Distribution of Ribosomal Ribonucleic Acid Cistrons Among Yeast Chromosomes

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High-molecular-weight deoxyribonucleic acid (DNA) of Saccharomyces carlsbergensis has been fractionated by sucrose density gradient centrifugation. The main DNA fraction has an average molecular weight of about $500 \times 10^6$. A major fraction of the DNA molecules containing sequences homologous to ribosomal ribonucleic acid (RNA) sediments as material of this molecular weight. The remainder sediments as material of a molecular weight of about $250 \times 10^6$. The latter fraction contains relatively more ribosomal RNA cistrons than the former. Studies on the buoyant density of high-molecular-weight DNA homologous to ribosomal RNA have led to the conclusion that the ribosomal RNA cistrons occur in groups attached to a relatively large amount of nonribosomal RNA and suggest that ribosomal RNA cistrons are distributed over a number of yeast chromosomes.

Recent studies (3, 16) have shown that undegraded nuclear chromosomal deoxyribonucleic acid (DNA) of Saccharomyces cerevisiae sediments as heterogeneous material of very high molecular weight (between $300 \times 10^6$ and $800 \times 10^6$). Other investigations have shown that yeasts like S. carlsbergensis and S. cerevisiae contain about 140 ribosomal ribonucleic acid (RNA) cistrons per haploid genome complement (7, 17, 19). Since the molecular weight of the ribosomal RNA precursor in yeast is about $2.5 \times 10^4$ (20), the molecular weight of the minimum amount of DNA homologous to ribosomal RNA must be in the order of about $700 \times 10^6$, a value well within the range of the molecular weight of undegraded yeast DNA. The question can then be asked whether all the ribosomal RNA cistrons indeed occur on one contiguous chromosome or whether they occur scattered over many chromosomes, associated with smaller or larger amounts of additional RNA sequences not homologous to ribosomal RNA.

To investigate these possibilities, the sedimentation pattern as well as the buoyant density of intact yeast DNA homologous to ribosomal RNA has been studied.

The results of these studies show that ribosomal RNA cistrons in yeast sediment as distinct peaks of material with a molecular weight of approximately $250 \times 10^6$ and $500 \times 10^6$. The results of the buoyant density gradient experiments show that, in undegraded yeast DNA, ribosomal RNA cistrons are associated with a relatively large amount of DNA of relatively high adenylic acid + thymidylic acid content and are scattered over a number of chromosomes.

MATERIALS AND METHODS

The yeast strain used in these investigations was S. carlsbergensis NCTC 74. Cells were grown and converted into protoplasts as described previously (10). $^3$H-thymidine-labeled phage T4 was a generous gift of R. Clarke.

For the incorporation of $^1$C-labeled adenine into DNA, yeast was grown in 100 ml of complete medium (10) at 27 C in the presence of 15 $\mu$Ci of adenine-$^1$C (sp act 41 mCi/mmole). The total amount of nucleic acid in a protoplast suspension was estimated from the amount of hot trichloroacetic acid-soluble material absorbing at 260 nm by using a value of 25 for the absorption of 1 mg of nucleic acid per ml. The amount of DNA was taken as 1% of the total amount of nucleic acids in the protoplasts (1). The specific activity of the DNA was then calculated from the amount of cold trichloroacetic acid-insoluble radioactivity in a sample of the protoplasts.

Mitochondrial DNA was labeled preferentially by incubating cells in the presence of cycloheximide prior to the conversion into protoplasts (9, 13). Cells were grown in 100 ml of complete medium until late log phase. Cycloheximide was added (50 $\mu$g/ml), followed immediately by 15 $\mu$Ci of adenine-$^3$H (sp act 41 mCi/mmole), and the incubation was continued for 2 h. Cells were converted into protoplasts as described above.

For the preparation of $^3$H-labeled ribosomal RNA,
yeast protoplasts (A490 nm = 1) were incubated for 60 min at 30 C in 30 ml of medium containing per ml: 20 μmol of potassium phosphate buffer (pH 6.2), 2 μmol of MgCl2, 20 mg of glucose, 2 mg of Casamino Acids, 200 mg of sorbitol, and 15 μCi of adenine-8-3H (sp act 20 Ci/mmol). The yeast protoplasts were collected by centrifugation and reincubated for 120 min in unlabeled medium containing, in addition, 50 μg of unlabeled adenine per ml. Ribosomal RNA was isolated by the cold phenol extraction procedure described before (6). For the preparation of [H]-labeled messenger RNA, protoplasts were incubated in the medium as described above in the presence of cycloheximide (20 μg/ml). Messenger RNA was extracted by the hot phenol procedure described in an earlier paper (7). The RNA preparations were freed from low-molecular-weight material by chromatography on Sephadex G-200.

Sucrose density gradient centrifugation of ungraded yeast DNA was carried out as follows, 0.05 to 0.1 ml of a protoplast suspension, containing not more than, at most, about 4 μg of DNA, were carefully mixed with 0.15 ml of a solution containing 1 M NaCl, 0.02 M tri(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.9), 0.02 M EDTA, 1% sodium Sarkosyl, and Pronase (1 mg/ml) and incubated in a centrifuge tube of the IEC SB 283 rotor for 4 h at 45 C. After cooling to room temperature, an exponential 10 to 25% sucrose gradient containing 1 M NaCl, 0.02 M Tris-hydrochloride buffer (pH 7.9) and 0.02 M EDTA was made under the lysis. Gradients were spun for about 15 h at 15,000 rpm at 20 C. Fractions were collected after puncturing the bottom of the tube. DNA was denatured and DNA hydrolyzed by the addition of 0.1 ml of 1 M NaOH and incubation overnight at 30 C. The fractions were neutralized by the addition of 0.2 ml of 1 M NaH2PO4.

Hybridization of the DNA fractions with ribosomal RNA and messenger RNA was carried out as described before with some minor modifications (7). A 5-μg amount of [H]-labeled ribosomal RNA (sp act 15,000–20,000 counts per min) and 2 μg of messenger RNA (sp act 5,000 counts per min) and water were added to a final volume of 1 ml. Samples containing ribosomal RNA were incubated for 4 to 6 h, those containing messenger RNA were incubated for 24 h at 65 C. After cooling and treatment with pancreatic and T1 ribonuclease, the hybrids were collected on Millipore HAWP filters as described earlier (7).

Centrifugation of DNA in sodium iodide gradients was carried out as described earlier (8). A protoplast lysate prepared as described above was mixed carefully with 6 ml of a sodium iodide solution containing 0.02 M Tris-hydrochloride buffer (pH 7.9), 0.01 M sodium bisulfite, 0.01 M EDTA, and ethidium bromide (20 μg/ml). The final density was about 1.53 g/cm3. Gradients were generated by centrifugation in the IEC A 321 rotor at 45,000 rpm and 25 C for 64 h. Tubes were punctured, and 0.2-ml fractions were collected. DNA was denatured with alkali, and the fractions were neutralized with sodium phosphate as described for the sucrose gradients. For hybridization with ribosomal RNA, 5 μg of [H]-labeled ribosomal RNA (sp act 15,000–20,000 counts per min) and 2 x SSC (0.15 M NaCl plus 0.015 M sodium citrate) were added to a final volume of 1 ml, and the samples were incubated for 4 to 6 h at 65 C. Hybrids were collected as described before (7).

Radioactivity of all samples was determined in a Beckman scintillation counter (LS 230) in 10 ml of toluene containing 0.4% Omnifluor.

RESULTS

When protoplasts of S. carlsbergensis are carefully lysed in a centrifuge tube, the released DNA sediments as heterogeneous material considerably faster than the DNA of phage T4 (Fig. 1). The molecular weight of DNA samples in the range of 106 and higher can, however, be seriously underestimated as a result of a considerable dependence of the sedimentation coefficient on the rotor speed (Rubenstein and Leighton, 1971, Biophys. Soc. Annu. Meet. 11:209a). In agreement with Blamire et al. (3) and Peters and Fangman (16), we found that rotor speeds of 12,000 rpm and lower, using the IEC SB 283 or SB 110 rotor, yield, constant values for the sedimentation rate of yeast DNA relative to T4 DNA. Taking 60S as the sedimentation coefficient of phage T4 DNA, the results in Fig. 1 show that yeast DNA sediments mainly as material with a sedimentation coefficient between 90 and 110S with a shoulder extending into the 70S region. With a value of 120 × 104 for the molecular weight of T4 DNA application of Freifelders equation [S30w = (Mw/Mr)0.34 (8)] yields a value of about 250 × 104 to 700 × 104 for the molecular weight of yeast DNA with the

![Fig. 1. Sedimentation pattern of high-molecular-weight DNA of Saccharomyces carlsbergensis. A yeast protoplast suspension containing about 2 μg of [14C]-labeled DNA (sp act 400 counts per min per μg) was mixed with [H]-labeled T4 phage (sp act about 1,000 counts per min per μg of DNA), lysed and centrifuged as described in Materials and Methods. Cold trichloroacetic acid-insoluble, alkali-stable radioactivity is plotted.](http://jb.asm.org/)

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main fraction having a molecular weight of about 500 \times 10^4.

To obtain such high-molecular-weight DNA, it is necessary to process the protoplasts as soon as possible. In agreement with Blamire et al. (3) it was found that storage of the protoplasts for more than a few hours leads to a reduction of the amount of material with molecular weight of 500 \times 10^4 and a concomitant appearance of material sedimenting at about 45S. In disagreement with these authors we have found, however, that lysis of protoplasts prior to centrifugation by incubation for a few hours at 45 C in the presence of EDTA, Pronase, and sodium sarkosylate yields identical sedimentation profiles as obtained by direct lysis of the protoplasts on top of the gradients. The latter method frequently yielded, however, a considerable amount of DNA sedimenting to the bottom of the centrifuge tube. The lysate prepared by the method described in the present communication can be stored for at least a day at room temperature without significant deterioration of the sedimentation pattern of the DNA. Slow application of the lysate from a wide-bore pipette on top of a preformed gradient was found not to affect the sedimentation pattern of the DNA. Furthermore, the method can be applied to other yeast strains like *Kluyveromyces fragilis*, which cannot be transformed easily into protoplasts. The cells of such organisms often lyse readily after freezing, thawing, and incubating under the same conditions as described above (de Kloet, unpublished results).

Quantitatively, not more than 2 \mu g of yeast DNA can be centrifuged in a single gradient made in centrifuge tubes of the SB 283 rotor. Larger amounts gave rise to aggregation of the DNA and a very high rate of sedimentation. The sedimentation patterns were found to be reproducible for DNA amounts smaller than 2 \mu g. The high-molecular-weight DNA most likely represents true continuous DNA molecules and not low-molecular-weight material kept together by protein. Control experiments with protoplasts labeled with 14C-labeled amino acids showed that the Pronase treatment left only 2% of the protein as hot trichloroacetic acid-precipitable material. All this material was found to remain at the top of the gradient, and no radioactivity was found in the region of the gradient where the DNA is found. Considering the specific activity of the protein in the protoplasts used for these experiments, this means that 2 \mu g of DNA contained less than 0.001 \mu g of protein.

Figure 2 shows the results of the experiments in which high-molecular-weight yeast DNA was examined for sequence homology with yeast ribosomal RNA. Since no more than 2 \mu g of DNA can be centrifuged per gradient and since the highest specific activity of the ribosomal RNA obtained in these experiments was 20,000 counts per min per \mu g, larger fractions had to be collected than in the experiments described in Fig. 1 in order to obtain meaningful results in the hybridization experiments. Consequently, the resolution of the DNA in components of different molecular weight was considerably less. The results show clearly, however, that the ribosomal cistrons sediment as two different components, one coinciding with the main DNA component of a molecular weight of about 500 \times 10^4, the other with a molecular weight considerably lower, of the order of 250 \times 10^4 and coinciding with the shoulder in the DNA sedi-
mentation profiles. Since these hybridization experiments were carried out under saturation conditions (7), it can be concluded that about 30% of the ribosomal RNA cistrons are concentrated in the DNA of the shoulder and that this DNA fraction contains relatively more ribosomal RNA cistrons than the DNA of the main peak. This latter result shows also that the DNA in the shoulder does not arise by random degradation of the high-molecular-weight material. When similar experiments are carried out with yeast messenger RNA, the hybridization pattern follows the DNA profile, closely indicating that messenger RNA is not transcribed preferentially from DNA of any particular size.

Earlier studies (7, 19) had shown that, in yeast DNA of relatively low molecular weight (about 10 × 10⁶), the ribosomal RNA cistrons have a slightly higher buoyant density in CsCl than the main DNA fraction (1.703 versus 1.698). In terms of base composition this corresponds to a guanylic acid + cytidylic acid (G + C) content of about 43% for the DNA homologous to ribosomal RNA (7). Since yeast ribosomal RNA contains about 47% G + C (6), the ribosomal RNA cistrons are apparently associated with nonribosomal sequences of lower G + C content. Figure 3a shows that a similar result is obtained when sodium iodide density gradients are used for the banding of DNA of relatively low molecular weight (about 15 × 10⁶ as estimated by sucrose density gradient centrifugation) obtained by forcing a protoplast lysate through a 25-gauge needle. Whereas in this salt the main yeast DNA component has a buoyant density of 1.526 g/cm³ and a G + C content of 38% (10), the ribosomal RNA cistrons have a buoyant density of 1.530 g/cm³ again corresponding to a G + C content of about 43%.

When undegraded yeast DNA is centrifuged in a sodium iodide gradient (Fig. 3b), the sequences homologous to ribosomal RNA band at a buoyant density coinciding or slightly lower than the buoyant density of the main DNA fraction. Control experiments (Fig. 4) showed that, under the conditions of the experiment, mitochondrial DNA bands separately from the main band DNA, indicating that trapping of the ribosomal cistrons in the main DNA fraction is not the reason for the shift in density of the DNA homologous to ribosomal RNA. These results lead, therefore, to the conclusion that in undegraded yeast DNA the ribosomal RNA cistrons are associated with a substantial amount of DNA of a G + C content lower than the mean G + C content of yeast DNA. A more systematic investigation of the relationship between the molecular weight of purified yeast DNA and the buoyant density in sodium iodide density gradients (Lusby and de Kloet, unpub-

Fig. 3. Fractionation of DNA of Saccharomyces carlsbergensis by banding in sodium iodide density gradients. A yeast protoplast lysate containing about 2 μg of ¹⁴C-labeled DNA (sp act 400 counts per min per μg) was centrifuged in sodium iodide, and the fractions were hybridized with ³H-ribosomal RNA as described in Materials and Methods. a, After shearing of the DNA by forcing the sodium iodide lysate mixture through a 25-gauge needle prior to the centrifugation; b, without shearing.
published data) showed that the ribosomal cistrons still band at a density slightly different from the density of the main DNA fraction when the molecular weight of the DNA is about $80 \times 10^6$, a result in agreement with that obtained recently by Cramer, Bhargava, and Halvorson (5), and suggesting fairly extensive clustering of the ribosomal RNA cistrons.

**DISCUSSION**

The results of experiments described in this paper show that high-molecular-weight DNA of *S. carlsbergensis* sediments mainly as a material of an average molecular weight of about $500 \times 10^6$. This agrees well with the results obtained by others (3, 16) in studies on *S. cerevisiae*. Genetic studies (15) have shown that *S. cerevisiae* contains about 17 to 20 chromosomes. The total molecular weight of the DNA in haploid cells in this organism has been estimated to be between $8 \times 10^8$ and $12 \times 10^8$ (14). The close relationship between *S. cerevisiae* and *S. carlsbergensis* (2) makes it likely that the same values for the number of chromosomes and the total amount of DNA hold true also for the latter organism. The average molecular weight of the individual chromosomes should therefore be about $600 \times 10^6$, a value in reasonable agreement with the value determined by sucrose density gradient centrifugation of intact yeast DNA and indicating, as has been concluded by others (3, 16), that yeast chromosomes contain single DNA molecules.

The results presented in this study show that intact high-molecular-weight yeast DNA homologous to ribosomal RNA sediments as two distinct components, one of molecular weight of about $250 \times 10^4$, the other with a molecular weight of approximately $500 \times 10^4$. This result has recently been confirmed by others for *S. cerevisiae* (Finkelstein et al. [5a]). In this organism the majority (70%) of the ribosomal RNA cistrons sediments, however, as the lower-molecular-weight component, whereas in *S. carlsbergensis* only 30% of the ribosomal RNA cistrons is found in this region. As has been pointed out in the introduction, the total molecular weight of yeast DNA homologous ribosomal RNA amounts to about $700 \times 10^6$. Therefore, two chromosomes with a combined molecular weight of $750 \times 10^4$ would suffice to code for all 140 ribosomal RNA cistrons, provided there is no “spacer” between the precursor RNA cistrons (18).

The studies on the buoyant density of yeast DNA in sodium iodide density gradients suggest however that ribosomal RNA cistrons are associated with DNA of rather low G + C content and that, therefore, more chromosomes than just two have to be involved. Firstly, in DNA of relatively low molecular weight ($15 \times 10^6$) the fraction hybridizing with ribosomal RNA has a G + C content somewhat lower than expected for a DNA component homologous to ribosomal RNA (43% G + C versus 47% G + C). This suggests that ribosomal RNA cistrons are associated with some DNA of low G + C content. The nature of the DNA of low G + C content associated with the ribosomal RNA cistrons is not known. It might be that the nonconserved part of the ribosomal precursor is transcribed from a segment of low G + C content. It may also be that ribosomal RNA cistrons are separated from each other by spacers of low G + C content. An anomalous relationship between base composition and buoyant density as has been described for a number of satellite DNA species (4) cannot be ruled out, however, and therefore conclusions with regard to the base composition will have to await actual isolation of the DNA homologous to ribosomal RNA. Our observations (Lusby and de Kloet, to be published) that the ribosomal RNA cistrons band to the heavy side of the main DNA component even when the DNA has a comparatively high molecular weight (about $80 \times 10^6$) agrees with the results obtained recently by Cramer et al.
(5) and indicates that in yeast clusters of 15 ribosomal RNA cistrons may occur. The probable existence of spacer regions between the ribosomal RNA cistrons (18) might make this number of course considerably less.

The results of experiments on the banding of yeast DNA of very high molecular weight show, however, that any cluster of ribosomal RNA cistrons must be attached to a relatively large amount of DNA of average or below average G + C content. These data lead, therefore, to the conclusion that the 140 ribosomal RNA cistrons in yeast are scattered in clusters over a considerable number of chromosomes, a conclusion similar to that reached by Goldberg et al. (12) in recent studies using disomic strains of *S. cerevisiae*.

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LITERATURE CITED