Molecular recombination and transcription are proposed mechanisms to initiate mitochondrial DNA (mtDNA) replication in yeast. We conducted a comprehensive analysis of mtDNA from the yeast Candida albicans. Two-dimensional agarose gel electrophoresis of mtDNA intermediates reveals no bubble structures diagnostic of specific replication origins, but rather supports recombination-driven replication initiation of mtDNA in yeast. Specific species of Y structures together with DNA copy number analyses of a C. albicans mutant strain provide evidence that a region in a mainly noncoding inverted repeat is predominantly involved in replication initiation via homologous recombination. Our further findings show that the C. albicans mtDNA forms a complex branched network that does not contain detectable amounts of circular molecules. We provide topological evidence for recombination-driven mtDNA replication initiation and introduce C. albicans as a suitable model organism to study wild-type mtDNA maintenance in yeast.

INTRODUCTION

Mitochondrial DNA (mtDNA) maintenance is essential because key proteins of the respiratory chain are encoded by the mt genome. An early replication model for vertebrate mtDNA proposed theta replication (Kirschner et al., 1968). Further research suggested a transcription-initiated strand-displacement model employing specific unidirectional origins of leading- and lagging-strand DNA synthesis (Clayton, 1982). Studies of vertebrates have added models of strand-coupled replication (Holt et al., 2000), RNA incorporation throughout the lagging strand (RITOLS) (Yang et al., 2002; Yasukawa et al., 2006), and initiation from various sites along mtDNA (Reyes et al., 2005). A recent study of the mtRNA polymerase in mammalian mitochondria has further elucidated initiation of the lagging strand in uncoupled replication (Vannrooj et al., 2008; Fusté et al., 2010).

Most studies on mtDNA replication in yeast have focused on Saccharomyces cerevisiae, in which many DNA maintenance factors were identified, and the majority of these analyses were performed in rho− cells in which only a small fragment of the 80 kb wild-type (WT) mt genome is maintained (Contamine and Picard, 2000; Chen and Butow, 2005). Although rho− cells are a valuable tool to identify maintenance factors, they may hinder study of WT mtDNA topology in S. cerevisiae.

Seven to eight putative replication origins (“ori/rep”) have been assigned to the mtDNA of S. cerevisiae (de Zamaroczy et al., 1981; Schmitt and Clayton, 1993; Fournier et al., 1998), but topological evidence for their involvement in replication initiation similarly to the vertebrate system is lacking. Three ori/rep elements are associated with a promoter and are believed to initiate replication (de Zamaroczy et al., 1984). It was therefore proposed that transcription primes replication of yeast mtDNA (Baldacci and Bernardi, 1982; Baldacci et al., 1984). While RNA primed DNA strands have been detected in rho− mtDNA (Baldacci et al., 1984; Xu and Clayton, 1995; Graves et al., 1998), transcription is dispensable for rho− mtDNA maintenance (Fangman et al., 1990).

Homologous recombination (HR) is being discussed as a likely process for mtDNA replication initiation in yeast and plant (Ling et al., 2000; Ling and Shibata, 2002; Manchekar et al., 2006; Shibata and Ling, 2007). Replication in protist mitochondria utilizes recombination (Preiser et al., 1996) and recent findings for human heart mtDNA also suggest a role for HR in non-theta replication (Pohjoismäki et al., 2009). To date, recombination-driven DNA replication (RDR) initiation has been best studied in phase T4 (Mosig, 1998; Kreuzer, 2000), and it has been proposed that mtDNA replication in yeast could employ a similar mechanism (Ling et al., 2007).

While S. cerevisiae tolerates loss of mtDNA, C. albicans belongs to the group of yeasts that cannot afford it, and therefore it provides a different system for wild-type mtDNA maintenance analyses. Close homologs to S. cerevisiae mtDNA maintenance proteins are found in C. albicans, suggesting close mechanistic similarities in both yeasts (Wardleworth et al., 2000; Nosek et al., 2006; Jöers et al., 2007; Visacka et al., 2009).

The mtDNA of C. albicans appears to consist of a network of complex branched molecules (Jöers et al., 2007). Unit-sized molecules of 40.4 kb are rare and circular molecules have not been detected. Possible replication origins and mechanisms in the mtDNA of C. albicans are unknown, but deletion of the mt helicase CaHm1p led to accumulation of mtDNA fragments harboring the cox2 gene. Therefore, enhanced replication initiation within or close to cox2 has been suggested (Jöers
et al., 2007). We recently identified the C. albicans mtDNA binding factor Gcf1p as a homolog of *S. cerevisiae* Abf2p (Diffley and Stillman, 1991). Gcf1p deletion reduced mtDNA copy numbers and formation of recombination intermediates suggesting close ties between replication and recombination (Visacka et al., 2009). The present work describes topological analyses of mtDNA in C. albicans by two-dimensional agarose gel electrophoresis (2D-AGE) to further clarify replication mechanisms of mtDNA in yeast. We present the data together with evidence from a *hmi1*-deletion strain underlining the major role of HR in the replication of mtDNA in yeast. We propose possible mechanisms based on HR for replication initiation. These include homologous invasions of 3’ single-stranded DNA (ssDNA) overhangs, suggesting special involvement of inverted repeat sequences, and annealing of free ssDNA to complementary stretches of the complex branched DNA network.

**RESULTS**

The mtDNA in *C. albicans* Consists of a Complex Branched DNA Network of Varibly Sized Subfragments

The genes encoded by the 40.4 kb *C. albicans* mtDNA (Anderson et al., 2001) are clustered into two major coding regions, large coding region (LCR) and small coding region (SCR) separated by a 2 × 7 kb inverted repeat (Wills et al., 1985; Shaw et al., 1989), inverted repeat a (IRa) and IRb (Figure 1A). In IRa and IRb, 5.6 kb are noncoding. Analyses with restriction enzymes that cut at one or two sites were performed (Figures 1B and 1D). Cleavage with Ncol-HindIII, Ncol-Lgul, or Ncol-Esp3 (Figures 1B and 1D) probed for cox3 (Figure 1A) was expected to produce two bands on radiographs (stars in Figure 1B). However, each cleavage revealed four prominent bands (Figure 1B). Alignment of the two additional bands to size markers indicated switched orientations of SCR relative to LCR (black arrows in Figure 1D, I and II). Further combinations of different restriction enzymes confirmed this finding (data not shown). The relative intensities of the four major bands in each lane had a ratio of 1:1 showing that both orientations of SCR occur at equal frequencies.
Restriction-fragment analysis of *C. albicans* mtDNA produces a circular map (Wills et al., 1985; Nosek et al., 2006), but pulsed-field gel electrophoresis (PFGE) analyses did not reveal the presence of circular DNA molecules (Figure 1C). To assess the quality of our mtDNA purification, we simultaneously analyzed agarose-embedded cells, embedded mitochondria, and purified mtDNA. As expected, the amount of well-bound mtDNA was less for purified mtDNA (Figure 1C, lanes 5, 6, and 7) than for embedded whole cells (lanes 1 and 2) and mitochondria (lanes 3 and 4). This reduction in the well-bound fraction is evidently due to breakage during preparation, resulting in more mtDNA migrating as a smear of smaller molecules. A similar observation has been made with PFGE data on in-gel and in-liquid preparation of *Neurospora crassa* mtDNA, which has a unit size of 67 kb and is thus comparable to *C. albicans* (Bendich, 1996). *N. crassa* mtDNA contains 1% or less circular molecules that are detectable as a faint signal. *C. albicans* mtDNA did not produce comparable signals even after 3 day exposure to phosphorimager screens. If a fraction of this mtDNA is in circular forms, it is a very minor fraction. Our analyses showed most of the mtDNA signal in the well-bound fraction and as a smear of molecules with sizes corresponding to linear 12.2 to 48.5 kb DNA fragments (Figure 1C, lanes 1–7, and Figure S1C available online). A smear of complex molecules resistant to restriction enzyme cleavage was observed not only after one-dimensional gel electrophoresis (e.g., Figures 1B and 1C), but also after 2D-AGE (as demonstrated later).

Cleavage with NcoI resulted in a shift of the hybridization signal (Figure 1C, lanes 2, 4, and 6). Compared to untreated mtDNA (lanes 1, 3, and 5), signal was now detected between 8 kb and ~40 kb, which equals the unit size (40.4 kb). An enhanced signal at ~40 kb indicates cleavage of head-to-tail concatemers from the complex well bound fraction. Head-to-head or tail-to-tail concatemers cleaved with NcoI would have produced linear fragments larger than unit size (~75 kb). Unit-sized supercoiled circular molecules that would migrate between the well and compression zone (cz) were not detected. The 40 kb band could therefore not be the result of linearized unit-sized supercoiled circular molecules. Treatment with topoisomerase I (TopoI), which catalyzes the relaxation of negatively supercoiled DNA (Kirkegaard and Wang, 1978) and therefore indicates the presence of circular molecules, did not lead to major changes in running patterns (Figure 1C, lane 7). If supercoiled circular molecules, which would migrate in the gel, were relaxed by TopoI, they would be immobilized and increase the signal of the well-bound fraction. In contrast, we observed a reduction of the signal in the well (Figure 1C, lane 7).

Significant changes were observed for purified mtDNA treated with phage T7 endonuclease I (T7EndoI; Figure 1C, lane 8), which recognizes and cleaves cruciform, branched, nicked, and imperfectly base-paired DNA structures (Guan et al., 2004). T7EndoI treatment degraded molecules of the well-bound fraction and of sizes larger than 20 kb. Remaining molecules of sizes between ~5 to 20 kb are assumed to be linear double-stranded DNA (dsDNA).

Circular dsDNA is a substrate for T7EndoI if it contains cruciform structures. Cruciform structures are discussed to be stably maintained only on supercoiled circular DNA, but supercoiled circular DNA was not detected in our experiments. Relaxed circular DNA would be cleaved at a nicked position. Cleavage of unit-sized, single-nicked circular molecules would result in a 40 kb band of linear DNA, which also was not observed. It is formally possible that circular molecules containing multiple irregular nicks are degraded to fragments of 20 kb and smaller. However, we cannot attribute any hybridization signal to relaxed or supercoiled circular molecules into which multiple nicks could be introduced. Since after T7EndoI treatment no residual signal could be observed in the well, molecules of 20 kb and smaller are most likely generated by cleavage of frequently branched molecules. The majority of *C. albicans* mtDNA thus forms a complex and branched network containing head-to-tail concatemers.

**Analyses of mtDNA Replication Intermediates Revealed Y-Shaped, X-Shaped, and Complex Branched Molecules**

The topology of *C. albicans* mtDNA raised the question of how it is replicated. The entire mtDNA was analyzed by neutral 2D-AGE, and restriction fragments of various size were chosen to overlap by several kb (Figure S1A, Figure 2). In general, restriction fragments containing a replication origin of strand-coupled synthesis comparable to e.g., mammalian mtDNA O briefly produce “bubble arcs” (Figure 2F, “B,” gray dotted line). Passing replication forks result in Y arc patterns (Figure 2F, “Y”) extending from the 1N spot of nonreplicating molecules to the 2N spot of almost fully replicated molecules. X arcs emerging vertically from the 2N (Figure 2F, “X”) spot have been shown to consist of four-stranded DNA structures (Holliday junctions), e.g., on mtDNA of *S. cerevisiae* (Lockshon et al., 1995; MacAlpine et al., 1998).

Systematic analysis of the entire *C. albicans* mtDNA revealed no bubble arcs but showed Y arcs on all restriction fragments. Examples presented here are fragments of BamHI-EcoRI probed for *cox3* (nt 8277–11724), EcoRV-atp6 (nt 12220–15748), EcoRV-nad2 (nt 19173–23049), SnaBI-cob (nt 21827–25069), and SnaBI-nad5 (nt 27735–31334) (Figures 2A–2E). In addition to Y arcs, prominent X arcs were detected, indicating HR throughout the mtDNA. A blurred arc extending from Y and X arcs up to the well of the former first-dimension gel was detected in all regions (Figure 2F, “C”). Similar cloud arcs (C arcs) of complex and branched molecules were detected in studies on mtDNA in yeast and plants and are rich in ssDNA stretches (Han and Stachow, 1994; Manchekar et al., 2006). An arc running below the dsDNA was detected with all probes. It was resistant to RNaseA treatments (data not shown) and consisted of ssDNA as revealed by S1 nuclease treatment (Figure 4).

The lack of bubble-shaped intermediates, even after heavy overexposure of radiographs, excluded classical strand-coupled replication initiation, as observed for mammalian mtDNA (Holt et al., 2000; Bowmaker et al., 2003; Reyes et al., 2005). On circular DNA, detection of Y arcs was proposed to indicate RC replication (Han and Stachow, 1994; Backert et al., 1997), which is one form of strand displacement (SD) synthesis. In the absence of circular molecules, Y arcs, X arcs, complex branched molecules (C arcs), and ssDNA could indicate recombination initiated SD synthesis on a linear template.
It is generally accepted that single-stranded 3’ DNA termini serve as precursors for strand invasion during HR (Mosig, 1998; Kowalczykowski, 2000; George et al., 2001). These termini may be products of targeted processes or can be generated from dsDNA breaks or ends. Assuming that recombination depends on dsDNA ends acting as precursors, large amounts of shorter dsDNA molecules should be observed after restriction digests. Indeed, as seen on all 2D-AGE radiographs (Figures 2, 3, and 4), linear dsDNA molecules shorter than the 1N fragment always make a significant contribution to the detected hybridization signal. These molecules might have random ends, as demonstrated elsewhere (Oldenburg and Bendich, 2001) and as suggested by PFGE in this study (Figure 1C). Radiographs in Figures 2A–2E were quantified and the results expressed as percentage of total detected hybridization signal per membrane (Figures 2G and 2H).

DNA was prepared from exponentially growing cells that actively synthesize mtDNA. Thus, replication intermediates should constitute a significant portion of the detectable mtDNA. This is shown by an average relative signal distribution of 13% into the area between 1 and 2N spots including Y and X arcs and 21% of relative C arc signal of molecules larger than 2N and X arcs. 1N spots corresponding to regular double-stranded molecules provide precursors for RDR of mtDNA in vivo, their uniform presence on all 2D-AGE radiographs could suggest that replication initiates randomly across the mtDNA of C. albicans.

**Analysis of IRa/IRb Revealed a Specific Type of Y Arc-like Intermediates**

The proposed ori/rep elements in S. cerevisiae are located in noncoding regions of the mt genome (de Zamaroczy et al., 1981; Schmitt and Clayton, 1993; Foury et al., 1998), and also in vertebrate mtDNA the noncoding intergenic region is associated with replication initiation (Clayton, 1982; Holt et al., 2000; Bowmaker et al., 2003; Yasukawa et al., 2005). Although in silico analyses of the C. albicans mtDNA sequence revealed no ori/rep-like sequences, cumulative GC skew analysis showed local minima in IRa (nt 7310) and IRb (nt 38580) (Figure S1B). Such local minima are indicative of replication initiation sites, because extremities in the GC skew profiles are detected at the replication origins of bacterial chromosomes (Grigoriev, 1998). Strikingly, upon cleavage of mtDNA, faint bands were observed forming in addition to expected products. These bands may represent preferential breaks or ends of linear DNA molecules. Among those bands, specific
fragments could be identified (Figure 1B, arrows) that match with breaks or molecule ends approximately at positions of GC skew minima (Figure S1B). If a specific break or end occurs at position 7310, fragments with sizes of 5.6 kb (nt 7310–HindIII), 22.5 kb (nt 7310–LguI) and 24.7 kb (nt 7310–Esp3) are detected by probing for cox3. A break or end at position 38580 produces accordingly fragments of 14.3 kb (nt 38580–HindIII), 8.7 kb (nt 38580–LguI), and 6.6 kb (nt 38580–Esp3). In addition, a band of 6.4 kb was detected in Ncol-digested mtDNA separated by PFGE (Figure S1C), which corresponds to a cox2-probed fragment spanning from Ncol to position nt 7310. 2D-AGE mapping involving IRa/IRb revealed specific Y-like arcs on fragments that cover parts of IRa/IRb and SCR (Figures 3A–3D and 3F). The specific extra-small or extra-large Y arcs (YES or YEL) occurred depending on the restriction enzymes used. As shown in Figures 3A and 3B, mtDNA cleaved by Dral (nt 38293–2391) or Dral-NcoI (nt 38293–929) probed for rRnL produced regular Y arcs and the specific type of YES arcs. Cleavage with BamHI-NcoI (nt 38238–929), also probed for rRnL, resulted in formation of specific YEL arcs and regular Y arcs (Figure 3C). Y-shaped molecules forming YES and YEL arcs can be attributed to strand invasion by homologous sequences of IRa into IRb (Figures 3G and 3H). As depicted in Figure 3H, strand invasion involving an ssDNA overhang is predicted to lead to the formation of a Y-shaped recombination intermediate that can be cleaved to result in the observed YES or YEL forms (Figures 3G and 3I). Single-end invasion intermediates in S. cerevisiae nuclear DNA were shown to comigrate with Y arcs (Hunter and Kleckner, 2001), and similar intermediates discussed here can be converted directly into regular Y-form replication intermediates.

Cleavage of C. albicans mtDNA with EcoRI-BamHI (nt 3665–7717) probed for nad1 produced an YES pattern as expected and thus provides further evidence for homologous strand invasion between IRa and IRb (Figure 3D).
Structures Containing ssDNA and RNA:DNA Duplexes Are Detected in C. albicans mtDNA

Recent studies of mammalian mtDNA have shown replication intermediates of strand-coupled synthesis (Holt et al., 2000; Bowmaker et al., 2003; Yasukawa et al., 2005) in addition to partially single-stranded intermediates supporting the orthodox model of DNA replication (Clayton, 1982; Brown et al., 2005). Careful analyses of the latter intermediates have revealed a mechanism involving incorporation of ribonucleotides (Yang et al., 2002; Yasukawa et al., 2006). To address the possibility of a similar mechanism in yeast, we treated purified C. albicans mtDNA with RNaseH, which degrades RNA:DNA duplex molecules, and ssDNA-degrading S1 nuclease.

2D-AGE of different mtDNA regions revealed remarkable features as demonstrated on a 2.3 kb EcoRV fragment (nt 2444–4807) probed for cox2 (Figures 4A–4D, interpreted in Figure 4E) located at the center of SCR. S1 treatment removed the ssDNA arc (Figure 4B) but also revealed strong effects on molecules running in the C arc indicating exposed ssDNA stretches, overhangs, or nicks (Figures 4A and 4B). A quick moving arc (Q) that is of higher mobility than dsDNA and that extends from the 1N spot (Figures 4A and 4E, also Figures 2B and 2D) was also found to be highly S1 sensitive (Figures 4B and 4E). Removal of complex and branched molecules revealed X-like arcs extending from two discrete spots on the dsDNA arc at ~9 and ~11 kb (Figures 4B and 4E, Figure S2).

Molecules migrating between the dsDNA and the Y arc (Figures 2 and 3) were generally found to be S1 sensitive. On the cox2-probed EcoRV fragment a blurred triangular arc was observed resembling a flag with molecule sizes between 2.3 and 3.5 kb (Figure 4) that was not S1 sensitive (Figure 4B) but was removed by RNaseH (Figure 4C) and led to formation of a S1-sensitive spot migrating below the dsDNA arc at ~9 to ~11 kb (Figures 4B and 4E, Figure S2).

Identification of long ssDNA molecules and nicked DNA further supports a SD replication mechanism.

Opposing Replication Forks Pass the mtDNA of C. albicans

Randomly initiated RDR might lead to either bi- or unidirectional replication forks which eventually could oppose each other (Figure 5A). The polarity of replication forks was analyzed by modified 2D-AGE (Friedman and Brewer, 1995; Reyes et al., 2005). Digested DNA was separated on a first-dimension gel and cleaved again in gelo followed by separation in the second dimension. Polarity of forks was determined according to Figure 5D. Unidirectional forks that enter any restriction fragment from either end will lead to formation of Y	extsubscript{a} or Y	extsubscript{b} arcs. Different fragments were analyzed, and examples from LCR and SCR are presented (Figures 5B and 5C). BglII-EcoRI (nt 40023–3665) cleaved mtDNA was treated in gelo with EcoRV (nt 2444) and probed for rRnL (Figures 5B and 5C). Two Y arc patterns could be observed. A Y arc extending from the 1N spot and ending well before reaching the 2N spot indicated a fork moving...
in 3′-5′ direction of the rRnL gene. A second Y arc starting from the horizontal arc indicated a fork moving in 5′-3′ direction. A Dral fragment (nt 28329–30681), in gelo treated with SpeI (nt 27816) and probed for nad5 (Figure 5C) revealed similar Y arc patterns, thus indicating replication forks moving in both directions on this fragment as well. Analyses of a Dral (nt 38293–2391, in gelo Ncol, nt 929) fragment in SCR and a Bgill (nt 14400–18855, in gelo BshNI, nt 13647) fragment in LCR showed the same result suggesting replication forks of both polarities pass the entire C. albicans mtDNA. These findings lead to two possible interpretations. First, forks passing the fragments may originate from one or several bidirectional initiations. Second, independent initiations could produce unidirectional forks of different polarities either on the same DNA molecule or on separate sister molecules.

**Relative DNA Copy Numbers in a C. albicans Mutant Suggest Elevated Levels of Replication Initiation from IRA/IRb and in SCR**

We previously characterized a C. albicans mutant strain (PJ387) lacking the mt helicase CaHmi1p, which supports WT mtDNA maintenance. Null mutants show severe mtDNA fragmentation (Joers et al., 2007). Relative DNA copy numbers of 20 loci on the mtDNA in PJ387 were analyzed in comparison to the WT. This revealed significant elevation of mutant strain DNA levels in SCR with a significant peak at nad1 (Figure 6A). Levels higher than WT were also observed in a defined region of IRA/IRb from positions nt 5540–6776 and 39210–40420. IRA/IRb showed a drop in DNA copy numbers to WT level between nt positions 6776–7565 and 38395–39210 (Figure 6B). Further analysis of IRA/IRb and LCR revealed progressively decreasing copy numbers significantly below WT level.

The differences in DNA accumulation in PJ387 that suffers mtDNA fragmentation suggest a special role for SCR in replication initiation. In addition, elevated DNA levels in defined regions of IRA and IRb suggest that these sequences serve as zones of elevated replication initiation. The specific YES and YEL arc patterns covering these sequence stretches (Figure 3) support this assumption. Strand invasions providing primers for DNA synthesis would lead to elevated DNA accumulation at or close to initiation sites. The significant DNA copy number elevation at nad1 would accordingly suggest especially frequent initiation within or nearby the fragment.

**DISCUSSION**

Our analysis demonstrates that recombination plays a key role in mtDNA maintenance in C. albicans. We propose that mtDNA replication initiation utilizes recombinative end invasions of homologous DNA fragments and show that most of C. albicans mtDNA forms a complex branched network containing a significant fraction of ssDNA regions. We suggest that the topology is directly linked to RDR mechanisms.

Transcription and recombination have been thought to initiate mtDNA synthesis in S. cerevisiae. According to the transcription based model, synthesis initiates from specific ori/rep elements. This model is supported mostly by experiments conducted in rho− strains (Baldacci et al., 1984; Xu and Clayton, 1995; Graves et al., 1998). Several observations question the role of transcription in yeast mtDNA maintenance. rho− genomes are replicated in strains lacking the mtRNA polymerase Rpo41p (Fangman et al., 1990) and clear topological evidence showing involvement of S. cerevisiae ori/rep sequences in initiation of replication is lacking.

In C. albicans, potentially hairpin-forming sequences, GC clusters and nonanucleotide promoter-like sequences that might serve as sites for RNA priming can be found in silico. These separate elements are scattered across the mtDNA sequence and do not cluster into S. cerevisiae-like ori/rep structures. Bubble arcs that would be diagnostic of strand-coupled replication initiation are absent from C. albicans mtDNA (Figures 2 and 3).
As an alternative to transcription, recombination was proposed for initiation of yeast mtDNA replication (Ling et al., 2007). Again, however, most underlying studies were done in rho− strains of S. cerevisiae. Interpretation of data obtained in these strains suffers from the fact that several factors required for WT mtDNA maintenance do not influence the stability of rho− genomes (e.g., Chen and Butow, 2005). It is thus unclear whether generally valid models can be inferred from these analyses.

Our data demonstrate that specific Y-shaped mtDNA intermediates in C. albicans are generated via a process that involves recombination (Figure 3). Since Y-shaped molecules are most likely replication forks, we provide a direct link between recombination and replication in yeast mtDNA maintenance. Recent findings in mitochondria of plants (Oldenburg and Bendich, 2001; Manchekar et al., 2006) and human heart (Pohjoismaa et al., 2009) suggest that HR is a universal mechanism in mtDNA maintenance.

Similarly to vertebrates, yeast mtDNA was assumed to form circular molecules (Williamson, 2002), and the RC model has been proposed as replication mechanism (Maleszka et al., 1991). Topological studies of mtDNA from different yeasts showed that circular molecules form a minor fraction in some species and are absent in others (Maleszka, 1993; Jacobs et al., 1996; Oldenburg and Bendich, 1996; Oldenburg and Bendich, 1998; Oldenburg and Bendich, 2001; Oldenburg and Bendich, 2004a; Oldenburg and Bendich, 2004b; Manchekar et al., 2006). The complex branched structure of maize chloroplast DNA (cpDNA) is believed to result from RDR leading to head-to-tail concatenemerization and branching. Similarly, the topology of the mtDNA in C. albicans implicates RDR as the major replication mechanism in yeast mitochondria.

Absence of bubble or double Y arcs but detection of simple Y arc patterns (Figures 2 and 3) was interpreted as supporting evidence for RC in mtDNA of the yeast S. pombe and also for mt plasmids in plant (Han and Stachow, 1994; Backert et al., 1997; Backert et al., 1998). RC replication is a form of SD synthesis on a circular template DNA. Our analysis did not reveal detectable amounts of circular mtDNA molecules in C. albicans (Joers et al., 2007 and Figure 1C). We did, however, observe Y arcs and complex branched molecules that are rich in ssDNA stretches (C arcs). These structures suggest parallel occurrence of uncoupled and coupled DNA synthesis on noncircular templates. Uncoupled leading-strand replication initiating from strand invasions could lead to SD, thus resulting in ssDNA-containing branched structures (Figures 2, 3, and 4). A nick encountered during SD replication could release ssDNA fragments to account for the formation of ssDNA-arcs. Released ssDNA fragments may anneal to homologous single stranded stretches. These could provide new primers initiating DNA synthesis, ssDNA annealing could therefore provide a replication initiation mechanism in addition to strand invasion.

Upon initiation of second-strand synthesis, coupled leading- and lagging-strand synthesis would generate Y arcs of replication forks (Figures 2, 3, and 4). On the basis of data for human mtDNA (Fusté et al., 2010), we speculate that priming of lagging strand synthesis involves Rpo41p, the only known mtRNA polymerase in yeast.
The accumulation of mtDNA at and near IRa/IRb in the mutant several specific sites spread throughout could also be derived from nearby strand invasion. This suggests Y-shaped molecules spanning from 1N to 2N in any fragment invasion leads to formation of the specific Y structures, classical that contain the GC skew minima. Since the assumed strand minima (Figure S1B). Formation of extra Y arcs at IRa/IRb initi- maps to putative replication origins as indicated by GC skew larly detect ends in IRa/IRb of proposed origins near the ends of the molecules (Oldenburg leads to RDR in a transcription-independent manner using the sequences that are separated by unique small and large regions. and branched molecules. Both DNA carry inverted repeat DNA Modifications are coding regions, and in an earlier study replication origins were mapped to their ends (Kunnimalaiyaan et al., 1997). It was later proposed that the homology of the cpDNA repeats leads to RDR in a transcription-independent manner using the proposed origins near the ends of the molecules (Oldenburg and Bendich, 2004a; Oldenburg and Bendich, 2004b). We similarly detect ends in IRa/IRb of C. albicans mtDNA (Figure 1) that map to putative replication origins as indicated by GC skew minima (Figure S1B). Formation of extra Y arcs at IRa/IRb initi- ated by RDR (Figure 3) is observed on restriction fragments that contain the GC skew minima. Since the assumed strand invasion leads to formation of the specific Y structures, classical Y-shaped molecules spanning from 1N to 2N in any fragment could also be derived from nearby strand invasion. This suggests that RDR could either initiate randomly or initiation occurs from several specific sites spread throughout C. albicans mtDNA. The accumulation of mtDNA at and near IRa/IRb in the mutant strain (Figure 6) would suggest that RDR initiates more frequently in SCR and close to the end of IR, and less frequently elsewhere. Our data emphasize HR as a key mechanism in C. albicans mtDNA replication. IRa/IRb could represent a recombination hotspot with a major function in RDR initiation, but initiation in other parts of the mtDNA cannot be ruled out. Although mechanistic details may differ, the common features of DNA replication in mitochondria and plastids of distantly related eukaryotes suggest that RDR is used to maintain organ-ellar chromosomes among evolutionarily diverse organisms. A comprehensive study of wild-type mtDNA from S. cerevisiae should now show whether RDR is the major replication mecha- nism in yeast.

EXPERIMENTAL PROCEDURES

Strains, Growth Conditions, and mtDNA Purification

C. albicans strains CAI4 (Δura3::im3434/Δura3::im3434) (Fonzi and Irwin, 1993) and PJ387 (CAI4-Δaehm11/Δaehm11) (Jörs et al., 2007) were grown in 1% yeast extract, 2% peptone, and 2% glucose at 30°C. mtDNA was purified from isolated mitochondria as previously described (Visaca et al., 2009).

Purification of Total Cellular DNA

Total DNA was isolated as described earlier (Sedman et al., 2005). DNA was denatured in 0.3 M NaOH for 15 min at 37°C and dot blotted to nylon membranes (Appligene). Each DNA sample was analyzed in triplicate and at three different concentrations (5, 10, and 15 ng/µL). Radioactive nuclear and mitochondrial probes were PCR generated with the primers listed in Table S1.

DNA Modifications

Ten micrograms of mtDNA (3 µg for PFGE) were digested with 3 units/µg of restriction enzymes (Fermentas) for 3 hr at 37°C. RNaseH (Fermentas) treat- ment was 2 units for 30 min at 37°C. S1 nuclease (Fermentas) was used at 1 unit for 1 min at 37°C. Phage T7 endonuclease I (NEB) was used at 1.5 units per 3 µg of mtDNA for 45 min at 37°C. Topoisomerase I (NEB) was used at 6 units per 3 µg of mtDNA for 15 min at 37°C.

Electrophoresis, Hybridizations, and Quantifications

Neutral 2D-AGE was carried out as previously described (Bell and Byers, 1983; Brewer and Fangman, 1991). Restriction fragments of mtDNA were separated on first-dimension 0.5% agarose gels at 0.9 V/cm and 21°C for 24 hr, and second-dimension gels were 1% agarose run at 3 V/cm and 4°C for 15 hr. Fork direction gels were performed as described in Reyes et al. (2005). In gelo digests were 3 x 2 hr at 37°C and 150 U of enzyme applied each time. First-dimension fork direction gels were 0.4% agarose run at 0.9 V/cm and 21°C for 22 hr, and second-dimension gels were 1.5% agarose run at 3 V/cm and 4°C for 16 hr. PFGE analyses were essentially carried out as described (Idanato and Gnikr, 1996) considering Levene and Zimm (1987) for separation settings. Agarose embedded whole cells or mitochondria were incubated for 12 hr at 37°C with 0.1 mg/ml proteinase K and 1% SDS and washed with 50 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.2 mM PMSF. Embedded and extracted mtDNA samples were separated on 0.5% agarose gels with a Bio-Rad CHEF II apparatus set to 6 V/cm, pulse 1–10 s, for 15 hr at 14°C.

Gels were blotted to nylon membranes by alkaline transfer. Southern blots of 1D, 2D, PFGE, and dot blots were hybridized to specific PCR probes as indicated (Table S1) by incubation for 3 hr at 65°C in 7% SDS, 250 mM NaHPO4 (pH 7.2), 1 mM EDTA, and 0.1% BSA (Church and Gilbert, 1984) and washed 2 x 15 min at 65°C with 5% SDS, 40 mM NaHPO4 (pH 7.2), 1 mM EDTA. Blots were exposed to X-ray films for 1–14 days and to Storage Phosphor Screens for 12–72 hr. Signals were detected with a Typhoon Trio Phosphor Imager (GE Healthcare) and quantified with ImageQuant TL (GE Healthcare).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one table and can be found with this article online at doi:10.1016/j.molcel.2010.09.002.

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