Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus

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How eukaryotes specify their replication origins is an important unanswered question. Here, we analyze the replicative organization of yeast rDNA, which consists of ∼100–200 identical copies of a 9.1-kb unit, and present the first quantitative analysis of the distribution of replication origins in the repeated rDNA. We use novel technologies based on BrdU incorporation in yeast and DNA combing to visualize replication origins on single rDNA molecules. Their distribution is nonrandom and dependent on the conserved histone deacetylase Sir2p, which is known to silence transcription and extend life span [Defossez et al. 2001; Gasser and Cockell 2001]. Because yeast aging is linked to rDNA replication via the formation of extrachromosomal rDNA circles [Guarente 2000], our results suggest that Sir2p may promote genome stability and yeast longevity by modulating the number and distribution of rDNA replication origins.

Results and Discussion

We present here the first quantitative analysis of the distribution of replication origins in the repeated rDNA array of S. cerevisiae. A combination of bromodeoxyuridine (BrdU) incorporation [Lengronne et al. 2001] and the recently developed technique of dynamic molecular combing [DMC; Michalet et al. 1997; Herrick et al. 2000] was used to visualize replication origins on individual rDNA fibers. To this end, thymidine-kinase-expressing (TK+) yeast cells were arrested in G1 with 2-fluoro-deoxyuridine (2-FdU) and DNA synthesis (BrdU) was then arrested with deoxythymidine to label newly replicated DNA. Using DNA combing and single-molecule imaging, we show that functional rDNA origins are clustered and interspersed with large domains where initiation is silenced. This repression is largely mediated by the Sir2p histone deacetylase. Increased origin firing in sir2A mutants leads to the accumulation of circular rDNA species, a major determinant of yeast aging. We conclude that rDNA replication is regulated epigenetically and that Sir2p may promote genome stability and longevity by suppressing replication-dependent rDNA recombination.

Eukaryotic chromosomes are replicated from multiple origins, which are activated sequentially throughout the S phase of the cell cycle. In the yeast Saccharomyces cerevisiae, as probably in other eukaryotes, most origins are not used by every cell cycle, some of them being totally silent in normal growth conditions [Fangman and Brewer 1991; Pasero and Schwob 2000]. A growing body of evidence indicates that chromosomal context is a major determinant of origin activity [Cimbora and Groudine 2001; Heun et al. 2001; Méchali 2001; Gerbi and Bielinsky 2002]. However, how chromatin structure modulates origin activity is still poorly understood, and the biological significance of this regulation remains to be established.

The ribosomal DNA cluster of S. cerevisiae, which consists of 100–200 identical copies of a 9.1-kb unit, is an interesting model system to address these questions. Indeed, every unit contains a potential origin of DNA replication [Fig. 1A,B], but only ∼20% of these elements were shown to be active in a single cell cycle [Linskens and Huberman 1988; Fangman and Brewer 1991]. Because sequences are identical, epigenetic mechanisms likely control origin activity in the rDNA. However, owing to its repeated nature, the organization of replicons in this locus has been difficult to address using conventional approaches and leads to divergent interpretations [Walmsley et al. 1984; Saffer and Miller 1986].

Here we use novel technologies based on BrdU incorporation in yeast and DNA combing to visualize replication origins on single rDNA molecules. Their distribution is nonrandom and dependent on the conserved histone deacetylase Sir2p, which is known to silence transcription and extend life span [Defossez et al. 2001; Gasser and Cockell 2001]. Because yeast aging is linked to rDNA replication via the formation of extrachromosomal rDNA circles [Guarente 2000], our results suggest that Sir2p may promote genome stability and yeast longevity by modulating the number and distribution of rDNA replication origins.
these conditions, forks stall ~9 kb away from origins in genomic DNA [Fig. 2A]. Because replication is monodirectional in the rDNA [Fig. 1B], we anticipated that a single fork would not replicate more than one repeat (9.1 kb) before stalling. However, significantly longer signals [18 ± 14 kb; Fig. 2B] were detected, suggesting that active origins are clustered in the rDNA. Statistical analysis of the size distribution of BrdU tracks and gaps is shown in Supplementary Figure 1 (see Supplementary Material at http://www.genesdev.org).

In HU-arrested cells, we observed that 28% of these gaps are larger than 60 kb and represent more than 70% (in size) of unreplicated rDNA [Fig. 2B]. This pattern differs significantly from that expected if origins were randomly distributed (P < 0.05). It also reflects a unique property of rDNA, as under the same experimental conditions, large gaps represent only 3% of unreplicated DNA in other regions of the genome [Fig. 2A]. To check whether large gaps might contain late-firing origins, we recorded how the rDNA replication pattern evolves during a synchronous S phase. To this aim, we measured the lengths of BrdU tracks and gaps and plotted them against the extent of rDNA fiber replication. We reasoned that if rDNA is only replicated from early origins, the mean length of BrdU tracks should increase regularly as S phase proceeds, whereas gap size should decrease proportionally. Indeed, this showed a linear relationship between the extent of replication of rDNA fibers and the length of tracks and gaps [Fig. 3B], indicating that most rDNA origins fire in early S phase. However, we also noticed that the mean gap size shrank slightly faster than the growth of BrdU tracks [slopes: -0.9 and 0.8, respectively], suggesting that a few late origins might get activated from within the large gaps. The fraction of rDNA origins (~25%) that we found activated in HU-arrested cells also corresponds to the total number of rDNA origins estimated by other methods [Linskens and Huberman 1988; Fangman and Brewer 1991]. We can therefore conclude that S. cerevisiae rDNA is mostly replicated from early origins, as recently shown in Schizosaccharomyces pombe (Kim and Huberman 2001).

Because initiation at most rDNA ARS elements appeared to be down-regulated, we next addressed the nature of the mechanism involved in this repression. The silencing protein complex Sir2/3/4p together with Rap1p represses both the transcription of adjacent genes and the initiation of adjacent origins at telomeres (Stevenson and Gottschling 1999; Gasser and Cockell 2001). One of these factors, the histone deacetylase Sir2p, is also found in the nucleolus, where it modulates chromatin structure and represses reporter gene expression [Fritze et al.
Because Sir2p is physically bound to the region containing the rDNA ARS (Gotta et al. 1997; Hoppe et al. 2002), we have tested whether it could affect initiation of DNA replication at this locus (Fig. 4A). To this aim, the distribution of active rDNA origins was analyzed in wild-type and \textit{sir2}/H9004 cells. This analysis revealed that almost twice as many rDNA origins fired in HU-arrested \textit{sir2}/H9004 cells relative to wild-type cells (+80%; Fig. 4A,B). Strikingly, BrdU tracks were not significantly longer in \textit{sir2}/H9004 cells, but the proportion of large (>60 kb) unreplicated gaps dropped from 28% in wild-type cells (Fig. 2B) to only 4% in the mutant (Fig. 4B; Supplementary Table 1, see Supplementary Material at http://www.genesdev.org). We can therefore conclude that, in \textit{sir2}/H9004 cells, additional origins are activated from within the large gaps normally repressed by Sir2p (Fig. 4C). To confirm the effect of Sir2p on rDNA replication by an independent method, we monitored initiation at rDNA origins in wild-type and \textit{sir2}Δ cells by 2D gel electrophoresis (Brewer and Fangman 1987). The quantitation of the bubble to Y-arc ratio in seven independent experiments revealed a 62% (+21%) increase of origin activity in the \textit{sir2}Δ mutant relative to wild-type cells (Fig. 5A,B,E). Moreover, the ectopic expression of \textit{SIR2} in \textit{sir2}Δ cells restored the repression at rDNA origins (Fig. 5B). Taken together, these data indicate that Sir2p affects both the number and the distribution of active origins in the rDNA by maintaining large domains of rDNA in a state refractory for the initiation of DNA replication.

Sir2p is also known as a key regulator of life span in different species, but it is not clear exactly how Sir2p promotes longevity at the molecular level (Defossez et al. 2001). In yeast, the short life span of \textit{sir2}/H9004 mutants is attributable mainly to the accumulation of extrachromosomal rDNA circles (ERCs; Kaeberlein et al. 1999). Because deletion of the \textit{FOB1} gene, which is required for fork arrest at RFBs (Kobayashi and Horiuchi 1996; Defossez et al. 1999; Johzuka and Horiuchi 2002), suppresses both the accumulation of ERCs and the premature aging of \textit{sir2} mutants, it has been proposed that Sir2p somehow stabilizes stalled forks to prevent DNA breaks and recombination (Kaeberlein et al. 1999). Our finding that Sir2p controls the frequency of origin firing within the rDNA sheds new light on this process. Indeed, given that RFBs are located immediately leftward of every active origin (Fig. 1A), more initiation will inevitably translate into more arrested forks, which are highly prone to recombination (Defossez et al. 1999; Ivessa et al. 2000; Rothstein et al. 2000). Although the causal link between RFB activity and ERCs formation remains to be established (Ward et al. 2000; Johzuka and Horiuchi 2002), it is therefore tempting to speculate that...
Sir2p acts indirectly to limit the formation of ERCs by reducing the number of active origins, and consequently of stalled forks. To test this model, we monitored the amount of stalled forks and rDNA circles in wild-type cells. To test this model, we monitored the reduction in the amount of ERCs (Fig. 5F), as shown earlier (Kaeberlein et al. 1999). To substantiate an eventual causal link between increased origin firing and accumulation of rDNA circles, we then tested whether an unrelated mutation that lowers replication initiation frequency (such as in the origin recognition complex, ORC) might suppress the accumulation of ERCs in sir2Δ cells. Hence, sir2Δ orc2-1 cells were grown at permissive temperature, conditions that reduce origin activity by ~50% (Liang et al. 1995). Interestingly, this led to a threefold reduction in the amount of ERCs [Fig. 5G], suggesting that the formation of circular rDNA species is coupled to the frequency of origin firing. Therefore, we propose that Sir2p suppresses rDNA recombination and extends yeast life span at least partly by lowering the number of active origins within the rDNA. Furthermore, because its deacetylase activity is regulated by NAD+ levels (Imai et al. 2000; Defossez et al. 2001), an attractive possibility could be that Sir2p modulates rDNA replication and recombination (generating ERCs as a by-product) to expand/contract the size of the rDNA array according to the metabolic activity of cells (Kobayashi et al. 1998).

In most eukaryotes, the rDNA is composed of long tandem repeats that are replicated pseudo-monodirectionally because of the presence of polar RFBs next to the 35S transcription termination site. It is generally believed that RFBs evolved to avoid or limit collision between RNA and DNA polymerases, however at the cost of increased recombination at arrested forks (Hyrien 2000). One way to dampen the latter would be to organize the replication of this locus so as to limit in space or time the number of arrested forks. We report here that replication origins are not distributed evenly within the rDNA, but form clusters of 2–3 consecutive units that are separated by large regions where origins are silenced. This organization differs from the rest of the yeast genome and depends on the histone deacetylase Sir2p. The increased number of active origins and shorter distance between arrested forks in sir2Δ mutants may largely account for some of their phenotypes. Indeed, the link between rDNA replication, recombination, and genomic instability is corroborated by other mutations, such as in the Rm3p DNA helicase, which also induce replication pausing and rDNA recombination (Ivesa et al. 2000). Furthermore, our results indicate that even in yeast, the choice of replication origins can be dictated by epigenetic mechanisms, which are susceptible to rapid and metastable variations. In this context, it is interesting to note that phenotypic switching in Candida albicans is controlled by an Sir2p ortholog and via events that include rDNA recombination and chromosome rearrangements (Perez-Martin et al. 1999). More practically, we believe that the single-molecule approach described here will be especially valuable to map replication origins in higher eukaryotes, where cell-to-cell variations of origin usage might be common and epigenetic selection the rule rather than the exception.

Materials and methods

**Strains and synchronization procedures**

Congenic E1000 (MATa, ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 GAL, ura3::URA3/GPD-TK+), E1244 (MATa, sir2::HIS3, TK+), E1385 (MATa, sir2::HIS3, hml::LEU2, TK+), E1410 (MATa, fob1::URA3, TK+), E1314 (MATa, orc2-1, TK+) and E1612 (MATa, sir2::HIS3, hml:: LEU2, orc2-1, TK+) cells were grown in complete synthetic medium at 25°C (Rose et al. 1990). Cells were synchronized in G1 with 2 µg/mL 5-FOA (Calbiochem) and released into S phase in the presence of 0.4 mg/mL BrdU by the addition of 50 µg/mL pronase (Calbiochem). Incubation in 0.2 M hydroxyurea for 90 min after release from the G1 block was used to block elongation (Lengronne et al. 2001). To induce sir2Δ in sir2Δ cells, the E1244 strain was transformed with a plasmid bearing SIR2 under the control of a galactose-inducible promoter [gift of M. Cockell | Institut Suisse de Re-
Sir2p silences replication origins in yeast rDNA

Figure 4. Sir2p affects both the firing efficiency and distribution of replication origins in the rDNA. Wild-type and sir2Δ hmlΔ cells [E138S] were synchronized in G1 with α-factor and released into S phase in the presence of HU. Ribosomal DNA fibers were analyzed by DMC as described previously. (A) Representative rDNA fibers. (Red) FISH; (green) BrdU. Bar, 50 kb. (B) Size distribution of BrdU tracks [b] and gaps [(a) in sir2Δ rDNA fibers. (C) Model of the distribution of active origins in the rDNA of wild-type and sir2Δ cells [see text].

Figure 5. 2D gel analysis of rDNA ARS activity in wild-type and sir2Δ cells. Genomic DNA was prepared from congenic wild-type [E1000] and sir2Δ [E1244] cells, and replication intermediates were examined by 2D gel electrophoresis. (A) Schematic presentation of replication intermediates expected for a Sir2-Xbal fragment (3.5 kb) containing the ARS element. (Bubble arc) active origins; (Y-arc) passive replication. [B] The proportion of active origins (arrowhead) increases by 60% in sir2Δ cells, but wild-type levels are restored when SIR2 is ectopically expressed in sir2Δ cells from a galactose-inducible promoter. Recombination intermediates are 20 times less abundant than replication bubbles in wild-type cells [Zou and Rothstein 1997; Ivessa et al. 2000] and are not detectable here. (C) Quantiﬁcation of autoradiograms was performed by storage phosphor imaging [Amersham].

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