserved mechanism of replication for vertebrate mtDNA.

In the 1970s and 1980s, a series of studies of mammalian mtDNA gave rise to a strand-asynchronous model of mtDNA replication (14). The model proposed that replication of the two strands of the circle arose in each case from a single initiation site. These sites were designated the heavy and light strand origins of replication (O_H and O_L). The site of second-strand synthesis, O_L, is a sequence element that can theoretically form a hairpin stem-loop and to which abundant free 5′ ends map. Its primary sequence is poorly conserved in mammals, yet the ability to form a stem-loop is conserved (15).

In its simplest or most literal form, this model cannot apply to all vertebrates. Avian mtDNAs lack a convincing stem-loop structure that might act as a dedicated initiation site for second-strand synthesis, begging the question by what mechanism is avian mtDNA replicated? Birds do share with mammals the abundant short displacement or D-loop form of mtDNA (16, 17), which until recently was widely regarded as supporting the strand-asynchronous model. As in mammals, the 3′ end of the D-loop is close to one end of the major non-coding region (NCR), and the 5′ end of the D-loop defines O_H. At 16,775 nucleotide pairs (np), the mitochondrial genome of Gallus gallus is slightly larger than that of most mammals (18). Avian D-loops, which typically span almost 800 nucleotides (np 75–855 in G. gallus), account for much of the length difference. The high degree of similarity in gene content and arrangement between avian and mammalian mtDNA is illustrated in Fig. 1.

The strand-asynchronous model of mammalian mtDNA replication has been challenged in recent years (19, 20). Duplex replication intermediates were identified that could not be reconciled with the strand-asynchronous model (19), and the co-existing population of partially single-stranded molecules was subsequently shown to arise from RNase H degradation occurring during isolation of replication intermediates, which contain extensive tracts of RNA-DNA hybrid (20). Extensive ribonucleotide incorporation has not been reported previously, and it was not known whether this feature was unique to mammalian mtDNA.

The contentious nature of recent findings in mammals (21, 22)
RESULTS

Origin Detection and Mapping—In a previous study, we reported that initiation of mtDNA replication encompasses a broad zone downstream of the 3′ end of the D-loop, in mammals (10). Therefore, a series of fragments of chick mtDNA was separated by neutral two dimensional AGE and examined for evidence of initiation arcs, with particular emphasis on the region downstream of the 3′ end of the D-loop. Restriction sites for the fragments analyzed in this study are illustrated on a schematic map of the *G. gallus* mitochondrial genome (Fig. 1C).

Analysis of a 7.8-kb AflIII fragment of *G. gallus* mtDNA, spanning np 10,540–1,803 and including O1h, revealed a prominent initiation arc and a weak simple Y arc terminating in a weak simple Y arc terminating in a weak initiation arc (Fig. 2). A prominent initiation arc and a weak simple Y arc terminating in a weak initiation arc (Fig. 2A), and interpreted in supplemental information). The prominent initiation arc suggests the majority of initiation events occur within the fragment, via a α mechanism. The prominent fragment arc reveals the presence of two initiation arcs within the 3′ fragment of the AflIII fragment (Fig. 2B). The prominent fragment arc reveals the presence of two initiation arcs within the 3′ fragment of the AflIII fragment (Fig. 2B).

A shorter DraI fragment (np 14,075–9,881), including O2h, also gave rise to an initiation arc more prominent than the simple Y arc (Fig. 2C), whereas a 7.4-kb fragment lacking O2h yielded a Y arc considerably stronger than the initiation arc.
FIG. 1. Organization of the mammalian and avian mitochondrial genomes. The gene content of mammalian (A) and avian (B) mitochondrial genomes is the same. Each encodes 13 polypeptides and the RNA elements necessary for their translation. ND, NADH dehydrogenase; CYTB, cytochrome b; CO, cytochrome c oxidase; rRNA, ribosomal RNA genes. The 22 transfer RNAs are denoted according to the single letter amino acid code. The D-loop region of G. gallus is 5'-3' np 855–75 (18). The arrangement of tRNA<sup>Asu</sup> and ND6 genes means that tRNA<sup>Asu</sup> (E) marks the end of the NCR and the 3' end of the D-loop in birds, whereas these elements abut the tRNA<sup>Asu</sup> gene in mammalian mtDNA. The only other notable difference in structure is the absence of a putative stem-loop structure (O<sub>L</sub>) in the 5 tRNA gene cluster (YCNAW) of birds. C, schematic map of chick mtDNA, indicating the restriction sites related to the fragments analyzed by two-dimensional AGE in Figs. 2 and 4. The position of the NCR (np 1–1227) is marked as a broad, solid gray line on the outside of the circle. Regional probes 1–5 are depicted as broad, solid black lines on the inside of the circle.

(Fig. 2D). Hence, some initiation events must arise at sites other than O<sub>H</sub>, which excludes the unidirectional replication from a discrete origin (O<sub>H</sub>) as the sole mechanism of replication for G. gallus mtDNA. Still weaker initiation arcs were associated with FauI (np 8,831–16,351) and MscI (np 8,334–15,947) restriction fragments (Fig. 2, E and F) in which the O<sub>H</sub> proximal ends are more distant from O<sub>H</sub> than that of the BsmBI fragment (Fig. 2D). These data can be interpreted in one of two ways. Either G. gallus mtDNA contains a series of unidirectional origins of decreasing strength, extending from O<sub>H</sub> into the cytochrome b gene, or G. gallus mtDNA contains an initiation zone of bidirectional replication whose center lies close to the 3' end of the D-loop region.

Direction of Fork Movement—If the replication of chick mtDNA initiates bidirectionally across a zone adjacent to the 3' end of the D-loop, and replication terminates in the NCR, then replication forks will travel in one direction only through fragments outside the initiation zone. To test this proposition, fork-direction gels were produced. These gels require an in-gel restriction enzyme digestion treatment between the first and second electrophoresis steps (23, 29). Fragments from several regions of the genome, including ones distant from the 3' end of the D-loop, gave rise to pairs of Y arcs (Fig. 3, A, D, G, and J) indicating that replication forks enter these fragments from both ends. Therefore, replication forks travel in both directions on G. gallus mtDNA.

In general, one arc was more prominent than the other, and wherever this was the case it was indicative of a majority of replication forks traveling away from the 3' end of the D-loop (Fig. 3, A, D, G, and J) (see supplemental information). The major arc is consistent with bidirectional replication arising from a zone proximal to the D-loop 3' end and terminating at or near O<sub>H</sub>, so that most of the genome is replicated by the counterclockwise-moving fork. It is equally consistent with counter-
clockwise unidirectional replication originating in the NCR. In contrast, the other, fainter arc comprising forks traveling toward the 3’ end of the D-loop (Fig. 3, A, D, and G) fits neither model, rather it necessitates initiation at sites far from the proposed initiation zone.

Unidirectional Versus Bidirectional Replication—Unidirectional replication originating in the NCR would contribute forks traveling exclusively in one direction, whereas a bidirectional initiation zone would generate forks traveling in two directions. Fork-direction analysis of a BsoBI-BsaHI fragment spanning np 12,777–15,152 indicated that most forks travel away from the 3’ end of the D-loop (Fig. 3G). In contrast, fork-direction analysis of a smaller fragment with one end closer to the 3’ end of the D-loop (MboI fragment np 14,866–16,476) revealed two simple Y arcs of approximately equal intensity (Fig. 3J). The difference between the MboI/HincII (Fig. 3J) and the BsoBI-BsaHI (Fig. 3G) fragments is decisive. It indicates that in a fragment spanning np 15,405–16,476, replication forks exit the fragment at the OH proximal end as frequently as the OH distal end, whereas in a fragment spanning np 12,777–15,152 most forks exit at the OH distal end. This is entirely consistent with bidirectional initiation at dispersed sites, whereas unidirectional replication from the NCR would yield only one simple Y arc on fork direction gels, and there would be no difference in the results from the BsoBI/BsaHI and MboI/HincII fragments.

FIG. 2. G. gallus mtDNA replication initiates across a region of several kilobases downstream of the 3’ end of the D-loop. Gradient-purified chick liver mtDNA was digested with a series of restriction enzymes, and the products were separated by two-dimensional AGE. A 7.8-kb AfIII fragment (np 10,540–1,603) revealed by hybridization with probe 1, without (A) and with S1 nuclease treatment (B). C, probe 1 was also used to reveal a 4-kb DraI fragment (np 14,075–988) containing O$_H$ (np 855) near one end. Cleavage with BsmBI yielded a fragment (np 10,092–768) with one end close to but not including O$_H$ (D). The samples in E and F were digested and hybridized with probes that detected, respectively, a Faul fragment np 8,831–18,351, and an MscI fragment np 8,334–15,947. G, the fragments analyzed in A–F are shown aligned with the relevant section of the G. gallus mitochondrial genome (np 7,000–1,000). The D-loop region is depicted as a broad line whose 5’ end is O$_H$, the intensity of each initiation arc is indicated by a number of ‘+’ symbols to the right of each restriction fragment.
Mapping of the Initiation Zone Based upon Fork-direction Gel Data—Comparison of the set of fork-direction gels (A, D, G, and J) interpreted in B, E, H, and K, C, F, I, and L, standard single digestion Brewer-Fangman gels. Generally the most prominent arc was associated with replication forks traveling away from the 3' end of the D-loop, a notable exception was the MboI fragment spanning np 14,866–16,476, where in-gel digestion with HincII gave rise to arcs of roughly equal prominence (J). Restriction enzymes are indicated on each panel, or the adjacent panel. Restriction fragments are depicted as lines beneath the related gel images, the enzymes applied, restriction sites, and fragment lengths are marked, together with the position of the probe (filled black box).

**Fig. 3.** Replication forks travel in both directions in avian mtDNA, yet most forks travel away from the 3' end of the D-loop. Both potential Y arcs were detectable in fragments of chick mtDNA from around the genome analyzed on fork-direction gels (A, D, G, and J) interpreted in B, E, H, and K. C, F, I, and L, standard single digestion Brewer-Fangman gels. Generally the most prominent arc was associated with replication forks traveling away from the 3' end of the D-loop, as denoted by 3' above the arc; a notable exception was the MboI fragment spanning np 14,866–16,476, where in-gel digestion with HincII gave rise to arcs of roughly equal prominence (J). Restriction enzymes are indicated on each panel, or the adjacent panel. Restriction fragments are depicted as lines beneath the related gel images, the enzymes applied, restriction sites, and fragment lengths are marked, together with the position of the probe (filled black box).
Hence, θ replication can account for all the replication intermediates of chick liver mtDNA.

Initiation arcs were faintest in fragments far from the initiation zone defined above, such as a DraI fragment spanning np 3,448–10,746 and an XhoI fragment spanning np 5,456–12,777 (Fig. 4, A and B). Analysis of fragments of G. gallus mtDNA that included the NCR and the ribosomal RNA genes also yielded an initiation arc (Fig. 4, C and D), which was S1 nuclease-resistant (Fig. 4E). Although these initiation arcs were less prominent than in fragments extending from the NCR in the opposite direction (e.g., Fig. 2A), they were more intense than for fragments far from the NCR (Fig. 4, A and B). Fragments including the rRNA genes, but lacking the NCR had a much reduced initiation arc (Fig. 4F). These data are again compatible with bidirectional initiation across a zone, accompanied by fork arrest in the NCR. In this case, the zone is located upstream of the D-loop 5′ end (Ω̅) and includes the ribosomal RNA genes.

The absence of a descending Y arc in fragments in which the NCR is centered (Fig. 4G) indicates that replication forks rarely traverse the NCR in either direction. Therefore the NCR acts as a bidirectional, or bipolar, replication fork barrier.

In the light of these findings it was important to determine whether we had overlooked initiation upstream of the 5′ end of the D-loop in mammals. Analysis of a Bsu36I fragment of mouse mtDNA spanning np 16,022–6,350 revealed a very faint initiation arc (Fig. 5A) as did another similar restriction fragment (MacI np 15,190–4,130) (Fig. 5B). NspI cleaves mouse mtDNA at three sites yielding three fragments of roughly equal size. The most prominent initiation arc was associated with the fragment spanning np 10,758–114 (Fig. 5C), which includes the NCR and the previously defined initiation zone (10). However, the NspI fragment of mouse mtDNA spanning np 6,092–10,758 also yielded an initiation arc, albeit a weak one (Fig. 5D). Nevertheless, unlike chick, some fragments of mouse mtDNA were not associated with an initiation arc, including the np 114–6,092 NspI fragment (Fig. 5E). Faint termination arcs (33) were also detected in a number of fragments of G. gallus mtDNA (Figs. 2E, and 4, A, B, D, and F) suggesting that termination events may be as dispersed as initiation events.

Replication Initiation Point Mapping—LM-PCR has been used widely to map free 5′ ends of DNA. These species are candidates for transition points from RNA to DNA synthesis and can thereby define sites of initiation of replication (34). λ exonuclease pretreatment degrades spurious ends created by nicking, making replication initiation point mapping a more reliable method for detecting start sites (35). Attempts to apply replication initiation point mapping to mammalian mtDNA have been confounded by the high ribonucleotide content of a majority of mammalian mitochondrial replication intermediates; however, conventional ribonucleotide-sparse replication intermediates were more abundant in G. gallus mtDNA, making the technique potentially applicable.

Although initiation zones allow origin firing at multiple positions, there are recognized preferred start sites in nuclear DNA (36–38). Therefore, we focused on the ND6 gene, which appeared to be the center of the initiation zone (see “Discussion”). Standard LM-PCR revealed a host of free 5′ ends (Fig. 6, lanes 1 and 3). Replication initiation point mapping simplified the results, revealing persistent prominent free 5′ ends, at np 16,184, 16,257, 16,492, 16,567, and 16,581 of the ND6 gene, and np 16,122, and 16,126 of tRNA<sup>Pro</sup> gene (Fig. 6, lanes 2 and 4). Note that these free ends are on the L-strand, which will often be the leading strand for the early arresting fork in our

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2 M. Yang and I. J. Holt, unpublished experiments.
Thus, the prominent free 5' ends detected on the L-strand are consistent with these loci being preferred sites of initiation within the zone defined by two-dimensional AGE mapping. Replication fork arrest or pausing will also generate prominent free 5' ends of DNA; however, frequent fork arrest can be discounted as an explanation of the prominent free 5' ends, as there was no evidence of replication pausing in this region of the *G. gallus* mitochondrial genome (Fig. 2).

**DISCUSSION**

Initiation arcs are diagnostic of origins of replication in all systems examined to date (8, 39–43). The initiation arcs detected in a variety of fragments of *G. gallus* mtDNA (Figs. 2 and 4, A–F) account for a substantial majority of mitochondrial replication intermediates. Therefore, the major mechanism for propagating mtDNA in chick liver is strand-coupled replication, as per mammalian liver and placenta (10). Accordingly, higher vertebrates appear to employ a common mechanism of mtDNA replication.

The first model of strand-coupled mtDNA replication in mammals erroneously inferred that the association between initiation arcs and fragments containing O_H was indicative of unidirectional replication from O_H (19). Subsequently, initiation arcs were found in fragments lacking O_H, and this led to characterization of an initiation zone of bidirectional replication downstream of the 3' end of the D-loop (10). The latest findings necessitate further revision of the model, as fork-direction gels indicate that replication occurs in both directions throughout the mitochondrial genome of *G. gallus* (Fig. 3), and moreover, standard two-dimensional gels revealed a fraction of initiation events mapping to the region immediately upstream of the 5' end of the D-loop in chick and mouse mtDNA (Figs. 4 and 5). Thus, there are two zones of initiation flanking the NCR, which can be represented as a single zone interrupted by a termination region, which may be synonymous with the NCR (Fig. 7A).

**Center of the Initiation Zone**—The decrease in initiation arc intensity with distance from O_H was more pronounced in chick mtDNA (Fig. 2) than that of mouse (10), suggesting that the center of the initiation zone is displaced toward the D-loop 3' end in *G. gallus* relative to mammals (Fig. 7B). There is one major structural difference between the mtDNA of birds and mammals, which could explain the different map positions of the center of the initiation zone. In birds, including *G. gallus*, *ND6* lies closer to the NCR than in mammals, because of the difference in gene order of *ND6* and cytochrome *b* (Fig. 1 and

**FIG. 5.** Initiation of replication in mouse mitochondrial DNA is almost as widespread as in chick. A and B, fragments of mouse mtDNA np 16,022–6,350 (Bsu36I), and np 15,190–4,130 (MscI), respectively. C, D, and E, NspI digested mouse liver mtDNA hybridized with probes that detect fragments spanning np 10,758–114 (C), 6,092–10,758 (D), or 114–6,092 (E). F, the restriction sites producing the fragments shown in A–E are marked on a circle that represents the mouse mitochondrial genome.

**FIG. 6.** Replication initiation point mapping. Standard LM-PCR was applied to chick liver mtDNA using primers (H4 and H3) covering the center of the initiation zone mapped by two-dimensional AGE (lanes 1 and 3). In lanes 2 and 4, the same samples were pretreated with λ exonuclease and alkali before LM-PCR. The *ND6* gene of chicken mtDNA spans np 16,184–16,705, tRNA proline spans np 16,108–16,177.
The initiation events will arise 150–650 np from the 3' end of the H11032 gene. Nevertheless, several fragmentation events will arise 150–650 np from the 3' end of the H11032 gene. Therefore, if the center of the initiation zone is located approximately to the center of the initiation zone maps approximately to the ND6 gene in both classes of organism, then most initiation events will arise 150–650 np from the 3' end of the D-loop in birds, whereas in mammals replication initiation events will be concentrated over a kilobase further downstream of the 3' end of the D-loop. Initiation point mapping in the region of the ND6 gene identified several prominent free 5' ends in the ND6 gene (see Figs. 3 and 6, and “Results” for details). The bidirectional initiation zone of avian and mammalian mtDNA. Circular maps of chicken and mouse mitochondrial genomes with the proposed prominent initiation zone (Ori-z) marked by an unfilled two-headed arrow inside the circle. The center of the initiation zone maps approximately to the ND6 gene in both classes of organism; however, the different arrangement of the cytochrome b gene and ND6 genes places the center closer to the NCR in birds than mammals. ND6, gene encoding a subunit of NADH dehydrogenase; cyt b, cytochrome b gene, 12S rRNA, 125 rDNA gene.

In conclusion, the replication of an avian mitochondrial genome occurs via \( \theta \) replication. The faint initiation arcs in fragments far from the center of the initiation zone can be regarded as the diminishing effect of the zone with distance from the center. The influence of the initiation zone extends throughout the mitochondrial genome of the chick and most of that of mouse.

**Mitochondrial DNA Replication in Other Animals**—The similarities between avian and mammalian mtDNA replication reported here suggest that \( \theta \) replication may well be the common mechanism for all vertebrate mtDNAs. As for invertebrates, it is known only that echinoderm (44) and malarial mtDNA (47) yield conventional simple replication fork arcs, implying that invertebrate mtDNA replication involves coupled leading and lagging strand synthesis. Therefore, it remains to be determined whether or not \( \theta \) replication is the universal mechanism of mtDNA replication of animals.

In conclusion, the replication of an avian mitochondrial genome occurs via \( \theta \) replication. G. gallus mtDNA replication is bidirectional and arises from an initiation zone straddling the NCR. The influence of the initiation zone pervades the entire genome of the chick and most of that of mouse.
mitochondrial genome in the chick and most of that of mouse. The center of the zone maps to the ND6 gene, and prominent free 5’ ends of DNA are found in this region, which represent candidate start sites of DNA replication.

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