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Genetics

Replication and meiotic transmission of yeast ribosomal RNA genes

(*Saccharomyces cerevisiae*/multiple-copy genes/sequence homogeneity/meiosis)

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ABSTRACT **The yeast *Saccharomyces cerevisiae* has approximately 120 genes for the ribosomal RNAs (rDNA) which are organized in tandem within chromosomal DNA. These multiple-copy genes are homogeneous in sequence but can undergo changes in copy number and topology. To determine if these changes reflect unusual features of rDNA metabolism, we have examined both the replication of rDNA in the mitotic cell cycle and the inheritance of rDNA during meiosis. The results indicate that rDNA behaves identically to chromosomal DNA: each rDNA unit is replicated once during the S phase of each cell cycle and each unit is conserved through meiosis. Therefore, the flexibility in copy number and topology of rDNA does not arise from the selective replication of units in each S phase nor by the selective inheritance of units in meiosis.**

The genome of the yeast *Saccharomyces cerevisiae* contains approximately 120 copies of the genes for ribosomal RNA (rRNA genes or rDNA) (1). Individual repeating units contain coding sequences for the 5S, 5.8S, 18S and 25S RNAs (2, 3), and within a strain the units are homogeneous in both size and sequence (4). Restriction enzyme and genetic analyses indicate that the rRNA genes are organized in tandem (4, 5) on chromosome XII (6, 7).

At the level of individual repeats, little is known about the replication and inheritance of yeast rDNA units. However, some observations suggest that rDNA behaves in ways that are unusual for chromosomal DNA. (*i*) The inheritance of restriction enzyme variant forms of rDNA is almost always 2:2 (8), the segregation pattern expected for a single Mendelian locus. This observation suggests either that meiotic recombination in the rDNA is drastically reduced compared to other chromosome regions (8) or that only one or a few rDNA repeats are transmitted through meiosis. (*ii*) Electron microscopy of meiotic cells indicates that a portion of the parental nucleolus is excluded from the four haploid spores which independently generate new nucleoli (9, 10). A possible extrapolation from this observation is that rDNA units are discarded during meiosis and are replaced at a later time. (*iii*) Strains monosomic for chromosome I have fewer rDNA units than diploids (11, 12), and amplification of the rDNA back to the normal level occurs during vegetative growth (13–15). (*iv*) Circular extrachromosomal copies of rDNA, which are one or a few units in length, are found at low frequencies in vegetative yeast cells (16, 17). Extrachromosomal circular rDNA molecules are found in many other systems, including *Xenopus* oocytes, where amplification of rDNA occurs by a rolling circle form of replication (18). The circular rDNA molecules found in yeast may reflect a mode of replication that is responsible for the normal duplication of rDNA or for the amplification of rDNA units after meiosis. These observations imply a flexibility of number and topology for yeast rDNA, which may reflect important aspects of its replication and inheritance.

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We have examined some features of rDNA metabolism in *S. cerevisiae* that are relevant to the replication and inheritance of the individual rDNA units. We find that all rDNA units are transmitted through meiosis to the haploid generation and that each unit serves as a template for one replication event in each S phase of the mitotic cell cycle.

EXPERIMENTAL PROCEDURES

Density Transfer Experiments. The strain used was a diploid uracil auxotroph, A364A D5 U^- A^+ (called D5). The ^{13}C glucose/$(^{15}NH_4)_2SO_4$ medium and the cell transfer and cell lysis procedures have been described (19). CsCl equilibrium centrifugation was carried out in a Spinco VTi65 rotor. Lysates (1.0 ml, about 3×10^8 cells) were added to 1.23 g of CsCl, and enough CsCl solution (1.23 g of CsCl per 1 ml of 10 mM Tris·HCl/100 mM EDTA, pH 8) was added to fill 5.3-ml VTi65 heat-seal tubes. Gradients were formed by centrifugation at 30,000 rpm for 60 hr and then collected by bottom puncture into microtiter plates with fractions of 0.11 ml. The distribution of total DNA in the gradients was determined by sampling each fraction for alkali-stable, acid-precipitable ^3H cpm.

Quantitative Spot Hybridization. The density distribution in CsCl gradients of the repeated rRNA genes was determined by hybridizing ^{32}P-labeled, nick-translated cloned DNA fragments to samples of each gradient fraction that had been spotted on nitrocellulose sheets. A portion of each CsCl gradient fraction was transferred to a microtiter plate, adjusted to 0.6 M NaOH, and incubated for 16 hr at 37°C to hydrolyze RNA and denature DNA. Drops (5 μl) were spotted onto dry nitrocellulose sheets and permitted to dry in air. To remove CsCl and NaOH from the spotted samples, the sheets were placed on a piece of filter paper on top of a sponge standing in 0.60 M NaCl/0.060 M Na citrate, pH 7, for 1 hr. After air drying, the sheets were baked in an 80°C oven for 2 hr. The rDNA repeating unit was cleaved with *Sma* I from the recombinant plasmid pBD4 (20) (obtained from G. Bell and W. Rutter) purified by electrophoresis, and nick translated (21). Hybridizations were carried out as described (22). Hybridization filters were placed over x-ray film (Kodak XR-2) with an intensifying screen (DuPont) and stored at −70°C. Developed autoradiograms were scanned with a densitometer, and the peak height corresponding to each spot on the autoradiogram was measured. Several quantitative features of this method of hybridization were examined. (*i*) The relative peak heights from the autoradiogram scans were identical to the relative ^{32}P cpm. (*ii*) The ^3H cpm profiles for the filter-bound DNA were found to be identical to the original gradient profiles. (*iii*) Several film ex-

Abbreviations: rDNA, DNA coding for ribosomal RNA; kb, kilobase pair(s).

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posures of each filter were made and scanned to be certain that the film darkening was proportional to ^{32}P decays. (*ib*) The extent of hybridization in each experiment was proportional to the amount of DNA per spot, as determined with a series of dilutions of total DNA.

Cell Synchronization. For cell cycle arrest and synchronization experiments, a uracil auxotroph containing a temperature-sensitive mutation in gene *cdc7* (strain 4008, from L. H. Hartwell) was used. The strain, culture media, and procedures have been described (19).

Meiotic Transmission Experiments. The strain used was D5. Conditions for isotopic labeling, cell growth, induction of meoisis, and spore purification have been described (23). Cell lysis procedures were the same as in the density transfer experiment (19).

rDNA Isolation. For cell synchronization and meiotic transmission experiments, the rDNA was isolated from total DNA by isopycnic centrifugation in CsCl followed by agarose gel electrophoresis. CsCl fractions containing the dense shoulder of chromosomal DNA, which is enriched in rDNA (24), were pooled and cleaved with the restriction enzyme *Sma* I, which cleaves once in each rDNA repeat unit to yield an 8.6-kilobase-pair (kb) DNA fragment (4, 19). The *Sma* I-digested DNA was electrophoresed in 0.4% agarose gels (19) to separate the rDNA from contaminating sequences.

RESULTS

Each rDNA Unit Replicates Each Cell Cycle. The distribution of replication events among the approximately 120 copies of the rRNA genes was examined by density transfer experiments. A culture of strain D5 grown at 30°C in isotopically dense medium with 20 μCi of [^{3}H]uracil per ml (1 Ci = 3.7×10^{10} becquerels) was transferred to light medium with the same specific activity of [^{3}H] uracil. At intervals after the transfer to light medium (0.0, 0.5, 0.9, 1.6, 2.1, and 2.5 doublings), cells were harvested and total cellular DNA was centrifuged to equilibrium in CsCl. The zero-time sample of dense cells was mixed with an equal number of cells grown in light medium containing [^{3}H]uracil. Fig. 1*A* illustrates the resolution obtained with the vertical tube rotor for dense compared to light DNA ($\Delta\rho$ = 0.03 g/cm^3) and for chromosomal compared to mitochondrial DNA ($\Delta\rho$ = 0.015 g/cm^3). Fig. 1 *B–F* shows the shift of total cellular DNA from fully dense (HH) to hybrid (HL) and then to fully light (LL) as a function of growth in light medium.

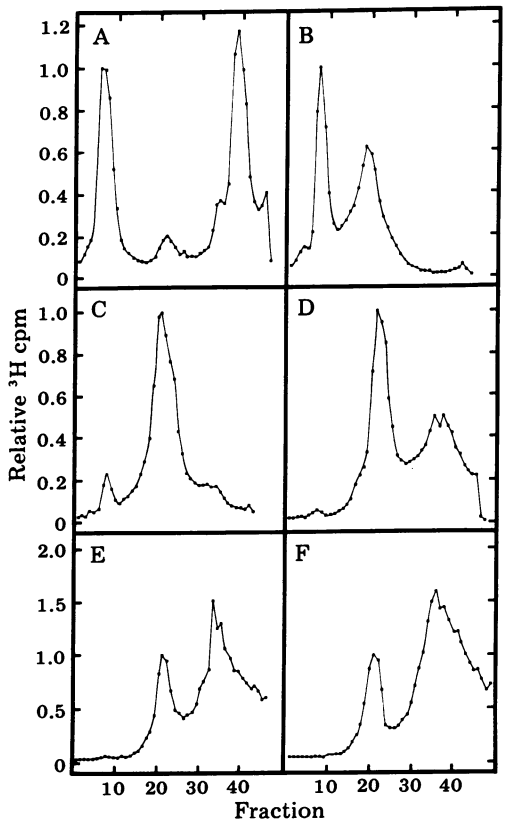

FIG. 1. CsCl equilibrium density banding of total yeast DNA from a density transfer experiment. (*A*) Cells containing ^{3}H-labeled fully heavy (HH) and fully light (LL) DNAs were mixed and the DNAs were centrifuged to equilibrium in the VTi65 rotor. Density decreases to the right. HH chromosomal DNA is found predominantly in fractions 6 and 7 and LL chromosomal DNA in fractions 38–40. The density difference is ≈0.03 g/cm^3. HH mitochondrial DNA is in fractions 20–24; LL mitochondrial DNA is at the top of the gradient and not well resolved from LL chromosomal DNA. The ordinate is a scale of ^{3}H cpm relative to the ^{3}H cpm in the HH peak (fraction 6 contained 4245 cpm). (*B–F*) ^{3}H-Labeled DNA isolated from cells harvested at 0.5, 0.9, 1.6, 2.1, and 2.5 doublings, respectively, after a transfer from dense to light medium. The same specific activity of [^{3}H]uracil was present in both heavy and light media. ^{3}H cpm are normalized to maximal cpm in HH DNA at 0.5 doubling and HL DNA at 0.9, 1.6, 2.1, and 2.5 doublings. The actual cpm in these fractions were 1977, 2808, 3987, 4793, and 3851, respectively.

The replication pattern of rDNA was determined by hybridizing ^{32}P-labeled cloned rDNA to the CsCl fractions spotted on nitrocellulose sheets. The extent of hybridization was quantitated by autoradiography and densitometry (Fig. 2). The hybridization data for the zero doubling sample (Fig. 3*A*) show that the densities of HH and LL rDNA are slightly greater than the corresponding densities of HH and LL chromosomal [^{3}H]DNA as expected from the high G+C content of rDNA (24). The results for each sample time (Fig. 3 *B–F*) show that rDNA sequences occur predominantly in discrete density classes (HH, HL, and LL) whose relative abundances are very similar to chromosomal DNA. HH rDNA disappears as HL rDNA accumulates, and it disappears completely (>98%) before LL rDNA begins to accumulate. These results mean that all rDNA units replicate during the mitotic cell cycle.

The density transfer data also provide information about the temporal control of rDNA replication. If replication of the rDNA units were to occur in a random order over the entire cell cycle, the ratio of HL/HH (or HL/LL) would be 6.7 at one doubling (19). The shorter the cell cycle interval for rDNA replication, the larger the HL/HH ratio would be. The proportion of HL/HH rDNA molecules measured at one doubling in the density transfer experiment is greater than 50, indicating that replication of a given rDNA unit occurs during an interval of ≤17% of the cell cycle (for calculations, see ref. 19). There are two alternative explanations for this result: either all rDNA units replicate within the same 17% of the cell cycle or different rDNA units replicate in different 17% intervals, but in either case the temporal order is maintained in subsequent cell cycles. In an attempt to distinguish between these two alternatives, we determined the length of the S phase in strain D5 by autoradiography of pulse-labeled cells (25). Cells grown under conditions that mimic the density transfer experiment were found to have an S phase length of 18% ± 4% of the cell cycle. It therefore seems possible that all rDNA units replicate in a single specific ≤17% interval, which corresponds to the S phase in strain D5.

rDNA Units Replicate in the S Phase. The time of rDNA synthesis was examined directly in synchronous cultures. *cdc7* cells, which are defective in the initiation of DNA synthesis at 36°C (26), were sequentially arrested in the G1 phase with α-factor (mating pheromone) at 23°C followed by removal of

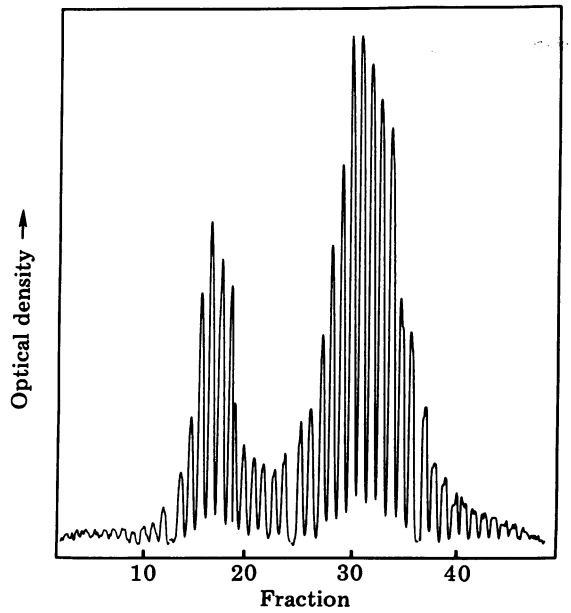

FIG. 2. Densitometer scan of rDNA autoradiogram. A densitometer scan of an array of spots from an autoradiogram in which [^{32}P]rDNA was hybridized to fractions of a CsCl gradient containing total yeast DNA isolated 2.5 doublings after a transfer from dense to light medium. The peak heights are proportional to the amounts of [^{32}P]rDNA hybridized.

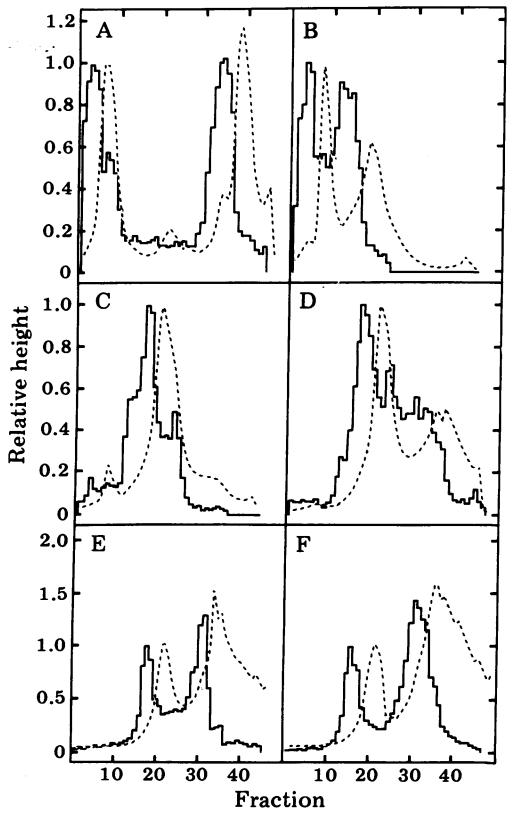

FIG. 3. Quantitation of rDNA hybridization to CsCl gradient fractions. —, Densitometer scans of autoradiograms of ^{32}P-labeled rDNA hybridized to the six gradients in Fig. 1 were measured and plotted as relative peak heights against fraction number. ---, The ^{3}H profiles of the six gradients from Fig. 1 are reproduced for comparison. The size of the DNA fragments containing rDNA was found to be about 55 kb (equivalent to about seven rDNA units), based on gel electrophoresis of the total DNA in each sample and hybridization of the gel-separated DNA to ^{32}P-labeled rDNA probe. Some of the gradient profiles show a peak of hybridization between the major density classes of HH, HL, and LL. Because such a peak is found on the light side of the HH chromosomal DNA in gradient *A*, in which no density transfer was involved, these intermediate-density classes of rDNA are unlikely to be related to replication.

α factor and temperature arrest at 36°C. Cells arrested in this way undergo a synchronous round of DNA synthesis when subsequently transferred to 23°C (19, 27). Evidence for synchrony comes from the pattern of [^{3}H]uracil incorporation into total DNA (see, for example, figure 5 of ref. 19). During incubation at 36°C, there is little incorporation of isotope into DNA. However, when cells are transferred to 23°C, the rate of incorporation increases sharply. This rapid increase in incorporation lasts about 75 min and is followed by an interval of about 100 min during which there is little incorporation of isotope into DNA. Data on incorporation of [^{3}H]uracil into total DNA were obtained for each experiment and used to estimate the times for the beginning and the end of S phase.

In order to monitor DNA synthesis during the synchronous S phase, cells were prelabeled with [^{14}C]uracil for about five generations prior to and during treatment with α factor. [^{14}C]Uracil and α factor were removed, and the cells were transferred to 36°C medium containing [^{3}H]uracil. rDNA was purified by CsCl isopycnic centrifugation and agarose gel electrophoresis (Fig. 4). The ^{3}H/^{14}C ratios for rDNA were determined at intervals throughout the synchronous S phase at 23°C and used as a measure of DNA synthesis (Fig. 5). For comparison, we have included our published data for 2-μm DNA, mitochondrial DNA, and single-copy chromosomal DNA (19) from the same experiments. At the beginning of S phase the rDNA has a low ^{3}H/^{14}C ratio, like single-copy chromosomal and 2-μm DNAs but unlike mitochondrial DNA. This low ratio shows that the rDNA is not synthesized in the absence of an active *cdc7* gene product. Moreover, extensive synthesis of rDNA did not occur during α-factor arrest because the relative amount of rDNA did not increase during this treatment (data not shown). During the synchronous S phase in four separate experiments, the patterns of increase in the ^{3}H/^{14}C ratios for single-copy chromosomal DNA and rDNA were indistinguishable and reached the same final value. Thus, rDNA is doubled by synthesis throughout the S phase.

All rDNA Units Are Transmitted Through Meiosis. The fraction of rRNA genes transmitted from the diploid cell, through meiosis and sporulation, to the haploid generation was determined in double-label experiments. The diploid strain D5 was grown in acetate presporulation medium containing [^{3}H]uracil before meiosis. After meiosis, which occurred in the absence of a radiolabel, the resulting haploid spores were purified, germinated, and grown in glucose medium containing [^{14}C]uracil. The ^{3}H/^{14}C ratio for the purified 8.6-kb rDNA units was normalized to the chromosomal DNA ratio. The normalized ratios from two experiments (Table 1, column A) were not significantly different from 1.0, suggesting that all the rRNA gene copies are transmitted through meiosis. In contrast, rRNA molecules exhibit a low ^{3}H/^{14}C ratio (0.20) compared to chromosomal DNA, indicating that they are inefficiently transmitted.

The normalized ^{3}H/^{14}C ratios could reflect changes in copy number as well as transmission efficiencies. For example, a normalized ratio of 1.0 for rDNA would result if only 50% of the rDNA copies are transmitted to spores and the spores maintain that lower rDNA content after germination. There is evidence for differences in the number of rRNA genes among various *S. cerevisiae* strains (28). Strain heterogeneity is inherent

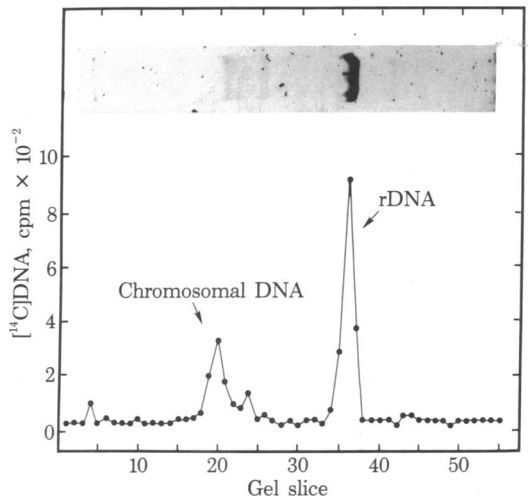

FIG. 4. Fractionation of rDNA from total chromosomal DNA by gel electrophoresis. Total cellular DNA was banded in CsCl and the dense shoulder of the main DNA band was recovered. This DNA was cleaved with *Sma* I and subjected to gel electrophoresis. The gel lane (*Inset*) was dried and sliced into ≈1-mm sections, and radioactivity was measured. The profile of ^{14}C cpm is shown. Electrophoretic migration is from left to right.

in this experiment because the presporulation population is a homogeneous set of diploid cells and the postgermination population is a culture of haploids of different genotypes plus some diploids (about 20%) formed by random matings. In addition, the presporulation medium contained acetate, a nonfermentable carbon source, whereas the postgermination carbon source was glucose. Differences in carbon metabolism or

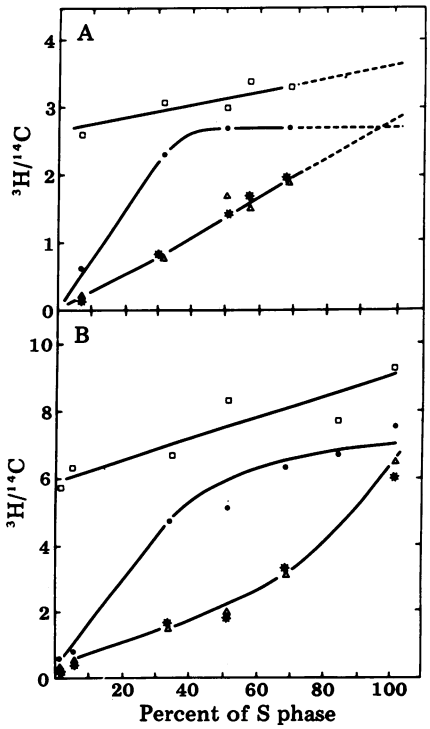

FIG. 5. $^{3}H/^{14}C$ ratios for various DNAs during a synchronous S phase. The results for two experiments (*A* and *B*) are shown. Percent of S phase refers to the time at which a sample was taken relative to the total length of S. The beginning and end of S phase were determined for each experiment from total DNA incorporation data (19). The mitochondrial DNA (□), 2-μm DNA (●), and chromosomal DNA (△) data are from ref. 19. The rDNA (*) data are from the same experiments.

Table 1. Meiotic transmission efficiencies for rDNA and rRNA

Nucleic acid	$^{3}H/^{14}C$ ratios relative to chromosomal DNA (A) Meiotic transfer	(B) Copy number control	(C) Meiotic transmission (A/B)
Chromosomal DNA	1.00 ± 0.06 1.00 ± 0.16	1.00 ± 0.05	1.00
rDNA	0.97 ± 0.06 0.94 ± 0.15	0.98 ± 0.05	0.97
rRNA	0.19 ± 0.01 0.21 ± 0.01	0.68 ± 0.02	0.29

SD of the chromosomal DNA ratio was calculated for each of the two experiments by averaging the $^{3}H/^{14}C$ ratios from at least 10 slices through the chromosomal DNA region of the gel and comparing the average to each individual ratio. The normalized ratios for the rDNA are assumed to have similar errors; independent SDs were not calculated because the rDNA was concentrated in only two or three gel slices. The normalized ratios for rRNA are more reliable because there was a large amount of radiolabel in this material and it was distributed over many gel slices. The "rRNA" consists of the two large RNAs (which could be contaminated with, at most, 20% nonribosomal rRNA).

growth rate conceivably could affect the copy number of rRNA genes.

The copy numbers for rRNA genes in the presporulation and the postgermination populations were compared in a second double-label experiment. One culture of D5 was grown in acetate presporulation medium and sporulated, and the spores were isolated and germinated in glucose medium containing [^{14}C]uracil (in a manner and for a length of time identical to the previous experiment, but without the prelabeling with [^{3}H]uracil). This culture was then mixed and harvested with a second culture of D5 grown in acetate medium containing [^{3}H]uracil. The $^{3}H/^{14}C$ ratios of purified rDNA and rRNA were again normalized to the chromosomal DNA ratio. The results (Table 1, column B) indicate that there is no significant difference in rRNA gene number between the presporulation and postgermination cell populations. However, the presporulation cells possess about 32% fewer rRNA molecules per haploid genome. Comparison of the normalized ratios from the two experiments provides an estimate of meiotic transmission efficiency (Table 1, column C). For rDNA, the transmission efficiency (0.97) is not significantly different from 1.00.

DISCUSSION

Density transfer experiments were performed to determine the distribution of replication events among the rDNA units. If replication from an extrachromosomal rDNA molecule were responsible for the doubling of rRNA genes each cell cycle, then at one doubling in a density transfer experiment there would be very few HL units (template molecules) but nearly 50% HH and 50% LL units. The observation that all (>98%) rDNA units are HL at one doubling indicates that replication events are uniformly distributed; i.e., each unit replicates once each cell cycle. This result formally eliminates a master gene mechanism for rDNA replication.

The experiments with synchronized cells show that replication of the rRNA genes is indistinguishable from that of the rest of the chromosomal DNA. No synthesis occurs during G1 arrests produced by either α factor or the *cdc7* temperature-sensitive block. The $^{3}H/^{14}C$ ratio increases in parallel with chromosomal DNA, showing that rDNA replicates continuously through the S phase. Because we were able to detect preferential synthesis of 2-μm DNA during early S phase, we are confident that the experiments could have detected replication of rDNA during

a similar limited interval of S phase. Because the final $^3H/^{14}C$ ratios for rDNA and chromosomal DNA are the same, the rDNA doubles during S phase. Previous experiments (29) did not establish that rDNA replication is confined to the S phase of the cell cycle. Because the replication fork rate under the synchronous culture conditions is 3.6 kb per min (30), it would take only 1–2 min to replicate a single 8.6-kb rDNA unit, an interval much shorter than the length of the S phase (about 75 min). Therefore, rDNA replication cannot occur by the simultaneous activation of origins in each repeat unit. A few origins would be adequate to account for replication of the entire rRNA gene cluster.

The behavior of the rDNA in meiosis is also indistinguishable from the remainder of chromosomal DNA. Each unit is stably transmitted to the haploid generation. We therefore conclude from these results that the portion of nucleolus discarded in meiosis cannot contain more than about 5% of the rDNA units, and the 2:2 segregation of rDNA restriction variants (8) must be explained by a reduction in recombination frequency and not by a reduction in rRNA gene numbers during meiosis.

Our results have implications for possible mechanisms by which rDNA sequence homogeneity is maintained. One class of mechanisms suggests that rectification of diverging sequences occurs by the intervention of master genes (31–33). At regular intervals (either once per cell cycle or once per sexual cycle) the master gene is used as a template either to correct the rest of the units by base mismatch repair or to produce new units by replication. Master replication could ensure homogeneity by degrading the pre-existing sets of rDNA units or simply by diluting them out during subsequent generations. Because we find no turnover of rDNA in either the mitotic density transfer or the meiotic transmission experiments, we conclude that master replication with degradation of preexisting rDNA units does not maintain sequence homogeneity in either mitotic or meiotic cycles. (The small amount of degradation involved in a master mismatch repair mechanism would not be detected in our experiments and, therefore, is not ruled out by our results.) Furthermore, master replication accompanied by dilution of preexisting rDNA copies cannot be responsible for the doubling of rDNA in vegetative cell cycles because each rDNA unit serves as a template for replication. However, our data do not exclude a master replication mechanism without degradation of pre-existing units during the premeiotic S phase, nor do they rule out either form of master replication at any stage if the event is a rare one.

The major alternative mechanism for the maintenance of rDNA homogeneity involves repeated cycles of unequal sister chromatid exchange and chromatid segregation (34). Recent observations show that unequal sister strand crossing-over occurs within the tandem arrays of yeast rDNA during both meiosis (35) and the mitotic cell cycle (36). It has been estimated that the frequency of recombination would be sufficient to maintain sequence homogeneity (36). Our experiments eliminate the major forms of the master gene hypothesis for the maintenance of rRNA gene homogeneity in yeast and, therefore, indirectly support the unequal exchange mechanism.

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