Ribosomal RNA Genes in *Kluyveromyces marxianus*

By M. OJHA

Laboratoire de Microbiologie générale, Département de Biologie végétale,
Université de Genève, CH-1211 Genève 4, Switzerland

(Received 9 May 1979; revised 28 August 1979)

DNA from the yeast *Kluyveromyces marxianus* was studied for its heterogeneity and the multiplicity of rRNA cistrons. These cistrons banded at a slightly different density from the bulk DNA in preparative CsCl or Hg²⁺–Cs₂SO₄ equilibrium density gradients. The reassociation kinetics of the bulk DNA showed that the repetitive fraction represented a small amount of the total cellular DNA (10%) and that the single copy fraction had a complexity of 6.3×10^9 daltons. Approximately 2.2% of the DNA hybridized to ³H-labelled rRNA at saturation. On the basis of the above genome size, the multiplicity of the rRNA cistrons was calculated to be about 70 per haploid nucleus.

INTRODUCTION

Information concerning the physical properties, multiplicity and organization of ribosomal RNA cistrons in yeasts has been mainly derived from studies with *Saccharomyces cerevisiae* (Cryer *et al.*, 1973; Kaback *et al.*, 1973). These investigations have revealed that there are approximately 100 to 140 cistrons per haploid genome (the haploid genome size is approximately 5×10^9 to 8×10^9 daltons), coding for 25, 18, 5.8 and 5S rRNA each. The 140 cistrons are not contiguous but 10 to 32 cistrons are grouped in clusters on more than one chromosome. These conclusions are based on the appearance of rDNA peaks on equilibrium density gradient centrifugation of DNA fragmented to defined molecular weight (Cramer *et al.*, 1972). A *Saccharomyces cerevisiae* strain monosomic (2n-1) for chromosome I contains about 30% fewer genes coding for rRNA than the related diploid strain, indicating that about 70% of the rRNA cistrons are located on chromosome I (Øyen, 1973; Finkelstein *et al.*, 1972; Kaback *et al.*, 1973). In experiments with intact chromosomal DNA sedimented in sucrose gradients the rDNA was shown to be associated with two size classes of DNA molecules, one of which was linked to chromosome I (Finkelstein *et al.*, 1972).

Kluyveromyces is so closely related to *Saccharomyces* that Campbell (1972) has suggested the rejection of *Kluyveromyces* as a genus. However, studies of the nucleotide sequence homology (Bicknell & Douglas, 1970), genetic analysis (Tingle *et al.*, 1968) and the difference in the chromosome number (*K. lactis* has a haploid chromosome number of 9 compared to 15 for *S. cerevisiae*; Galeotti & Williams, 1978) justify the retention of *Kluyveromyces* as a separate genus. In view of these obvious similarities and differences we thought an investigation of the organization of rRNA cistrons in *Kluyveromyces* worthwhile. In this paper we report our results on the characterization of rRNA cistrons in *K. marxianus*.

METHODS

Organism, growth and DNA extraction. *Kluyveromyces marxianus* strain no. 712 CBS was maintained on malt agar slants at 25 °C. Yeast cells were scraped from malt agar slants (4 to 5 d-old), washed with distilled water by centrifugation, and inoculated in 1 l growth medium to give a density of about 1×10^9 cells ml⁻¹. The growth medium, YPG (containing, per litre, 10 g yeast extract, 20 g peptone and 40 g glucose), was prepared in a 2 l Erlenmeyer flask and the culture was grown at 25 °C with forced aeration. After 48 h, the cells were harvested by centrifugation, washed with distilled water and frozen in liquid nitrogen. The frozen cell clumps were broken and ground to fine powder in liquid nitrogen with a Braun homogenizer. The broken cell powder was suspended in a lysing buffer (8 M-urea/0.24 M-sodium phosphate buffer pH 6.8/1 % (w/v) sodium dodecyl sulphate/10 mM-EDTA/1 M-sodium perchlorate) and deproteinized twice with chloroform/isoamyl alcohol (24:1, v/v). The DNA from the deproteinized supernatant was extracted on hydroxyapatite and further purified as described by Dutta & Ojha (1972).

Analytical equilibrium density gradient centrifugation. DNA (5 µg ml⁻¹) was prepared in either CsCl or Cs₂SO₄ solution, the latter containing HgCl₂ or AgNO₃ at heavy metal ion to DNA ratios of 0.1 and 0.3, respectively, as described elsewhere (Ojha & Turian, 1977). The samples were centrifuged in an MSE analytical ultracentrifuge at 140000 *g* for 24 h at 25 °C. Ultraviolet photographs were traced using a Vernon photometric integrator recorder.

Preparative ultracentrifugation. CsCl, Hg²⁺- and Ag⁺-Cs₂SO₄ density gradient equilibrium centrifugation was done as described earlier (Ojha & Turian, 1977). CsCl-actinomycin D gradient centrifugation was done according to Peacock *et al.* (1973). The actinomycin D complexed to DNA fractions was removed by extracting the DNA/CsCl/actinomycin D solution with propan-2-ol. This procedure was repeated twice. The Hg²⁺ or Ag⁺ ions complexed to DNA fractions were removed with 0.001 M-EDTA as described elsewhere (Ojha & Turian, 1977).

Individual fractions were denatured, neutralized, fixed on Millipore filters and hybridized with ³H-labelled rRNA from an aquatic fungus, *Allomyces arbuscula* (Ojha & Turian, 1977).

Thermal denaturation. The procedure followed has been described previously (Ojha, 1978). The denaturation profile was analysed by the differential melting curve and the probability plot method of Knittel *et al.* (1968).

Reassociation kinetics. The fragmentation of the DNA samples, evaluation of the fragment length, denaturation and the procedure of reassociation have been described elsewhere (Ojha *et al.*, 1977).

A minimum of 1 unit of absorbance of the fragmented DNA was sealed in the reaction vials and incubated at 60 °C. After incubation to the desired *C₀t* value (*C₀t* is the concentration of nucleotide in mol s⁻¹ l⁻¹; Britten & Kohne, 1968), further reassociation was terminated by quickly plunging the vials into ice. The reassociated DNA was differentiated on hydroxyapatite columns as described elsewhere (Dutta & Ojha, 1972).

Isolation of ³H-labelled rRNA from K. marxianus. YPG medium (500 ml) containing 500 µCi (18.5 MBq) [³H]uridine was inoculated with 7 d-old cells grown on grape juice slants and incubated with forced aeration at 25 °C. After 24 h growth, 100 times the quantity of unlabelled uridine was added and the culture was allowed to grow for another 5 h. The ribosomes and rRNA were isolated according to Rubin (1975). The rRNA subunits (25+18S) were purified by polyacrylamide-agarose (acrylamide, 2.4 %, w/v; agarose, 0.5 %, w/v) electrophoresis and rRNA species were recovered as described by Rubin (1975).

RESULTS

Satellite DNAs

The presence of satellite DNAs was investigated by analytical equilibrium centrifugation in CsCl, Hg²⁺- or Ag⁺-Cs₂SO₄ and by analysis of the melting curve by the differential or probability plot method of Knittel *et al.* (1968). The equilibrium density gradient centrifugations did not reveal the presence of distinct satellites. However, the probability plot of the denaturation data showed the presence of two populations of molecules differing in their G+C values. The major and minor components had 43 and 23 mol % G+C, respectively.

Frequency of ribosomal RNA genes

Saturation hybridization. The number of ribosomal RNA gene copies in the *K. marxianus* genome was estimated by hybridizing denatured DNA immobilized on nitrocellulose filters

Fig. 1. Saturation hybridization of *K. marxianus*, *S. cerevisiae* and *N. crassa* DNA with ³H-labelled rRNA (25+18S) from *K. marxianus*. (a) Respective DNAs were fixed on nitrocellulose filters and hybridized with increasing concentrations of rRNA (sp. act. 7000 c.p.m. μg⁻¹) under the conditions described in Methods. (b) Double reciprocal plots of the saturation data. ●, *K. marxianus*; ■, *S. cerevisiae*; ▲, *N. crassa*.

Fig. 2. Reassociation kinetics of *K. marxianus* DNA. DNA was sheared (average fragment length 1000 base pairs), denatured and reassociated as indicated in Methods. DNA concentration, phosphate buffer molarities and temperatures of incubation were as follows: from C₀t 10⁻³ to 10⁰ – 40 μg ml⁻¹, 0.12 M and 60 °C; from C₀t 10⁰ to 10² – 850 μg ml⁻¹, 0.14 M and 60 °C; from C₀t 10² to 10³ – 1150 μg ml⁻¹, 0.48 M and 66 °C. The reassociated fractions were separated from the unreassociated DNA by hydroxyapatite chromatography as described by Dutta & Ojha (1972). ○, Experimental results; ●, theoretical curve of possible single copy sequences.

with increasing concentrations of combined ³H-labelled (25+18S) homologous rRNA (Fig. 1). The plateau value obtained revealed that 1.6% of the DNA was complementary to rRNA at saturation. The saturation value of 2.2% was obtained by the double reciprocal transformation (Bishop, 1972) of the above data and represents the percentage hybridization at infinite concentration of the rRNA. As a control, heterologous hybridization was done with *S. cerevisiae* and *Neurospora crassa* DNA; the saturation values obtained were 2.4 and 1%, respectively (Fig. 1).

Genome size. The genome size was determined from the rate of reassociation of the total cellular DNA. From the reassociation kinetics of total cellular DNA sheared to an average length of 1000 base pairs (Fig. 2), the following results were obtained. (1) Repetitive sequences represented a very small amount of the total DNA; approximately 10% of the DNA reassociated faster than expected from single copy sequences. (2) The slowly re-

Fig. 3. Banding profile of rRNA cistrons of *K. marxianus* in the preparative neutral CsCl equilibrium density gradient. Total cellular DNA (100 μg) was centrifuged to equilibrium at 95000 g for 60 h in an MSE 10 $\times$ 10 rotor. Individual fractions were collected and their A_{260} values were recorded. The DNA in each fraction was then denatured, neutralized, immobilized on nitrocellulose filters and hybridized with ^3H -labelled rRNA from *A. arbuscula* (sp. act. 8600 c.p.m. μg^{-1}) for 4 h in 6 $\times$ SSC buffer at 60 $^{\circ}\text{C}$ as indicated in Methods. $\circ$, A_{260} ; $\bullet$, ^3H hybridized.

Fig. 4. Banding profile of rRNA cistrons of *K. marxianus* in the preparative Hg^{2+} - Cs_2SO_4 equilibrium density gradient. The samples were prepared in 0.1 M-borate buffer pH 9.2. The Hg^{2+} to DNAp ratio was 0.1. The conditions of centrifugation, fractionation and hybridization were as indicated in Methods. $\circ$, A_{260} ; $\bullet$, ^3H hybridized.

associating component accounted for 90% of the total DNA and appeared to be kinetically homogeneous. (3) The $C_0t_{1/2}$ of the slowly reassociating component was 10, which is about 2.5 times that of *Escherichia coli* sheared to equivalent fragment length (Ojha *et al.*, 1977) and reassociated under comparable conditions (not shown in Fig. 2). Considering the genome size of *E. coli* to be 2.8×10^9 daltons the molecular mass of *K. marxianus* DNA was calculated to be approximately 6.3×10^9 daltons.

Separation of the rRNA cistrons

The total cellular DNA was fractionated on a preparative neutral CsCl gradient and the individual fractions were hybridized with ^3H -labelled rRNA from *Allomyces arbuscula*. The results indicated that most of the cistrons coding for rRNA banded at a slightly lower density than the main peak DNA where very little hybridization occurred (Fig. 3).

The banding pattern of rRNA cistrons in CsCl-actinomycin D and Ag^+ - Cs_2SO_4 gradients in cacodylate buffer pH 7.0 corresponded to the main peak DNA.

When bulk DNA was complexed with Hg^{2+} in borate buffer pH 9.2 in Cs_2SO_4 , centrifuged to equilibrium and individual fractions hybridized with ^3H -labelled rRNA, the cistrons were found to band in the heavier regions of the main band (Fig. 4). However, the cistrons did not resolve into distinct satellites.

DISCUSSION

In *S. cerevisiae* and *S. carlsbergensis*, cistrons coding for rRNA appear as distinct satellites – the Y satellites – in analytical equilibrium density gradient centrifugations in CsCl, Hg^{2+} - or Ag^+ - Cs_2SO_4 (Corneo *et al.*, 1966; Cramer *et al.*, 1972; De Kloet, 1970; Karijord & Øyen, 1975). In contrast, we have not found any satellite with *K. marxianus* DNA under similar conditions of centrifugation.

Saturation hybridization experiments demonstrated that 2.2% of the DNA codes for 25+18S rRNA in *K. marxianus*. This represents 1.4×10^8 daltons of coding sequences considering the genome size of *K. marxianus* to be 6.3×10^9 daltons (see below). The combined molecular mass of 25+18S rRNA in yeast has been shown to be 2×10^6 daltons (Udem & Warner, 1972). Therefore, 1.4×10^8 daltons of DNA should represent approximately 70 cistrons. This number is half of the 140 cistrons reported for *S. cerevisiae* (Schweizer *et al.*, 1969). The control saturation hybridization values obtained with heterologous DNA from *S. cerevisiae* (2.4%) and *Neurospora crassa* (1%) correspond to the published results (De Kloet, 1970; Chattopadhyay *et al.*, 1972) and attest to the validity of our method. The difference in chromosome number between *S. cerevisiae* (17) and *K. marxianus* (9) should not account for this difference, since *Neurospora crassa* with 7 chromosomes has 100 cistrons per nucleus (Chattopadhyay *et al.*, 1972). Furthermore, an increase in the level of ploidy does not lead to any increase in the number of cistrons per nucleus in *Physarum polycephalum* (Hall *et al.*, 1975).

The kinetics of reassociation indicated that repetitive sequences represent about 10% of the total DNA and that the $C_0t_{1/2}$ of the possible single copy sequence is 10. This is about 2.5 times that of *E. coli* measured under comparable conditions. Physical measurements of *E. coli* have given a genome size of 2.8×10^9 daltons (Cairns, 1963), and so we calculate the genome size of *K. marxianus* to be approximately 6.3×10^9 daltons, assuming that the non-repeated portion represents approximately 90% of the total genome ($2.8 \times 10^9 \times 2.5 \times 0.9$). This is in close agreement with the value published by Christiansen *et al.* (1971) for *K. lactis*.

CsCl gradient centrifugation results have shown that rDNA in *K. marxianus* is lighter than the main peak DNA, in contrast to *S. cerevisiae* where it is associated with a heavy satellite DNA (Schweizer *et al.*, 1969). However, we found no evidence of a light satellite peak with *K. marxianus*. The specificity of actinomycin D binding to G+C regions of the DNA has been used to separate the rRNA cistrons in higher organisms (Peacock *et al.*, 1973). In our experiments with *K. marxianus* DNA the rRNA cistrons did not react differentially with actinomycin D and so did not appear as a satellite. However, separation was achieved with Hg^{2+} - Cs_2SO_4 gradients where rRNA cistrons appeared in the heavier side of the gradient. The actinomycin D and Hg^{2+} binding experiments suggest that the rRNA cistrons in *K. marxianus* are A+T rich as in *S. cerevisiae* and *A. arbuscula* (Ojha & Turian, 1977).

The authors are grateful to Professor G. Turian, Department of Plant Biology, University of Geneva, for laboratory facilities and constant encouragement, Dr J. P. Tessier, Department of Medical Biophysics, University of Claude Bernard, Lyon, for analytical centri-

fugation, Miss A. Cattaneo for technical assistance and Mrs B. Duchoud for secretarial help.

REFERENCES

- BICKNELL, J. N. & DOUGLAS, H. E. (1970). Nucleic acid homologies among *Saccharomyces*. *Journal of Bacteriology* **101**, 505–512.
- BISHOP, J. O. (1972). DNA-RNA hybridization. In *Karolinska Symposia on Research Methods in Reproductive Endocrinology. 5th Symposium: Gene Transcription in Reproductive Tissue*, pp. 247–276. Edited by E. Diczfaluzy. Stockholm: Karolinska Institutet.
- BRITTEN, R. J. & KOHNE, D. E. (1968). Repeated sequences in DNA. *Science* **161**, 529–540.
- CAIRNS, J. (1963). The chromosome of *E. coli*. *Cold Spring Harbor Symposia on Quantitative Biology* **28**, 43–46.
- CAMPBELL, I. (1972). Numerical analysis of the genera *Saccharomyces* and *Kluyveromyces*. *Journal of General Microbiology* **73**, 279–301.
- CHATTOPADHYAY, S. K., KOHNE, D. E. & DUTTA, S. K. (1972). Ribosomal RNA genes of *Neurospora crassa*: isolation and characterization. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 3256–3259.
- CHRISTIANSEN, C., LEITH BAK, A., STENDRUP, A. & CHRISTIANSEN, G. (1971). Repetitive DNA in yeasts. *Nature New Biology* **231**, 176–177.
- CORNEO, G., MOOR, C., SANADI, L., GROSSMAN, I. & MARMUR, J. (1966). Mitochondrial DNA in yeast and some mammalian species. *Science* **151**, 687–689.
- CRAMER, J. H., BHARGAVA, M. M. & HALVORSON, H. O. (1972). Isolation and characterization of rDNA of *Saccharomyces cerevisiae*. *Journal of Molecular Biology* **71**, 11–20.
- CRYER, D. R., GOLDTHWAITE, C. D., ZINKER, S., LAM, K. B., STORM, E., HIRSCHBERG, R., BLAMIRE, J., FINKELSTEIN, D. B. & MARMUR, J. (1973). Studies on nuclear and mitochondrial DNA of *Saccharomyces cerevisiae*. *Cold Spring Harbor Symposia on Quantitative Biology* **38**, 17–30.
- DE KLOET, S. R. (1970). The formation of RNA in yeast: hybridization of high molecular weight RNA species to yeast DNA. *Archives of Biochemistry and Biophysics* **136**, 402–412.
- DUTTA, S. K. & OJHA, M. (1972). Relatedness between major taxonomic groups of fungi based on the measurements of DNA nucleotide sequence homology. *Molecular and General Genetics* **114**, 232–240.
- FINKELSTEIN, D. B., BLAMIRE, J. & MARMUR, J. (1972). Location of ribosomal RNA cistrons in yeast. *Nature New Biology* **240**, 279–281.
- GALEOTTI, C. L. & WILLIAMS, K. L. (1978). Giemsa staining of mitotic chromosomes in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. *Journal of General Microbiology* **104**, 337–341.
- HALL, L., TURNOCK, G. & COX, B. J. (1975). Ribosomal RNA genes in the amoebal and plasmodial forms of the slime mould *Physarum polycephalum*. *European Journal of Biochemistry* **51**, 459–465.
- KABACK, D. B., BHARGAVA, M. M. & HALVORSON, H. O. (1973). Location and arrangement of genes coding for ribosomal RNA in *Saccharomyces cerevisiae*. *Journal of Molecular Biology* **79**, 735–739.
- KARLJORD, Ø. & ØYEN, T. B. (1975). Study of haploid yeast with an unusually high rDNA content. II. Fractionation of the DNA in preparative Ag⁺-Cs₂SO₄ density gradient. *Biochimica et biophysica acta* **383**, 255–265.
- KNITTEL, M. D., BLACK, C. H., SANDINE, W. A. & FRASER, D. K. (1968). Use of normal probability paper in determining thermal melting values of deoxyribonucleic acid. *Canadian Journal of Microbiology* **14**, 239–245.
- OJHA, M. (1978). Characterization of spinach genome. *Cytobiology* **16**, 185–198.
- OJHA, M. & TURIAN, G. (1977). Ribosomal RNA cistrons in *Allomyces arbuscula*. *Biochimica et biophysica acta* **478**, 392–406.
- OJHA, M., TURLER, H. & TURIAN, G. (1977). Characterization of *Allomyces* genome. *Biochimica et biophysica acta* **478**, 377–391.
- ØYEN, T. B. (1973). Chromosome I as a possible site for some rRNA cistrons in *Saccharomyces cerevisiae*. *FEBS Letters* **30**, 53–56.
- PEACOCK, W. J., BRUTLAG, D., GOLDRING, E., APPELS, R., HINTON, C. W. & LINDSLEY, D. L. (1973). The organization of highly repeated sequences in *Drosophila melanogaster* chromosome. *Cold Spring Harbor Symposia on Quantitative Biology* **38**, 405–476.
- RUBIN, G. M. (1975). Preparation of RNA and ribosomes from yeast. *Methods in Cell Biology* **13**, 45–64.
- SCHWEIZER, E., MACKECHNIE, E. & HALVORSON, H. O. (1969). The redundancy of ribosomal and transfer RNA genes in *Saccharomyces cerevisiae*. *Journal of Molecular Biology* **40**, 261–277.
- TINGLE, M., HERMAN, A. & HALVORSON, H. O. (1968). Characterization and mapping of histidine genes in *Saccharomyces lactis*. *Genetics* **58**, 361–371.
- UDEM, S. A. & WARNER, J. R. (1972). Ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *Journal of Molecular Biology* **65**, 227–242.