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Hybridizable sequences between cytoplasmic ribosomal RNAs and 3 micron circular DNAs of Saccharomyces cerevisiae and Torulopsis glabrata

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ABSTRACT

We have shown that 2.8 and 3.1 μm circular DNA molecules, previously reported to be present in Saccharomyces cerevisiae and Torulopsis glabrata respectively, contain sequences hybridizing to cytoplasmic ribosomal RNAs. In S. cerevisiae the 2.8 μm circular DNA appears to be identical to the rDNA repeating unit from nuclear DNA, both in length (approximately 9000 base pairs) and in the location of the 25, 18 and 5.8S rRNA sequences on the large HindIII fragment (6500 bp) and the presence of the 5S rRNA sequence on the small HindIII fragment. The 3.1 μm molecule from T. glabrata is approximately 2000 base pairs longer than the S. cerevisiae molecule and in addition, one of the HindIII sites lies within the region hybridizing to 25, 18 and 5.8S rRNAs. In S. cerevisiae the 4-5 copies of the 2.8 μm circular DNA molecules per cell, which have an extra-nuclear location, do not appear to be essential for cell viability as in one strain they were undetectable.

INTRODUCTION

There are a number of size classes of circular DNA molecules in a petite mutant of Saccharomyces cerevisiae (strain 5 ep^-) which lacks mitochondrial DNA (1). Average lengths of the circular molecules in the size classes were 1.9, 2.8, 3.8 and 5.8 μm and number frequencies in each peak were 74, 4, 16 and 6% respectively. We have also found in a 5 ep^- strain resistant to copper, that there were increased numbers of molecules in the 2.8 and 5.8 μm classes which correlated with an increase in a 1.704 g/cm^3 component in the buoyant density profile of the circular DNA (2). Since yeast nuclear ribosomal DNA (rDNA) is known to have a buoyant density of 1.704 g/cm^3 (3), we suggested that the 2.8 and 5.8 μm molecules may encode ribosomal RNA genes (2).

In a separate study of circular DNA in another yeast, Torulopsis glabrata, we again found a class of non-mitochondrial molecules which had a length of 3.1 μm and a buoyant density of 1.709 g/cm^3 (4). By analogy with the heavy satellite in S. cerevisiae it can be inferred that the 3.1 μm circular molecules in this yeast code for ribosomal RNAs.

Recently we have recommenced studying the 3 μm molecules because of their potential for mediating inter-species transfer of genes. In the present report we show that the 2.8 and 3.1 μm circular DNAs in S. cerevisiae and T. glabrata do indeed have sequences hybridizing to cytoplasmic ribosomal RNAs.

MATERIALS AND METHODS

S. cerevisiae strain D22 a ade2 was obtained from D. Wilkie, and D243 a/α adel/adel, p7/+, tryl/+, lys2/+ was obtained from F. Sherman. Other strains, 5 eρ$^-$, (ATCC 26500) (1) 410 eρ$^-$ and ure3$^+$ (5) have been previously described. The notation eρ$^-$ indicates that the petite mutants have been generated with ethidium bromide by the 'margin-of-growth' technique (6) and lack detectable mitochrondrial DNA (mt DNA). T. glabrata CBS 138 was the same as that used previously (4).

Culture conditions, labelling of cells with [^{3}H] adenine, isolation of circular DNA and examination of circular DNA in the electron microscope, have been described in detail (1, 6). Preparation of whole cell DNA and analytical ultracentrifugation have also been described (6, 7).

For fractionation of DNA, approximately 200 μg of whole cell DNA from a petite mutant of T. glabrata lacking mt DNA, was adjusted to a density of 1.69 g/cm^3 CsCl in a final volume of 20 ml and centrifuged for 96 hr. at 30,000 rpm in a Beckman 60 Ti rotor operated at 20°. Absorption of fractions was measured at 260 nm and the ability to hybridize to [^{3}H] rRNA was examined after alkali denaturation and binding of single stranded DNA to nitrocellulose filters (8).

Preparation of 25S, 18S and 5.8S rRNA (insoluble rRNA or irRNA) was essentially as described by Azad (9). Briefly RNA was extracted from a cell lysate obtained by breaking

S. cerevisiae cells with glass beads. irRNA was purified after phenol extraction by 3 precipitations from 2.5 M NaCl (0°, 20 hr.). This NaCl-fractionation procedure leaves 5S rRNA and tRNA in solution. The irRNA pellet was washed three times with 67% ethanol then three times with absolute ethanol and then dried under vacuum before being dissolved in water.

5S rRNA was prepared from the large ribosomal subunit, as previously described (10). In brief, the S. cerevisiae ribosomes were dissociated in the presence of 0.5 M KCl and the subunits separated on a 10-30% linear sucrose gradient containing 0.5 M KCl. RNA was extracted by phenol treatment of the large subunits and precipitated by 2.5 M NaCl at 0°, 20 hr. This results in the precipitation of the 5.8S and 25S rRNA complex leaving the 5S rRNA in solution. 5S rRNA was recovered from the supernatant by ethanol precipitation, dissolved in a small volume of water and freed of salt by dialysis against water.

Labelling of irRNA with [^{32}P] was by reaction with γ[^{32}P]ATP (Amersham <2000 Ci/mmole) catalysed by polynucleotide kinase (15). 5S rRNA was labelled at the 3' end with T4 RNA ligase (P-L Biochemicals) and [5'-^{32}P]pCp from Amersham (>1500 Ci/mmole) as described by Bruce and Uhlenbeck (11).

Isolation of the rDNA repeating unit from nuclear DNA was by Sma 1 digestion, which results in the liberation of monomer units (12). Whole cell DNA from S. cerevisiae was digested with Sma 1 in 20 mM KCl, 6 mM Tris/HCl pH 8.0, 6 mM $MgCl_2$, 6 mM β-mercaptoethanol and 100 μg/ml bovine serum albumin for 2 hr. at 37°. After each digestion the DNA was electrophoresed in a 1% agarose slab gel and the band hybridizing to irRNA was excised and the DNA recovered from the gel by electro-elution for 36 hrs. into a dialysis sac suspended in tris-borate buffer pH 8.3 (25 mM Tris, 25 mM Na borate, 5 mM EDTA).

Restriction endonucleases Sma 1, HindIII and EcoR1 were obtained from New England Biolabs (Beverly) and digests were performed in buffers described by the supplier. Gel electrophoresis was performed in 0.7-1.0% agarose in a flat bed apparatus and the sizes of the DNA fragments were determined

from a graph calibrated by inclusion of a mixture of pBR322 fragments (13). This mixture was generated by separate digestion of pBR322 with S1 nuclease (Sigma) (which yields monomers and dimers) PstI/BamI, PvuII/EcoRI and AluI. Lengths of HindIIIλ DNA fragments were determined against the pBR322 standards.

Transfer of DNA fragments to nitrocellulose was by gel blotting (14). Hybridization of the filters to the labelled rRNAs (approximately 2-5 x 10^6 cpm [^{32}P] rRNA/filter) was performed in sealed plastic bags in 3 x SSC at 65° for 21 hrs. [for details see (15)]. After incubation, filters were given three 1 hr. washes in SSC at 37° prior to autoradiography at -80° with an intensifying screen, using X-Omat (Kodak) X-ray film. Rehybridization of filters with 5S rRNA was performed as above after heating the filter to 80° for 2 hrs.

End labelled HindIII fragments of λDNA and pBR322 plasmid DNA were gifts from J. Shine.

RESULTS

In S. cerevisiae about 8% of whole cell DNA consists of a satellite peak of buoyant density 1.704 g/cm^3 which hybridizes to cytoplasmic rRNA (3). With T. glabrata the satellite peak has a buoyant density of 1.709 g/cm^3 and comprises approximately 6% of the DNA (Fig. 1a). When [^{3}H] labelled rRNA was annealed to fractions from whole cell DNA centrifuged to equilibrium on a CsCl gradient, hybridization was found to occur on the heavy side of the main band (Fig. 1b), indicating that T. glabrata, like S. cerevisiae, has ribosomal RNA cistrons located in DNA with a higher buoyant density than main band DNA.

Circular DNA preparations from both yeasts were then examined for their ability to hybridize to labelled rRNA. When circular DNA from S. cerevisiae strain 5 eρ$^-$, containing approximately 8% of a 1.704 g/cm^3 density component (2) was electrophoresed in 0.7% agarose, the circular DNA was resolved into a number of bands (Fig. 2a). The major bands were due to supercoiled and relaxed monomers, and nicked circular dimers of 2 μm DNA. Minor components visible in the photo-

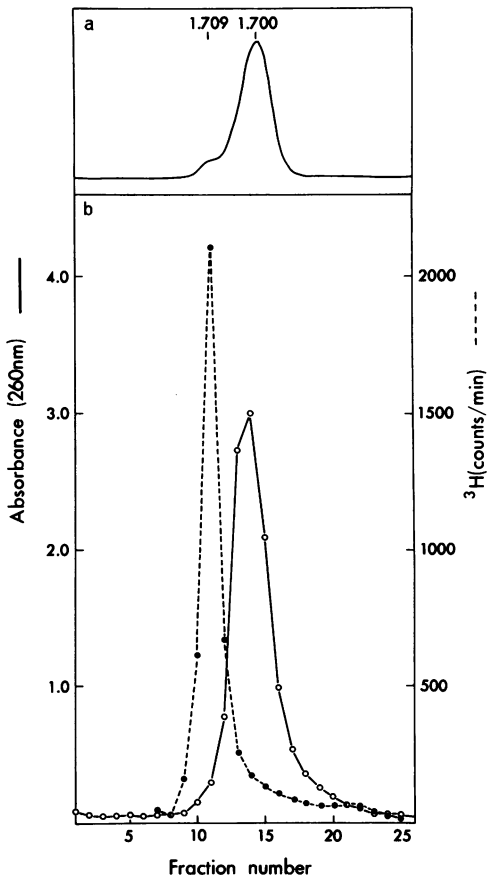

Fig. 1a Uviscan trace of a CsCl buoyant density gradient of whole cell DNA from a petite mutant of T. glabrata lacking mitochondrial DNA.

Fig. 1b Hybridization to [^{3}H] rRNA of fractions from a CsCl buoyant density gradient of T. glabrata whole cell DNA. In each case the left side of the figure represents the direction of centrifugation and the figures have been aligned so that the peaks coincide.

graph were nicked circular monomers and dimers of 2.8 μm DNA; these minor bands hybridized to [^{32}P] irRNA, whereas 2 μm DNA did not (Fig. 2b).

Upon digestion of circular DNA with Sma 1, the 2.8 μm nicked circular monomer and dimer forms were converted to a

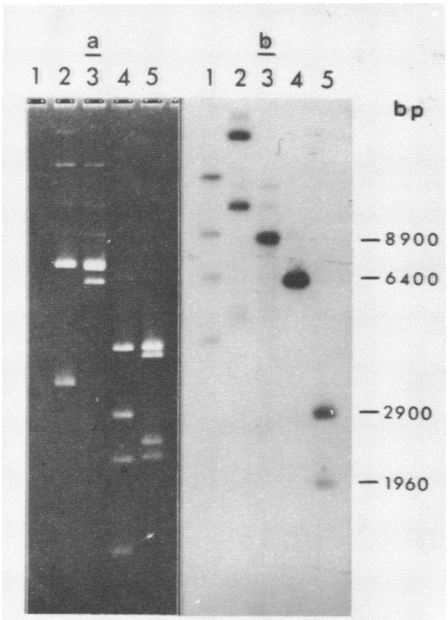

Fig. 2 Circular DNA from S. cerevisiae 5 $e\rho^-$ after gel electrophoresis (a) and autoradiography (b) following [^{32}P] irRNA hybridization of DNA bands transferred to a nitrocellulose filter. Channel numbers, which correspond in (a) and (b), represent 1, λDNA cleaved with HindIII and end labelled with [^{32}P], 2, circular DNA from S. cerevisiae 5 $e\rho^-$, 3-5, circular DNA after cleavage with Sma I, HindIII and EcoRl respectively. The sizes of the HindIII fragments of λDNA were determined to be 9100, 6430, 4270, 2250 and 1960 base pairs.

single molecular species of approximately 9000 bp which hybridized to the probe (Fig. 2b). Digestion with HindIII cleaved the 2.8 μm circles to yield a fragment of 6500 bp which hybridized to the [^{32}P] irRNA (Fig. 2b, Table I). Cleavage with EcoRl converted the 2.8 μm circular DNA to small products, only two of which hybridized to [^{32}P] irRNA. With 2 μm circular DNA, digestion with HindIII and EcoRl gave the expected 4 bands in each case (Fig. 2a). These bands, which do not hybridize to the probe, arise from the isomeric forms of this DNA (16-20).

When whole cell DNA from S. cerevisiae was cleaved with Sma 1 and the resulting digest electrophoresed in agarose, it

Table 1: Size of DNA fragments hybridizing to rRNA (base pairs)*

DNA	irRNA		5s RNA
	Sma 1	HindIII	HindIII
S. cerevisiae			
whole cell	9000	-	-
Sma 1 fragment	-	6500	1940
circular DNA	9000	6500	2600
T. glabrata			
circular DNA	8500	6400	-
	2400	4650	-

* Sizes were obtained from graphs of data from Figures 3 and 4 using pBR322 fragments for calibration (Fig. 4).

was observed that a [^{32}P] irRNA hybridizable component was released which had an identical mobility to the single band resulting from Sma 1 cleavage of 2.8 μm circular DNA (Fig. 3). Also present in the cellular DNA preparation was a hybridizable component with a slower electrophoretic mobility. In other experiments where Sma 1 digestion has gone to completion, this band is absent. In addition, a minor faster migrating band hybridized to the [^{32}P] irRNA.

Circular DNA from T. glabrata, consisting of a mixture of 3.1 μm circles and 6 μm circular mtDNA (4), was resolved into a number of components which possessed sufficient sequence homology to hybridize with the S. cerevisiae irRNA (Fig. 3b, channel 5). After digestion with Sma 1, the multiple bands were cleaved to yield two new hybridizable bands of 8500 and 2400 bp (Table 1). From this experiment it can be concluded that the mtDNA present in the sample did not hybridize to the probe because it lacks a Sma 1 recognition site (7).

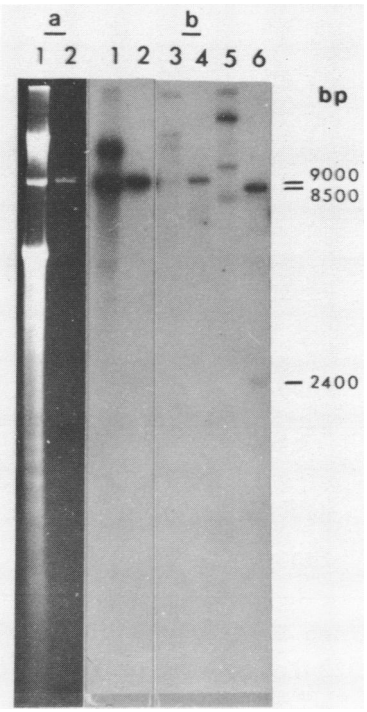

Fig. 3 Comparison of Sma 1 cleavage of S. cerevisiae nuclear DNA with circular DNAs from S. cerevisiae and T. glabrata by gel electrophoresis (a) and autoradiography (b) after [^{32}P] irRNA hybridization of DNA bands transferred to a nitrocellulose filter. Channel numbers, which correspond in (a) and (b) represent 1, nuclear DNA cleaved with Sma 1, 2, isolated Sma 1 fragment of nuclear DNA, 3 and 4, circular DNA from S. cerevisiae 5 $e\rho^-$ before and after Sma 1 digestion, 5 and 6, circular DNA from T. glabrata before and after Sma 1 digestion. The photograph of channels 3-6 has not been presented because DNA bands were too faint.

A comparison of the HindIII cleavage products of the isolated Sma 1 fragment from S. cerevisiae and the circular DNAs from S. cerevisiae and T. glabrata, is illustrated in Figure 4. Digestion of the Sma 1 linear DNA fragment liberated a [^{32}P] irRNA hybridizable fragment of 6500 bp which had an identical electrophoretic mobility to a HindIII fragment produced from the S. cerevisiae 2.8 μm circular DNA. In addition, HindIII digestion of the Sma 1 fragment produced a

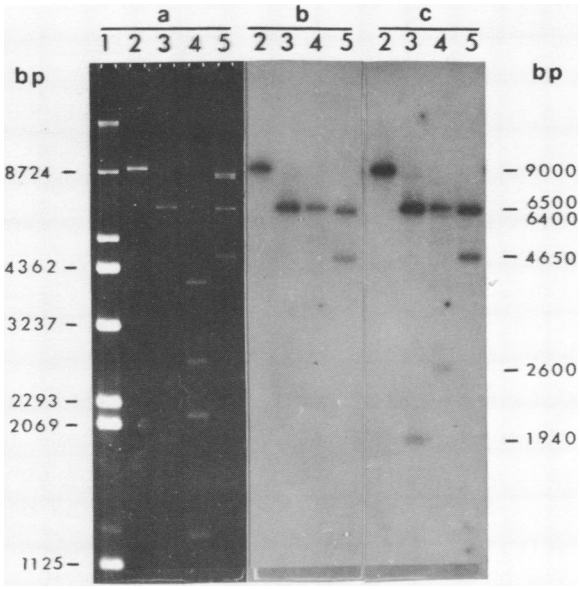

Fig. 4 Comparison of HindIII cleavage of the isolated Sma 1 fragment from S. cerevisiae nuclear DNA with HindIII digests of circular DNAs from S. cerevisiae and T. glabrata. The three panels of the figure correspond to (a) gel electrophoresis, (b) and (c) autoradiograms of DNA bands transferred to a nitrocellulose filter after hybridization with (b) [^{32}P] irRNA (c) rehybridization of the filter with [^{32}P] 5S RNA. Numbers represent 1, pBR322 fragments, 2 and 3, isolated Sma 1 fragment before and after cleavage with HindIII, 4, circular DNA from S. cerevisiae 5 eρ cleaved with HindIII and 5, circular DNA from T. glabrata cleaved with HindIII. Sizes of pBR322 fragments are on the left.

1940 bp band which only hybridised to 5s rRNA (Fig. 4c). On the other hand, cleavage of the 2.8 μm circular DNA with HindIII yielded a 2600 bp fragment which only hybridized to 5s RNA (Fig. 4c).

T. glabrata circular DNA digested with HindIII gave two fragments of 6400 and 4650 bp which hybridized to the [^{32}P] irRNA (Fig. 4b). No further hybridizable fragments were revealed by re-hybridization of the filter with [^{32}P] 5s RNA. The other 4 DNA bands visible in channel 5 (Fig. 4a) were produced by HindIII cleavage of mitochondrial DNA (7), these

bands did not hybridize to the rRNA probes.

The sizes of the circular DNAs from S. cerevisiae and T. glabrata obtained from the restriction endonuclease fragments were found to be approximately 9,000 and 11,000 bp respectively (Table 2). These values are in agreement with sizes calculated from the data obtained by electron microscopy where both molecules were measured in the presence of λDNA as an internal standard (1, 4).

Finally, to see if the quantity of 2.8 μm circles varied in relation to 2 μm DNA in other S. cerevisiae strains, we examined by electron microscopy the circular DNA size profiles from 4 additional cultures. Listed in Table 3 are the lengths and numbers of molecules in each size class in circular DNA preparations from the other S. cerevisiae strains. Three strains are similar to the 5 $e\rho^-$ used in the original study in possessing a small number of 2.8 μm circles. However, in D22 $e\rho^-$ no 2.8 μm circles were observed and only 1 molecule of 5.7 μm was present which is possibly a trimer of the 2 μm DNA.

Table 2. Comparison of circular DNA sizes from electron microscopy and restriction endonuclease digestion

		Electron microscopy			Restriction endonuclease digestion (Table 1)
	(†)	Length (μm)	MW x 10^6*	Base pairs (MW/660)	Base pairs
S. cerevisiae	(1)	2.85	5.8	8790	~9000
T. glabrata	(4)	3.08	6.65	10070	~11000

* λDNA MW 30.8 x 10^6 was used as the internal standard

† Reference

Table 3. Circular DNA profiles from different S. cerevisiae strains

l = length in m μ, n = number

Strain	Peak I	Peak II	Peak III	Peak IV
5 ep^-	l = 1.88 ± 0.12 n = 122	l = 2.82 ± 0.1 n = 7	l = 3.91 ± 0.17 n = 27	l = 5.82 ± 0.18 n = 9
410 ep^-	l = 1.96 ± 0.13 n = 114	l = 2.85 ± 0.26 n = 8	l = 3.98 ± 0.23 n = 12	l = 5.93 ± 0.29 n = 4
D243 ep^-	l = 1.91 ± 0.12 n = 88	l = 2.65 ± 0.12 n = 7	l = 3.89 ± 0.11 n = 7	l = 5.62 ± 0.19 n = 8
Ure3 ep^-	l = 2.02 ± 0.08 n = 155	l = 2.9 ± 0.1 n = 5	l = 4.12 ± 0.1 n = 19	l = 6.4 n = 1
D22 ep^-	l = 1.94 ± 0.01 n = 164	0	l = 3.83 ± 0.032 n = 14	l = 5.7 n = 1

DISCUSSION

The demonstration that 2.8 μm circular DNA molecules from S. cerevisiae hybridize to rRNA, confirms our previous suggestion that these molecules code for ribosomal RNA genes (2). Furthermore, the 2.8 μm circles appear to be equivalent to the rDNA repeating unit in nuclear DNA. Sma 1 digestion cleaved the 2.8 μm circles once to yield an approximately 9000 bp molecule which was identical in size to the Sma 1 released rDNA fragment from nuclear DNA. HindIII digestion liberated a 65000 bp segment from both the Sma 1 rDNA unit and the 2.8 μm circles. On the other hand, HindIII produced a 2600 bp fragment from the 2.8 μm circles which hybridized to 5S RNA, whereas only a 1940 bp hybridizable fragment resulted from the linear Sma 1 rDNA. The difference of 660 bp is due to the Sma 1 site in the 2600 bp HindIII fragment, this Sma 1 site remains uncleaved in the circular

DNA. These results agree with mapping studies which showed that the 25, 18 and 5.8S rRNA genes are confined to the 6500 bp HindIII fragment, whereas the 5S RNA is located in the small HindIII portion of the rDNA repeating unit (21, 22).

In T. glabrata we have shown that nuclear DNA of buoyant density 1.709 g/cm^3 codes for ribosomal rRNA and that the 3.1 μm circular DNA of this density does likewise. However 3.1 μm circular DNA from T. glabrata has some notable differences from its counterpart in S. cerevisiae. The 3.1 μm molecule is 2000 bp longer than the 2.8 μm molecule and contains an extra Sma 1 site. Moreover, unlike the 2.8 μm molecule, one of the HindIII sites lies within the segment coding for 25, 18 and 5.8S rRNAs.

Circular DNA coding for rRNA genes have been found in protozoa (23) insects (24-26) and amphibians (27-29) either in the nucleus or cytoplasm and, in some cases, they clearly represent gene amplification products. Although our results suggest that the 2.8 μm circles in S. cerevisiae are equivalent to the rDNA repeating unit in nuclear DNA, we would question their role in gene amplification. Firstly, we estimate from data in our earlier studies that there are only 4-5 copies of the 2.8 μm circles per cell (30). This hardly represents a significant amplification of the 100 or more rDNA units in the nuclear DNA (31, 32). However we consider it likely that the 2.8 μm circles have an extra-nuclear location. This belief stems from the fact that 2.8 μm circles are present in circular DNA prepared from cytoplasmically located particles free of nuclei (6). Additional support for an extra-nuclear location for 2.8 μm circles comes from our studies where we were able to exclude a nuclear location for 2 μm DNA (30). These data also rule out a nuclear location for the 2.8 μm circular DNA.

Our present results also suggest that 2.8 μm circular rDNA may not be essential for cell viability, as in one S. cerevisiae strain (D22 $e\rho^-$) we could not detect any 2.8 μm circles after measuring 164 of the 2 μm molecules. This observation raises a question concerning the automomy of the circular rDNA molecules. If they are continuously produced

from the nuclear rDNA then production must be blocked in D22 $e\rho^-$. Alternatively, 2.8 μm circles may replicate autonomously and only rarely arise from nuclear DNA, hence D22 $e\rho^-$ may represent a strain which has by chance lost this cytoplasmic element.

The question surrounding the propagation of the circular rDNA in S. cerevisiae also impinges on the phenomenon of the [psi] factor. If autonomous replication of the circular rDNA is the case then this would be compatible with the extrachromosomal inheritance of [psi] (33-36). It is conceivable that [psi], which affects aspects of translation, is a genetic manifestation of circular rDNA. In strains manifesting [psi], circular rDNA may differ from the nuclear rDNA repeating unit by encoding altered rRNA genes which in turn could cause altered translation by modification to some ribosomes.

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