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# Difference in Strength of Autonomously Replicating Sequences among Repeats in the rDNA Region of *Saccharomyces cerevisiae*

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**The rDNA region of *Saccharomyces cerevisiae* contains 100–200 tandemly repeated copies of a 9 kb unit, each with a potential replication origin. In the present studies of cloned fragments from the region involved in the regulation of replication of rDNA, we detected differences in autonomously replicating sequence (ARS) activity for clones from the same yeast strain. One clone, which showed very low ARS activity, carried a point mutation, a C instead of T, in position 9 of the essential 11 bp consensus ARS as compared to clones carrying the normal 10-of-11-bp match to the consensus. The mutation could be traced back to genomic rDNA where it represents about one-third of the rDNA units in that strain. Differences in ARS activity have implications for understanding the regulation of replication of rDNA, and the ratio of active to inactive ARS in the rDNA region may be important for potential generation of extrachromosomal copies.**


Autonomously replicating sequences (ARS) are fragments of genomic yeast DNA which allow plasmids to replicate as independent minichromosomes (1). Originally, these sequence elements were considered to consist of a 11 bp ARS consensus sequence (ACS) and flanking sequences of various length up to 200 bp (2–7). The ACS: WTTTAYRTTTW (where W is A or T, Y is T or C, and R is A or G) is necessary but not sufficient for ARS activity (3, 4). More recently it has been suggested that the ACS should be expanded to 17 bp: WWWWTTTAYRTTTWGTT (8). This expanded ACS (EACS) is more efficient in identifying essential sequences for ARS function than the ACS (8).

The rDNA region of *Saccharomyces cerevisiae* is located on chromosome XII (9) and is composed of 100–200 tandemly repeated copies of a 9 kb unit (Fig. 1) that alone spans the size of an average chromosome (10). In the published yeast genome sequence this region is represented by only two copies, the centromere-proximal and centromere-distal unit (11). Generally ARS occur about once for every 30 to 40 kbp (5, 7, 12), and efficient replication of the rDNA region would require the function of more than 30 replication origins. According to the sequence data (11, 13), each repeating unit carries a potential origin with four 10-of-11 matches to the original ACS, or 13-15 matches to the 17 bp EACS. Cloned rDNA sequences confer autonomous replication of plasmids (14–17). Yet, the rDNA ARS is relatively weak (15), and genomic rDNA replicons are opened in clusters of about 5 units in length separated by one to three units (18, 19), indicating that not all origins are used. Also, the replication occurs mainly unidirectionally due to a block at the end of the 35S transcription unit for the replication fork moving opposite to transcription (20–22).

Extrachromosomal rDNA circles excise from the rDNA locus and replicate via the ARS (16, 23, 24). These 3 $\mu$m circles are particularly frequent in stationary phase cells (25). Recently, it was shown that asymmetric segregation and thereby accumulation of rDNA circles in ageing mother cells leads to expansion and fragmentation of nucleoli (26). Accumulation of rDNA circles is then proposed to be a general cause of ageing in yeast and possibly also in higher eukaryotes, including man (26–29). Due to this there has been a renewed interest in the regulation of replication of rDNA in yeast and mammalian cells.

In earlier studies, we detected length heterogeneities in the NTS1 (Fig. 1) in clones of the *Eco*RI fragment B (Fig. 1), as well as in genomic rDNA, from different strains and from the same strain (30). In the present

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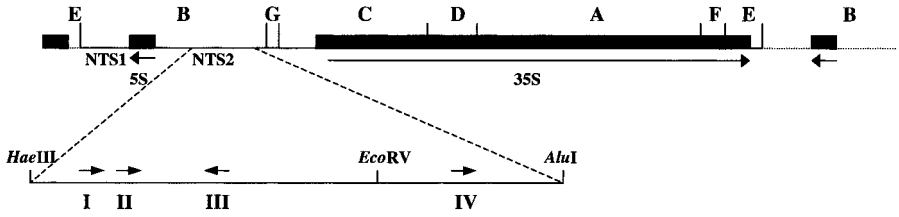


**FIG. 1.** The repeating rDNA unit of *Saccharomyces cerevisiae*. The upper part shows the *Eco*RI restriction map and coding functions. The lower part shows an enlarged map of the *Hae*III–*Alu*I subfragment spanning the potential ACS elements I, II, III and IV.

work we have analysed these clones further and studied the ARS activity in a 570 bp fragment from the non-transcribed region (NTS2) located upstream of the divergently transcribed 35S rRNA- and 5S rRNA-gene (Fig. 1). The ARS function has been mapped to a 139 bp fragment within this region (16). Contrary to others (16), we find marked differences in ARS activity for clones from the same strain. Sequence analysis on genomic rDNA and of individual clones of the actual rDNA region from this strain showed that a significant fraction of rDNA repeats had an ARS element leading to strongly reduced ARS activity.

## MATERIALS AND METHODS

*Materials.* [$\alpha$-$^{32}$P]dATP and [$\gamma$-$^{32}$P]ATP were from Amersham, reagents used for molecular biology from New England Biolabs or GibcoBRL, and culture media components from Difco Laboratories.

*Strains, transformation and culture conditions.* Plasmids were propagated in *E. coli* strains JA221 or JM103, using standard methods (31) and cultured in Luria Broth supplemented with ampicillin (100 $\mu$g/ml). The haploid strains 12B and 309, of *Saccharomyces cerevisiae* were as described (32, 33). The strain FL200 ($\alpha$, *ura*3, *leu*2) was transformed (34) with appropriate plasmids and used for measuring ARS activity. Yeast was grown in non-selective medium, YPD (2% glucose, 2% Bacto peptone, 1% Bacto yeast extract) or selective medium (6,7% yeast nitrogen base without amino acids, 2% glucose, supplemented with amino acids).

*Isolation of genomic rDNA.* The genomic $\gamma$-DNA satellite containing the rDNA was isolated by fractionation of total DNA from the strains 12B and 309 in $Ag^{+}$-$Cs_2SO_4$ density gradients as described (33).

*Plasmid constructs.* All plasmids used for measuring ARS activity were based on the pBR322-derivative pFL1 (35) which contains the *URA* 3 as a marker in the *Hind*III site and the endogenous 2-micron plasmid *Eco*RI fragment D for autonomously replication in the *Eco*RI site. To get pYB, the *Eco*RI fragment was replaced with cloned *Eco*RI fragment B of rDNA from the two yeast strains 12B and 309 (clone 12B-2 and -9 and 309-10 and -12 as in (30)). A fragment containing *CEN*4 was excised from YCp50 with *Tth*111I and *Xho*I, and subcloned into a filled in *Bam*HI site in pUC8 and re-excised with *Sal*I and *Xho*I prior to ligation into the *Sal*I site of pFL1 and pYB giving rise to the pCFL1 and pYCB respectively. To get pYCsubB, the *Eco*RI fragment of pYCB was replaced with a *Hae*III-*Alu*I sub-fragment containing the ARS near-consensus-matches (Fig. 1 and Fig. 3).

*Sequencing.* For determination of polymorphic sites in genomic rDNA, the large *Eco*RI-*Eco*RV fragment of *Eco*RI-fragment B was isolated after digestion of the $\gamma$-DNA fraction (Fig. 3, *Eco*RV cleaves 268 bp from the 3′ end of the 11 bp ACS element I, or 347 bp after the *Hae*III site), labelled with [$\gamma^{32}$P]ATP using T4 DNA polynucleotide kinase and then cut with HaeIII. The ARS-containing *Hae*III-*Eco*RV fragment was purified on agarose gel prior to sequencing reactions using the method of Maxam and Gilbert (31, 36, 37). Individual clones of the genomic *Eco*RI fragment B in pBR322 were subjected to sequencing between the *Hae*III and *Alu*I sites (Fig. 1) using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and primers: 5′-(Cy5)-GTAGCAAACGTAAGTCAAAG-3′ and 5′-(Cy5)-CTCCCA-TTACAAACTAAAATC-3′ (Eurogentec, Bel S.A.) recognising sequences near the outside of the *Hae* III and *Alu*I sites respectively. Automatic sequencing was performed on an ALFexpress DNA Sequencer (Pharmacia Biotech). Access to the *Saccharomyces* Genome Database was made through the Stanford Genomic Resources (http://genome-www.stanford.edu/Saccharomyces/).

*Assay for ARS activity.* To measure plasmid loss and thereby ARS activity, transformants were grown for 4–8 generations on non-selective medium prior to parallel plating on selective and non-selective plates. Plasmid loss was calculated using the formula $K(1 - X/100)^n = S$, where X is the percentage of plasmid loss per generation, n is the number of generations in non-selective growth medium, S is the number of colonies appearing on selective growth medium, and K is the number of colonies appearing on non-selective growth medium.

In one of the experiments, DNA was extracted from several yeast clones and used for transformation of *E. coli.* The plasmid was then isolated from *E. coli* and subjected to restriction analysis. The ARS-containing plasmid was present in all clones that appeared on plates within 3 days but not in clones appearing after 5–6 days. Hence, only clones appearing within 3 days were used for calculations of ARS activity.

As an alternative assay for ARS activity, the growth rate in selective growth medium was analysed by counting cells each hour during the exponential phase of growth. The data were fitted to a straight line using the method of least squares.

## RESULTS

### *Different ARS Activity in Clones of rDNA Sequences from the Same Strain*

To analyse the ARS activity, the plasmids pYCB and pYCsubB containing the rDNA *Eco*RI fragment B or subfragment thereof, respectively, were used to transform a *ura3* haploid yeast strain. Transformants were analysed for presence of the plasmid after growth for 4–8 generations on non-selective medium. Of four clones containing the *Eco*RI B-fragment, two from strain 12B and two from strain 309, all gave transformants and hence apparently conferred ARS activity. As expected, the stability of all four plasmids was less

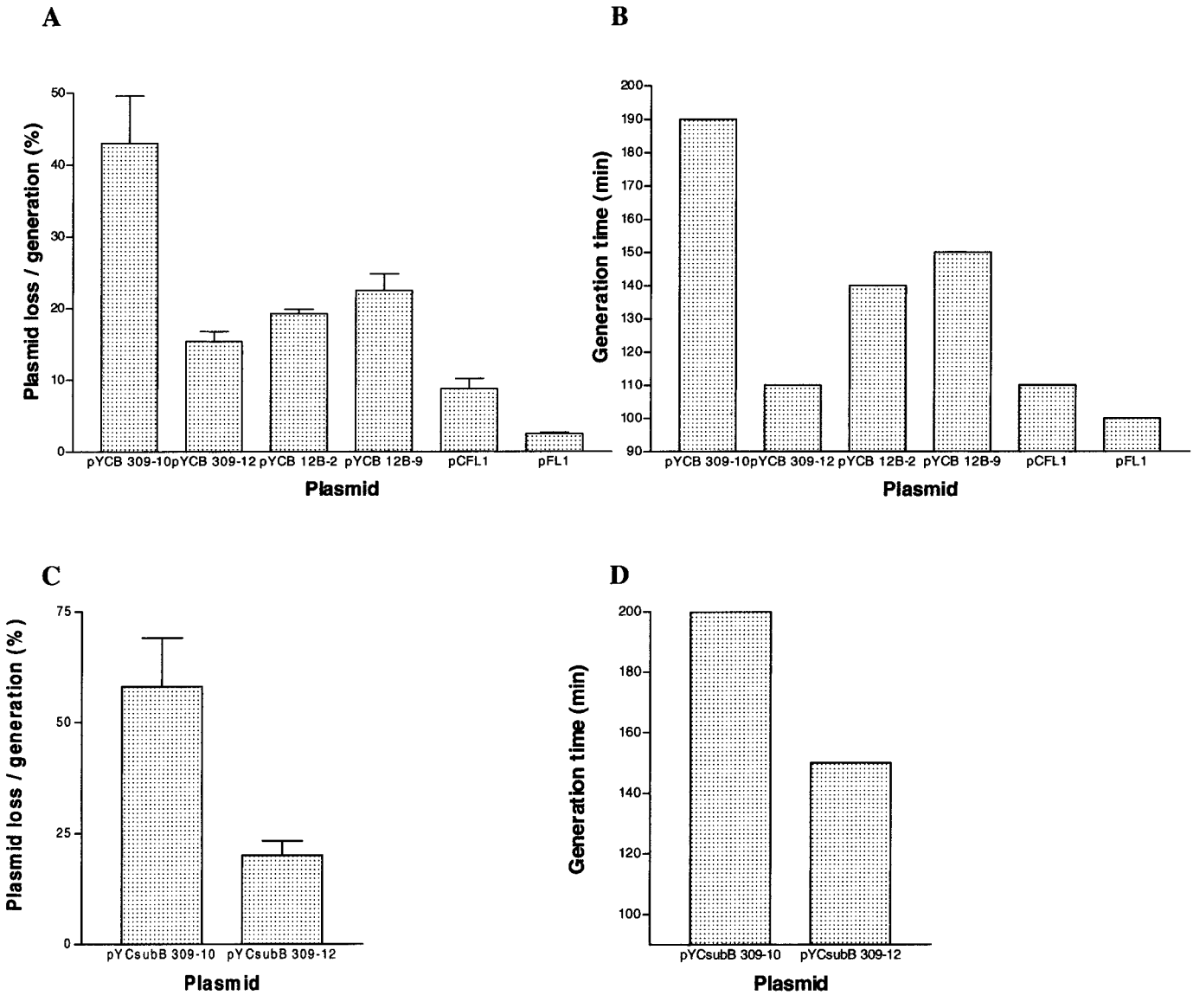


**FIG. 2.** Comparison of ARS activity. (A and B) Comparison of ARS activity measured as plasmid loss per generation (A) or as generation time (B) between plasmids containing *CEN*4 and the *Eco*RI fragment B from various clones of rDNA (pYCB 12B-9, pYCB 12B-2, pYCB 309-10 and pYCB 309-12) and in the similar plasmid with the *Eco*RI fragment D from the 2-micron plasmid as replication origin with and without *CEN*4 (pCFL1and pFL1 respectively). Error bars denote standard deviation from 5–10 tests on different transformants. (C and D) Comparison of ARS activity measured as plasmid loss per generation (C) or as generation time (D) between the 570 bp *Hae*III–*Alu*I subfragments. Error bars denote standard deviation from 10 tests on different transformants.

than that of pCFL1 containing the 2-micron plasmid replication origin (Fig. 2A and B). However, the results in Fig. 2A and B clearly demonstrate that the stability of the rDNA clones, and hence the ARS activity, differed significantly and that the largest difference was seen between the two clones from strain 309. Clone 309-10 gave a very high frequency of plasmid loss, more than 40% per generation compared to about 15% for clone 309-12, indicating insufficient replication of the plasmid during cell growth. From Fig. 2B and C it is seen that the difference in frequency of plasmid loss remained in the pYCsubB constructs. Hence, the difference in ARS activity can be ascribed to differences in the *Hae*III-*Alu*I fragment (Fig. 1).

### *Molecular Basis for the Difference in ARS Activity*

To find the molecular basis for the difference in ARS activity, the *Hae*III-*Alu*I subfragment of the two 309 clones, that should contain the four potential ACS or EACS ((13, 16) Fig. 3, element I–IV), was studied in detail and sequenced. Firstly, elimination of the element IV by deleting the *Eco*RV-*Sma*I-fragment did not significantly change the stability of the plasmids. By

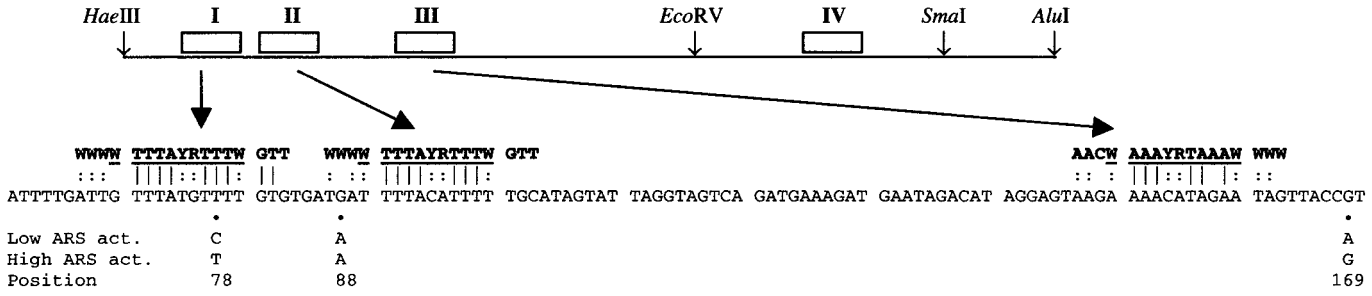


**FIG. 3.** Sequence differences between rDNA clones. Map of the *Hae*III-*Alu*I subfragment with the different ACS *and* EACS elements (I, II, III and IV), displaying sequence identity between the ACS (underlined), EACS (bold) and the sequence from of the complete yeast genome. The displayed genome sequence goes from 61 to 170 bp after the *Hae*III cleavage site. Bases in the 309 clones differing from the yeast genome sequence are shown at the lower part of the figure at indicated positions. Of 11 clones that were sequenced, 3 were of the low ARS activity-type (Low ARS act.) represented by the sequence of clone 309-10, while 8 clones were of the normal ARS activity-type (High ARS act.) represented by clone the 309-12.

sequencing, two single base mismatches were detected between the clones (displayed in Fig. 3), one within number I of the four ACS, the other outside any of the four elements (position 78 and 169, respectively, after the *Hae*III cleavage site). The low activity ARS of clone 309-10 carried a C in position 78 (position 9 of the 11 bp element I) and an A in position 169, while clone 309-12 that gave higher ARS activity had a T and a G in those positions, respectively, as does the yeast genome rDNA sequence (Fig. 3). Hence, element I of clone 309-10 deviates in two positions, position 1 and 9, from the 11 bp ACS and matches 14 of 17 bp of the EACS, while element I of 309-12 was identical to the genome sequence. On the other hand, both of the 309-clones deviated from the yeast genome rDNA sequence with an A instead of G in position 88 after the *Hae*III cleavage site. Away from these three positions (78, 88 and 169 after the *Hae*III site), the *Hae*III-*Alu*I-fragment of both clones was identical to the yeast genome sequence. Compared to the two earlier published sequences (13), there were several other deviations as well, but interestingly, one of the sequences in (13) shows the same three transitions, a T-to-C in position 78, and G-to-A in 169, as found in the low ARS activity clone 309-10, together with the G-to-A in position 88. Taken together, our data point to an essential role of element I in the replication of rDNA, in accordance with the findings of Miller and Kowalski (16). Furthermore, the T-to-C transition in position 9 of the ACS evidently destroys the replication origin of an affected rDNA unit.

### *Presence of Very Low Activity ARS Elements in Genomic rDNA*

Although the yeast genome has been sequenced, only two rDNA units are included in the chromosome XII sequence, both with the 10-of-11 bp match in ACS, and 15 of 17 match to the EACS in element I (11). Miller and Kowalski (16) studied four independent clones of rDNA and found that the DNA sequence spanning the ARS region was identical. They concluded that a low frequency of origin usage was not due to polymorphism within the rDNA ARS. To find whether clone 309-10 then represents a very rare mutation, we performed sequencing reactions directly on isolated genomic rDNA fragments. By labelling the 5′-end in the *Eco*RV-site (Fig. 3), the T- to C-transition in element I should appear as a G upon applying the Maxam-Gilbert sequencing method. Figure 4 shows the result of a G-reaction of the labelled *Eco*RV-fragment from genomic rDNA and from the two clones 309-10 and 309-12. The band corresponding to a G in position 9 of the ACS element I (position 270 from the *Eco*RV site) is clearly visible in the genomic fragment. Scanning of the film from the sequence gel indicated about 35% of the ACS to be of the low activity type.

With such high proportion of low activity ARS, one would also expect a high frequency of the same defect among clones of the rDNA *Eco*RI-fragment B. We therefore sequenced the *Hae*III-*Alu*I-part of nine additional clones. Two of these showed a sequence identical

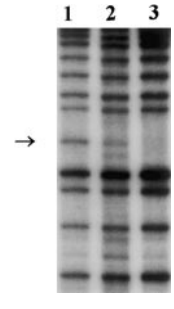


**FIG. 4.** Presence of low activity ARS in genomic rDNA. Band pattern from a G-specific cleavage sequencing reaction on fragments from clone 309-10 (lane 1), 309-12 (lane 3) and from genomic rDNA (lane 2) after labelling at the *Eco*RV site within rDNA. The arrow indicates position 270 from the *Eco*RV site, or position 78 from the *Hae*III site. The band below and above the arrow in lane 1 is at position 253 and 290 from the *Eco*RV site, respectively.

to that of 309-10 with the T-to-C- and the G-to-A-transition in position 78 and 169 respectively, while seven of the clones were found to be identical to 309-12. All the nine clones, together with 309-10 and 309-12, differed from the yeast genome sequence with the G-to-A-transition in position 88, evidently a strain feature that does not influence the ARS activity. No other polymorphic sites were detected. Consequently, of a total of eleven individual clones that were sequenced, three carried the low activity type origin with the T-to-C-transition in position 9 of the ACS element I. These data confirm our results from the G-reaction on the genomic fragment. Hence, about 1/3 of the rDNA repeats in this strain carries an NTS2 with very low ARS activity. This is of the same order as for length heterogeneities in the NTS1 in the same strain (30). The three low ARS-activity clones were identical, with the same transitions at position 78 and 169, suggesting these heterogeneities to be linked. In Fig. 4 another heterogeneity (just below that pointed to by the arrow) can be seen for the genomic fragment in lane 2 as a clear band that is absent for the clones in lane 1 and 3. This variant was not seen in any of the sequenced clones.

The mutation in element I reduces the EACS match from 15 to 14 bp (similar to element II and III), also indicating element I to be the principal determinant for ARS activity. However, the sequence bias in position 169 can not be totally excluded to contribute to varying ARS activity since origin specific replication enhancers have been found to affect the frequency of origin firing (38). Also this mutation is close to and upstream of element III and could therefore affect replication.

According to sequences in the database, it should be possible to take up the distal rDNA-ARS copy by PCR using a unique 3′-primer matching the flanking region together with an internal 5′-primer. Attempts to do this, with the purpose to determine the type of the distal copy, gave no PCR-product with 309-DNA while a correct size product was obtained with templates from both S288C and strain 12B. This indicates that the linking to flanking regions in strain 309 is different from that in S288C and strain 12B.

## DISCUSSION

The present work demonstrates unequal activity of ARS elements among the rDNA units in yeast and points to one of the putative ACS as the main determinant of the ARS activity. This brings new aspects for understanding the behaviour of the rDNA region with implications for the replication of genomic rDNA and for maintenance of extrachromosomal circles.

Accumulation of extrachromosomal rDNA circles in mother cells appears to be a major factor leading to ageing in yeast, and premature ageing is observed in the DNA helicase negative mutants *sgs1* where accumulation of extrachromosomal rDNA circles occurs more rapidly than in wild type cells (27). Obviously, the accumulation of extrachromosomal rDNA circles requires a functional ARS in each circle. Based on limited sequence data, it has so far been assumed that the rDNA ARS is invariant and weak and not used in every unit for replication of chromosomal rDNA (11, 13, 15, 16). Our present finding that in one strain as much as one third of the rDNA repeats contains an inactive ARS, clearly illustrates that polymorphism within this region also has to be taken into account. Furthermore, that the same mutations are present in other strains as well (13) indicates that low activity ARS may be a common phenomenon, although in (13) the ARS activity was not tested. The average life span is a characteristic feature of a yeast strain (reviewed in (39)), and the proportion of low activity ARS within the rDNA region might well be part of this feature. One would expect that extrachromosomal copies are more easily maintained when generated from rDNA units with high potential to replicate. Mutations leading to strengthening of ARS activity might therefore be selected against because of consequent reduced life span of the cells, while cells with a high proportion of rDNA units with low activity- or non-functional ARS could have an advantage. Since the rDNA contains an excess of potential replication origins, replication of chromosomal rDNA might not be affected unless units with low origin activity are not interspersed but organised as a long contiguous block. Lack of a suitable test system makes it difficult to determine the organisation, and attempts to identify the centromere-distal copy after PCR-amplification failed since no PCR-product could be obtained for strain 309, even with purified DNA as a template. But most likely, the rDNA units with defect ARS are grouped together, as proposed for length heterogeneities (30), because the two transitions, T78C and G169A, in the low-ARS clones appear together and are not likely to be the result of independent mutations in random repeats. Blocks of low-ARS rDNA units can be replicated from flanking origins. The size of such groups must be limited to an array of units that can be replicated in time during the S-phase. Thus, a dual selection system that keeps the rDNA ARS at low strength and adjusts the size of groups with nonfunctional ARS might operate in yeast.

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