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Mitochondrial DNA Replication

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Eukaryotic mitochondrial organelles contain a genome separate and distinct from that of the nucleus. Although limited in genetic content, expression of mitochondrial DNA (mtDNA) is an essential function in most eukaryotes, and therefore the genome is not dispensable. Strategies designed to define both *cis*-acting elements and *trans*-acting factors required for mtDNA replication have been developed over the last 25 years. Both in vivo and in vitro approaches have been employed to describe the mode of mammalian mtDNA replication. This chapter first reviews the basics of earlier works and then turns to more recent investigations that focus on early steps in the replication process. Finally, comparison is made to yeast mtDNAs. The novel kinetoplast DNA is reviewed by Torri et al. (this volume).

BASIC BACKGROUND

Although mtDNA comprises typically less than 1% of a metazoan cell's DNA population, the cellular copy number is 10^3 to 10^4, given the relatively small size of these genomes (~16 kb). It is usually assumed that mtDNA is present in several copies per mitochondrion, but the organelle population is likely dynamic in the living cell, and the distribution of mtDNA may therefore be variable. Replication is under relaxed control and there is no apparent accounting of DNA origins that ensures that each molecule is replicated once and only once per cell cycle, a requirement assumed to be strictly enforced in the case of chromosomal DNA origins of replication. Consistent with this is the lack of any sharp restriction on mtDNA replication with regard to cell cycle phase (Clayton 1982).

The signature form of mammalian mtDNA is the displacement-loop (D-loop) molecule, which maintains a short piece of nascent strand at the leading-strand origin of replication. The leading strand has historically

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been termed the heavy (H) strand because of its greater intrinsic buoyant density in alkaline cesium chloride gradients. Therefore, the opposite strand of the helix has been termed the light (L) strand. The D loop is thus defined as a three-stranded structure with the nascent leading H strand defining the origin of leading-strand replication (O_H) at its 5′ end. D-loop strands are variable in size in a species-specific manner and are turned over more rapidly than the rate of genomic replication would require. A commitment to replication is defined by a molecule in which DNA synthesis has proceeded past the D-loop region around the molecule; this occurs unidirectionally with continuing displacement of the parental H strand. After the growing H strand has elongated to two-thirds or more of its total length, the origin of lagging L-strand replication (O_L) is exposed on the displaced parental H strand and initiation of daughter L-strand synthesis begins. This asynchrony in the functioning of the two origins of mtDNA replication results in two distinct progeny circles being segregated, one of which requires single-strand gap filling prior to closure. Both newly synthesized circles are initially of zero superhelix density and undergo a stepwise introduction of negative superhelical turns. Synthesis of a new D loop then completes the cycle. The topological nature of replicative intermediates, their occurrence, and their form have been reviewed previously (Clayton 1982).

REPLICATION OF MAMMALIAN mtDNA

Productive mtDNA replication begins by an initiation of leading (H)-strand synthesis that results in strand elongation over the entire length of the genome. Since initiation of lagging (L)-strand synthesis only occurs after O_L is exposed as a single-stranded template, leading-strand synthesis is the dominant event in dictating genomic replication. Although the 5′-end map positions of short D-loop strands and elongated leading strands are the same, there has been no reported experiment that determines whether elongation occurs from preexisting D-loop strands or whether it requires initiation and uninterrupted synthesis through the D loop and then beyond.

Initiation of Heavy-strand Synthesis at the Leading-strand Origin

Experiments performed 10 years ago documented the 5′ and 3′ ends of both DNA and RNA species complementary to the D-loop region of human (Chang and Clayton 1985) and mouse (Chang et al. 1985) mtDNAs.

These data refined the map positions of these species because of improved sizing technologies for nucleic acids and were consistent with previous assignments (Gillum and Clayton 1979). In summary, there are several sites of likely transition from RNA synthesis to DNA synthesis. For both human and mouse species it is possible, in the majority of cases, to align RNA 3′ ends with DNA 5′ ends, consistent with the possibility that the RNAs are involved in priming replication. In the case of mouse, a species was isolated that consisted of RNA at its 5′ end and DNA throughout most of its distal portion (Chang et al. 1985). Importantly, the 5′ end of the RNA portion of a primed nascent leading strand mapped at the transcription promoter of this strand. The organization of the genome is such that all genes served by this promoter lie downstream from the origin. Thus, in principle, one round of transcription could be sufficient for a priming event and transcription of all of the genes on this strand.

Initiation of Light-strand Synthesis at the Lagging-strand Origin

The origin of lagging (L)-strand replication, O_L, located well away from O_H (Fig. 1), is approximately 30 nucleotides in size. It is interesting that this small noncoding region is tightly flanked by tRNA genes. The origin itself has the potential for forming a predictably stable stem-loop structure, and this feature is highly conserved among sequenced vertebrate mtDNAs, with the exception of chicken (Desjardins and Morais 1990).

Our current understanding of how O_L functions required the development of an in vitro replication system capable of initiation of L-strand synthesis that produced nascent strands which were the same as those that had been previously identified from in vivo mitochondrial nucleic acid isolates. The in vitro run-off replication assay (Wong and Clayton 1985a,b) revealed a human mtDNA primase with the ability to recognize O_L and initiate priming and DNA synthesis. Daughter L-strand synthesis is primed by RNA synthesis complementary to the T-rich loop structure of O_L. In turn, the transition from RNA synthesis to DNA synthesis occurs near the base of the stem. The site of transition from RNA synthesis to DNA synthesis is contained within an immediately adjacent tRNA gene. Furthermore, a mutational analysis demonstrated that although the small O_L sequence was necessary and sufficient to support initiation of DNA replication (Hixson et al. 1986), a short pentanucleotide sequence at the base of the O_L stem was required to maintain a correctly functioning origin. This appears to involve the ability of mtDNA polymerase to engage a primer, as the mutations examined by Hixson et al. (1986) were

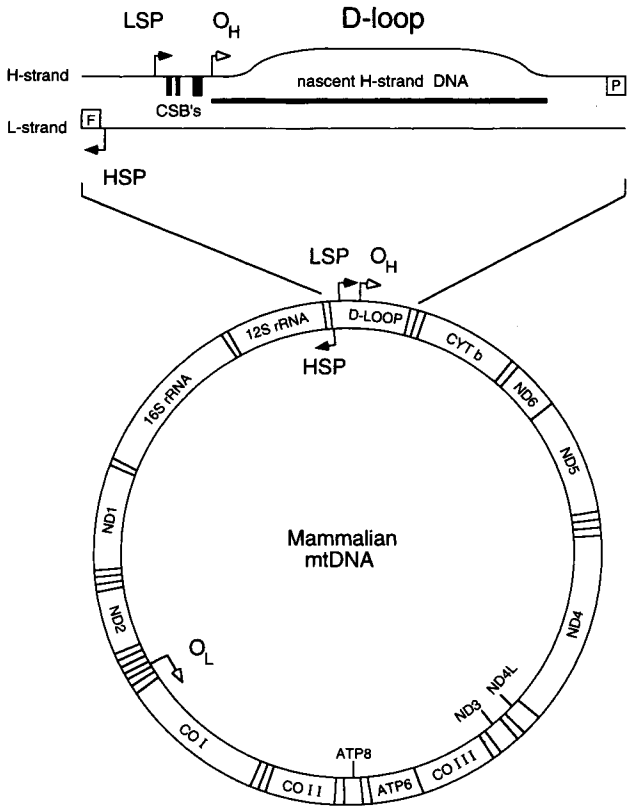

Figure 1 Representation of mammalian mtDNA. The small boxes represent the 22 tRNA genes (F, phenylalanine; P, proline). The 12S and 16S rRNA genes and D-loop region are shown. O_H and O_L are the respective origins of H- and L-strand synthesis. HSP and LSP are the respective promoters for transcripts copied from the H and L template strands. Arrows denote the directions of synthesis. The protein-coding genes are cytochrome *c* oxidase subunits I, II, and III (COI, COII, COIII); ATPase subunits 6 and 8 (ATP6, ATP8); cytochrome *b* (CYTb); and subunits 1, 2, 3, 4, 4L, 5, and 6 of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6). ND6 and eight tRNA genes are encoded by H-strand sequence (L strand is the template strand); all the other genes are encoded by L-strand sequence. (CSB's) Conserved sequence blocks.

not obviously impaired in priming function, but instead were deficient in the process of elongating primers into DNA strands.

Elongation and Maturation of Progeny Strands

The basic details on the mode of elongation and maturation of both leading and lagging strands of mammalian mtDNA have been determined by

isotopic labeling and electron microscopic visualization approaches (Clayton 1982). There has been no extensive further work along these lines, although there have been attempts to utilize isolated organelles as a way to study various steps of mtDNA replication (for examples, see Dunon-Bluteau et al. 1987; Enríquez et al. 1994). Although this approach may lead to insights on regulating replication, it has some limitations, since it is not possible to define the molecules responsible for various events in the process nor to test easily for the efficacy of altered reaction conditions, different templates, and requirements for known, purified entities.

TRANS-ACTING FACTORS INVOLVED IN REPLICATION

The close physical and functional relationships between leading-strand mtDNA replication and transcription have resulted in data from studies of each process that merge at the level of transcription initiation from the strong promoter upstream of O_H. Promoter-specific transcription requires the presence of the transcription factor mtTFA (formerly termed mtTF1) in addition to a mitochondrial RNA (mtRNA) polymerase fraction that alone is incapable of selective transcription (Fisher et al. 1987). Mature human mtTFA (h-mtTFA) is a small (204 amino acids) basic protein that binds immediately upstream of the mitochondrial promoters. There is an excellent correlation between DNA binding by h-mtTFA and the ability of that protein to support correct and efficient transcription initiation (Dairaghi et al. 1995 and references therein), and Fisher et al. (1992) were able to demonstrate that h-mtTFA has the capacity to unwind and bend DNA, thereby suggesting the manner in which at least one part of the transcription initiation process takes place.

Human mtTFA contains two domains characteristic of high mobility group (HMG) proteins, and these two domains (HMG boxes) comprise most of the molecule. The two HMG boxes are interrupted by a 27-amino-acid linker sequence, and the protein contains a 25-residue, carboxy-terminal tail. Mutational analysis of the protein has recently shown that this short tail is critical for DNA binding at the promoter and for transcriptional activation (Dairaghi et al. 1995).

Processing of Mitochondrial Transcripts for Replication

The first isolated mammalian mtRNA processing activity was RNase MRP (for *m*itochondrial *R*NA *p*rocessing), which was isolated from mouse and human mitochondrial fractions and shown to be a site-specific

endoribonuclease containing an essential RNA component (Chang and Clayton 1987). RNase MRP is a ribonucleoprotein with its RNA component and very likely all of its protein components encoded by nuclear genes. The sequence of the nuclear gene for the RNA component of RNase MRP has been obtained for vertebrates and fungi (Paluh and Clayton 1995; for sequence comparisons of earlier reported sequences, see Schmitt et al. 1993), and the genes are distinctly similar. Inspection of the coding region and surrounding sequences indicates that it is very likely that these genes function as polymerase II/polymerase III transcription units, which may represent an ancient class of nuclear gene. The genes are single copy in the cases of both mouse and human (Hsieh et al. 1990), and it is interesting to note that there is extensive sequence similarity in the 5′-flanking region that surpasses that typical for other small RNA genes of nuclear origin. There are two protein components identified for *Saccharomyces cerevisiae* RNase MRP: One is shared with RNase P (Lygerou et al. 1994) and the other appears unique to RNase MRP (Schmitt and Clayton 1994).

RNase MRP cleaves mtRNA transcripts that are complementary to the leading-strand origin of mammalian mtDNA, and therefore, it is a candidate for playing a role in primer RNA metabolism. The predominant site of cleavage on single-strand mtRNA is one that may occur in vivo as well, given the fact that transcript termini map at this position (Chang and Clayton 1985; Chang et al. 1985). RNase MRP could produce 3′-OH groups for extension by mtDNA polymerase or could be somehow involved in stabilizing primers at the 5′ end or in 5′-end editing of transcripts (Chang and Clayton 1987); new evidence is consistent with any or all of these roles (see section below on regulation).

Mammalian mtDNA Replication Proteins

mtDNA polymerase, commonly termed DNA polymerase-γ, has been studied sporadically over the years, but only recently has information become available on its actual size and some of its functional features. There are several reports of an associated 3′→5′ exonuclease activity being an intrinsic part of the polymerase; it likely confers a proofreading capacity consistent with studies on the error rate of DNA polymerase-γ (Kunkel and Soni 1988; Insdorf and Bogenhagen 1989b; Kaguni and Olson 1989 [for *Drosophila melanogaster*]). The catalytic subunit of the holoenzyme appears to be of significant size, approximately 140 kD in *Xenopus laevis* (Insdorf and Bogenhagen 1989a; a similar situation may prevail for *D. melanogaster* [Wernette and Kaguni 1986]); in both these

cases there appears to be a stoichiometric, smaller subunit present in the most highly purified preparations. Human mtDNA polymerase has been partially purified (Gray and Wong 1992) and has recently been cloned by virtue of its conservation to yeast mtDNA polymerases (W.P. Copeland, pers. comm.). This significant advance should permit a comprehensive study of the structure, functional parameters, and intermitochondrial distribution of this central activity in mtDNA replication.

There are several instances of the identification of mtDNA-binding proteins that display a preference for single-stranded DNA (Pavco and Van Tuyle 1985; Van Tuyle and Pavco 1985; Mignotte et al. 1988). These are likely to be standard single-stranded DNA-binding proteins, because their sequences closely resemble *Escherichia coli* SSB (Tiranti et al. 1993), which has facilitated their cloning. It will be of interest to learn the possible association of mitochondrial SSB with mtDNA polymerase in the replication complex and to determine whether mutations or deficiencies in mitochondrial SSB protein can cause mtDNA deletions in human disease. In the case of *Drosophila, E. coli* SSB is able to stimulate the rate of DNA synthesis in vitro by mtDNA polymerase (Williams and Kaguni 1995). The current working model for mammalian mtDNA replication is depicted in Figure 4 of the chapter by Brush and Kelly (this volume).

REGULATING mtDNA REPLICATION IN THE MAMMALIAN CELL

Regulation of mammalian mtDNA replication most likely involves molecular events surrounding initiation of leading-strand DNA synthesis at the D loop. As outlined earlier, O_L is well separated physically in the genome, and there is no evidence to indicate that a round of productive replication can begin there. Our knowledge of the exact characteristics of the DNA primase activity that recognizes O_L has not advanced beyond work described in an earlier review (Clayton 1991). This is due, at least in part, to the very low abundance of this activity in the cell. However, we can describe some of the fundamental aspects of initiation of leading-strand synthesis that enable, in turn, some predictions on how regulation of replication may occur.

Step one in the process is initiation of transcription at the LSP to provide primers. Initiation requires mtTFA and a partially purified mtRNA polymerase. There are currently no known differences in the requirements for initiation that reflect the eventual fate of the transcripts. That is, the nucleus-encoded transcription proteins, and their interactions with the template and each other, could be identical in producing RNA for

priming or for transcripts that represent tRNAs or that encode a protein. Thus, the absence or impairment of function of these proteins should affect both mtDNA replication and gene expression. It is known that h-mtTFA is greatly reduced in some human tissues and cell lines with little or no mtDNA (Larsson et al. 1994; Poulton et al. 1994), but a direct loss of mtDNA due to down-regulation of h-mtTFA remains to be shown.

The second important event in initiation appears to be the formation of stable RNA-DNA hybrids over the origin region (Fig. 2). The principal feature of mtDNA responsible for this is the highly conserved *c*onserved *s*equence *b*lock II (CSBII) element. This short (usually defined as between 15 and 20 nucleotides) sequence is atypical in two obvious ways. It is not only more than 90% G+C-rich, but all of the guanosines are in one strand; RNA transcribed across CSBII thus contains this virtual guanosine homopolymer run. The requirements for stable RNA-DNA hybrid formation are clearest for yeast putative mtDNA origin sequences (Xu and Clayton 1995). The yeast CSBII sequence homolog is the necessary and sufficient element for sponsoring RNA-DNA hybrid formation, and the natural polarity of the sequences (guanosines in the RNA strand, cytosines in the DNA strand) must be maintained. The same basic phenomenon is seen for mammalian mtDNA, and in this case, CSBII is required along with some dependence on the two other conserved sequences at the origin, CSBIII and CSBI, with respect to efficiency of hybrid formation (Xu and Clayton 1996).

Cleavage of D-loop region RNA-DNA hybrids has been achieved (D.Y. Lee and D.A. Clayton, unpubl.) with highly purified preparations of RNase MRP. These sites of cleavage align well with mapped termini of primer RNAs and nascent mtDNA 5′ ends at the leading-strand origin. Thus, a more refined model for initiation of leading-strand synthesis invokes stable RNA-DNA hybrid formation followed by R-loop processing to achieve synthesis by mtDNA polymerase (Fig. 3).

COMPARING MAMMALIAN AND YEAST mtDNA REPLICATION

Although the field of yeast mitochondrial biology and genetics has a longer history than that of mammalian studies, the molecular details of yeast mtDNA replication are largely unknown. This is due in part to the greater complexity of yeast mitochondrial genomes. In the case of wild-type mtDNAs, genome size is variable among strains and species. In cases where yeast mtDNA has been studied physically, it is apparent that the genome can be found in different circular and linear forms. This situation makes it difficult to perform the type of biochemical analyses

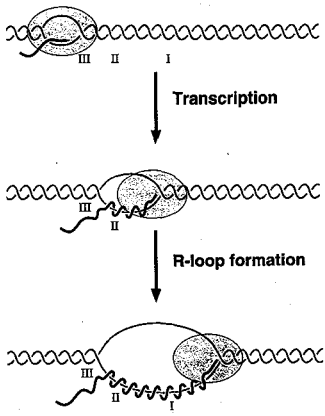

Figure 2 Diagram illustrating formation of a persistent RNA-DNA hybrid via transcription at the leading-strand origin. Roman numerals represent CSBIII, CSBII, and CSBI, respectively. The gray filled oval represents the mtRNA polymerase complex.

that have been employed for the mammalian systems, where the rate of replication is slow and where, in retrospect, the mode of replication (unidirectional synthesis from two widely spaced origins) has advantaged these studies.

Another issue is the aggressive rate of recombination in the fungal mtDNA systems, in contrast to mammals, where mtDNA recombination is at most a rare occurrence. Therefore, the problem of distinguishing between replication and recombination events is acute for yeast and essentially nonexistent for mammals. Finally, the small closed circular form of mammalian mtDNAs and a less aggressive background of nucleases have facilitated isolation of intact genomes.

Some parallels may exist between the yeast and mammalian systems. Very recent cloning efforts point to homologs between transcription, replication, and accessory protein sequences. Putative origin sequences in yeast have some conserved elements in common with mammals, and these possible shared features have been reviewed in detail previously (Schmitt and Clayton 1993). However, it is likely that yeast mtDNA can be propagated by multiple mechanisms, at least one of which is a rolling circle (Maleszka et al. 1991).

CONCLUSIONS AND PROSPECTS

The overall mode of mammalian mtDNA replication has been known for some time. During the last decade, the nature of both *cis*- and *trans*-

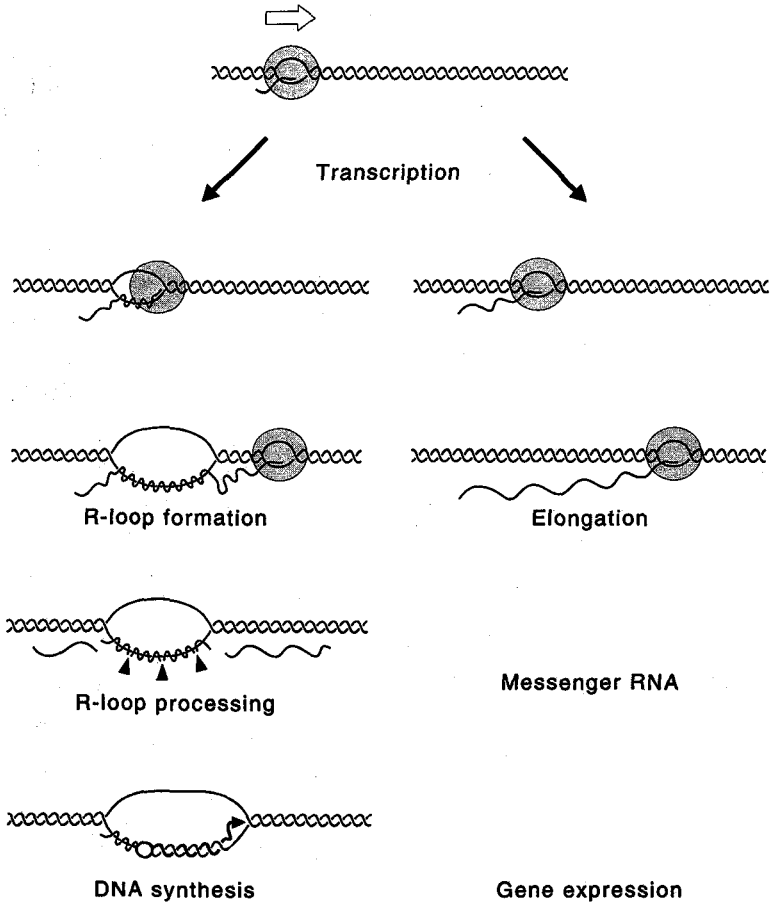

Figure 3 Model for transcript fates at the leading-strand origin. The leftward portion depicts RNA–DNA hybrid formation and subsequent processing of the RNA strand to form primers for replication. The rightward portion represents orthodox transcription, which results in release of the transcript for processing into eight tRNAs and one mRNA. The gray filled circle represents the mtRNA polymerase complex.

acting elements has been revealed for mammalian mtDNA, and the most recent efforts are elucidating the molecular details of nuclear gene-encoded proteins that form the set of activities required for mtDNA replication. Thus, new approaches to understanding mtDNA replication should now be available, and efforts to learn the full spectrum of protein-DNA interactions in the D-loop region should be revealing (see Cantatore et al. 1995 and references therein).

The possibility of developing an in vitro D-loop replication system is now realistic, given that both of the relevant polymerases and key additional activities are now, or soon will be, available as cloned entities. This should facilitate a good understanding of D-loop metabolism and could shed light on possible mechanisms of faulty replication that lead, for example, to deleted forms of mtDNA. Deleted forms of human mtDNA can cause human disease (for review, see Larsson and Clayton 1995), and at present their basis of formation is unknown. A new avenue of research is to study the effect of mutated mtDNA replication proteins in the context of the organism as a whole. Both human and mouse genes for relevant activities will soon be in hand and available for manipulation. It should be possible to introduce variants of mouse mtTFA, mouse polymerases, and other proteins in the animal itself and, thereby, to study altered function with all other components present. Such model systems should also provide the best opportunities to test cellular phenotypic consequences of deficiencies in mtDNA replication and genome maintenance.

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