Genetic organization of the ribosomal transcription units of the yeast *Saccharomyces carlsbergensis*

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ABSTRACT

The genetic organization of the multiple ribosomal transcription units (RTUs) on the genome of the yeast *Saccharomyces carlsbergensis* was studied by electron microscopy of purified ribosomal DNA hybridized to 26S rRNA using the R-loop technique (Thomas, M., White, R.L. and Davis, R.W. (1973) Proc. Natl. Acad. Sci. U.S. 73, 2294-2298). Plasmid pBR 322, the molecular weight of which is known, was used as a standard for converting contour length of double-stranded DNA into molecular weight.

The 140 yeast RTUs were found to be arrayed in tandem repeats, each repeat containing at most 0.4×10^6 D (about 6% of the length of the RTU) of non-transcribed spacer DNA. The repeats, in turn, are arranged in a number of clusters separated by much longer stretches of non-ribosomal DNA.

INTRODUCTION

A previous study on the denaturation and renaturation of purified ribosomal DNA (rDNA) led us to conclude that the ribosomal transcription units (RTUs) in *Saccharomyces carlsbergensis* are separated by stretches of non-transcribed spacer DNA varying in both length and sequence (1). Indeed, digestion of rDNA with various restriction enzymes produced a set of distinct fragments, adding up to the length of the RTU, superimposed upon a continuous background of DNA fragments. No partial digestion products exceeding the length of the RTU could be positively identified (2, 3). Moreover, the amount of heterogeneous DNA agreed well with the percentage of spacer DNA calculated from the melting studies (1).

We have re-examined the genetic organization of the yeast RTUs by applying the R-loop technique (4) to purified rDNA. Ribosomal 26S RNA was hybridized to rDNA displacing one of the DNA strands of the 26S RNA genes. The resulting structure (R-loop) can be seen by electron microscopy. Provided DNA of a sufficiently high molecular weight is used the degree of linkage between the RTUs can then be determined from the spacing of the R-loops. The results provide clear evidence for an arrangement of the yeast RTUs in

multiple clusters made up of tandem repeats which contain very little if any non-transcribed spacer DNA.

MATERIALS AND METHODS

Isolation of rDNA

High-molecular-weight yeast nuclear DNA from Saccharomyces carlsbergensis strain S74 was isolated as previously described (5). Ribosomal DNA was separated from the rest of the nuclear DNA by preparative $\text{Hg}^{2+}/\text{Cs}_2\text{SO}_4$ density gradient centrifugation (5).

Isolation of plasmid DNA

DNA from plasmid pBR 322 was isolated as described by Goebel et al. (6).

Isolation of rRNA

Unlabeled 26S rRNA was prepared from yeast ribosomes and purified by sucrose gradient centrifugation (7).

R-loop formation and specimen preparation

R-loops were formed by incubating 26S rRNA with purified rDNA in a ratio of 3:1 (w/w) in a solution containing 70% deionised formamide (8), 0.1 M Pipes (piperazine-N,N'-bis-2-ethanesulfonic acid, pH 7.8), 10 mM EDTA, 17 mM NaCl, 1.7 mM Tris-HCl (4). After 15 h incubation at 45°C 50 μl of the mixture was cooled on ice and added to 10 μl of a 0.06% (w/w) solution of cytochrome C in 2.4 M ammonium acetate. The final mixture was spread on distilled water (redistilled twice over quartz).

The protein-nucleic acid film was picked up on 400 mesh copper grids covered with a supporting film of carbon. Grids were dehydrated and stained by dipping into 0.05 M uranyl acetate, 0.05 mM hydrochloric acid in 90% (v/v) ethanol for 20 sec, followed by a brief rinse in isopentane. Finally they were rotary-shadowed with platinum at an angle of 5°.

Electron microscopy

The specimens were examined in a Philips EM 200 electron microscope. Photographs were taken on Kodak FRP 35 mm film at a magnification of 5000-9000 x. Magnification was calibrated with a grating replica (Ladd, 2160 lines/mm). Contour lengths were determined on prints with a Hewlett Packard 9864 A digitizer coupled to a 9820 A calculator at a magnification of 40,000-70,000 x.

Plate I. Electron micrographs of the various types of partial hybrids obtained after R-loop formation between rDNA and 26S rRNA (the bar represents 0.5 μm). For 1 C ammonium acetate was omitted from the mixture, while a supporting film of parlodion/carbon was used.

RESULTS AND DISCUSSION

The molecules observed in the electron microscope after R-loop formation between rDNA and 26S rRNA can be grouped in three classes.

Class I hybrids contain several R-loops spaced at regular intervals and have one double-stranded DNA tail the size of which exceeds the distance between two consecutive R-loops. The possibility that this long tail reflects incomplete R-loop formation can virtually be excluded. Hybridization was carried out under saturating conditions and in no instance was a partial hybrid observed in which an internal R-loop was missing from series of regularly spaced loops. Electron micrographs of class I hybrids can be seen in panels A and B of Plate I. Molecules having a similiary long DNA tail but containing only a single R-loop (not shown) were also assigned to this class.

Class II (Plate I, panel C) encompasses hybrids having one of more R-loops flanked on both sides by a short double-stranded DNA tail. The R-loops are spaced at the same distance as in class I hybrids as can be seen in the schematic representation of the partial hybrids in Fig. 1.

Finally class III (Plate I, panel D) consists of long double-stranded DNA fragments not containing any R-loops at all.

From these observations it follows that the majority of the yeast rDNA is arranged in a regularly spaced array. The amount of non-transcribed spacer DNA separating neighbouring RTUs can be determined by measuring the distance between the midpoints of two consecutive RTUs and comparing this distance to the size of the RTU determined by other means. Conversion of the measured

Fig. 1. Schematic representation of the partial hybrids depicted in Plate I. The lines represent double-stranded DNA, the blocks represent the R-loops. Measures are given in μm . The letters correspond to the panels in Plate I.

contour length into molecular weight, however, requires standardization of the length measured. For this purpose pBR 322 DNA with an estimated molecular weight of 2.60×10^6 D (9) was used. The contour length of the plasmid DNA (Table 1) when spread under the same conditions used for spreading of the partial hybrids was $1.37 \mu\text{m}$ with a standard deviation of $0.07 \mu\text{m}$ ($N=63$). Therefore, $1 \mu\text{m}$ of double-stranded DNA equals a molecular weight of $1.90 \pm 0.13 \times 10^6$ D (Table 1).

The length of an R-loop can, in principle, be converted into molecular weight of double-stranded DNA directly by considering the molecular weight of the 26S rRNA. This molecular weight is $1.19 \pm 0.03 \times 10^6$ D as determined by polyacrylamide gel electrophoresis under denaturing conditions (10). If we assume the distance between two adjacent bases in an RNA/DNA hybrid to be the same as in double-stranded DNA the length of an R-loop would be $1.25 \pm 0.14 \mu\text{m}$.

Fig. 2 shows a histogram of the length distribution of 60 R-loops measured. A mean length of $1.06 \pm 0.11 \mu\text{m}$ can be calculated from these data. A sizeable portion of the R-loops considered in Fig. 2, however, showed whiskers of unhybridized RNA, a phenomenon also described by White and Hogness (11). The calculated value will therefore be an underestimate of the length of an R-loop. When only those R-loops having no visible whiskers are taken into account (hatched part of the histogram in Fig. 2) a value of $1.20 \mu\text{m}$ with a standard deviation of $0.07 \mu\text{m}$ ($N=26$) is arrived at. This is within the standard deviation of the expected value. The assumption that, in terms of double-stranded DNA, the relation between contour length and molecular weight for an RNA/DNA hybrid is the same as for DNA/DNA hybrids thus appears to be valid.

Fig. 2. Length distribution of the R-loops observed in the electron microscope. The hatched part of the histogram was obtained by taking into account only those R-loops that showed no detectable whiskers of unhybridized RNA.

The distance b between the midpoints of 26 pairs of adjacent R-loops in class I and II hybrids was measured and the mean value calculated (Table 1). Although the occurrence of whiskers on either or both members of a pair of R-loops may influence the value of b for that particular pair, the absence of polarity in whisker formation (data not shown) ensures that there is no effect on the mean value of b . The value of b calculated in Table 1 corresponds to a molecular weight of double-stranded DNA of $6.23 \pm 0.23 \times 10^6$ D, which is very close to the molecular weight of the RTU determined from renaturation studies (6.2×10^6 D), digestion of rDNA with restriction enzymes (5.9×10^6 D), or the size of the primary transcript (6.2×10^6 D) (1-3, 12). Given the value of b (Table 1) and taking the molecular weight of the RTU as 6.1×10^6 D, two neighbouring RTUs can be separated by at most 0.4×10^6 D of non-transcribed spacer DNA which is equal to about 6% of the size of the RTU. The RTUs may however, well be linked head to tail. Retl and Planta (5) proposed, on the bases of sedimentation studies, that the individual RTUs were separated by stretches of non-transcribed spacer DNA having various lengths and sequences. This proposal clearly is no longer tenable.

The results described above provide clear evidence for an arrangement of, at least part of, the yeast RTUs in tandem repeats containing very little if any non-transcribed spacer DNA. In fact we could not detect in our preparations any partial hybrids containing a single R-loop bordered by two long double-stranded DNA tails suggesting that probably all RTUs are arranged in tandem repeats. The frequent occurrence of class I hybrids which have a single DNA tail longer than the mean length c ($2.18 \pm 0.15 \mu\text{m}$; $N=38$) of the double-stranded DNA separating two consecutive R-loops (Fig. 1) indicates that the tandem repeats are organized in a number of clusters.

Whether all clusters contain an equal number of tandem repeated RTUs or whether significant differences in size exist between individual clusters is a question which is more difficult to answer. In our experiments partial hybrids were observed containing up to six R-loops, the maximum number possible for the DNA preparation used, which originally had a molecular weight of about 40×10^6 D. This suggests that clusters of six or more tandem repeated RTUs may occur on the yeast genome. However, the number of these large clusters must be relatively small, since only a small number of DNA molecules containing 3 or 4 R-loops and with relatively short ($< c = 2.18 \mu\text{m}$) double-stranded DNA tails were found. In fact, at least 75% of these molecules with 3 or more R-loops appeared to have one double-stranded DNA tail exceeding length c . Therefore, it may be concluded that the majority of the tandem repeats are arranged in clusters, made up of not more than 4 to 5 RTUs.

TABLE 1. CONTOUR LENGTHS OF THE PARTIAL HYBRIDS AND pBR 322

	Length (μm)	S.D. (μm)	N	Calc. mol. weight $\times 10^{-6}$ D
pBR 322	1.37	0.07	63	2.60 [¶]
a [*]	1.20	0.07	26	2.28 ± 0.15
b	3.28	0.19	26	6.23 ± 0.23
c [¶]	2.18	0.15	38	4.14 ± 0.21

¶ This value was taken from ref. 9.

* For the calculation of the mean length of a, the hatched part of the histogram shown in Fig. 2 was used.

¶ This value was determined without taking into account the presence of possible whiskers on the R-loops.

In this respect the experiments published by Lusby and de Kloet (13) are relevant. These authors studied the sedimentation behaviour of DNA hybridizing to rRNA after shearing total *S. carlsbergensis* DNA to various molecular weights. They observed a separation of the hybridizable DNA into a heavy and a light peak of about equal size when the molecular weight of the sheared DNA had a value larger than 60×10^6 D (13). At a molecular weight of 30×10^6 D the DNA hybridizing to rRNA sedimented as a single heavy peak. The authors concluded that about half of the yeast RTUs are closely linked whereas the remaining 50% are more scattered. This conclusion is in line with the arrangement of the RTUs deduced above from the electron microscopic data.

The rDNA isolated by $\text{Hg}^{2+}/\text{Cs}_2\text{SO}_4$ density gradient centrifugation contains approximately 40% non-ribosomal DNA, which is (A+T)-rich (1). It seems plausible to assume that the long DNA fragments that do not contain any R-loops observed in the electron microscope (Plate I, panel D) are derived from this (A+T)-rich DNA although absolute proof for this assumption is not yet available. Most likely the (A+T)-rich fraction represents the DNA separating the various clusters of tandemly repeated RTUs. The coding properties of the (A+T)-rich fraction are largely unknown but the presence of about 30% of

the total number of yeast tRNA genes on this DNA has been established (5, J.H. Meyerink and H. van Keulen, unpublished results).

In summary, the genetic organization of the ribosomal transcription units of S. carlsbergensis shows an arrangement of the RTUs in multiple clusters made up of tandem repeats containing little or no non-transcribed spacer DNA. This organization is quite similar to that proposed by Cramer et al. (14) for the RTUs of S. cerevisiae on the basis of restriction analysis of purified rDNA. It is, however, quite different from the arrangement of the RTUs in higher eukaryotes such as Xenopus laevis (15) and Drosophila melanogaster (16) where individual RTUs are separated by long stretches of non-transcribed spacer DNA.

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