Circular single-stranded RNA replicon in *Saccharomyces cerevisiae*

*(yeast 20S RNA/electron microscopy/SKI genes/acetate induction)*

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**ABSTRACT**

Circular RNA replicons have been reported in plants and, in one case, in animal cells. We describe such an element in yeast. In certain yeast strains, a 20S RNA species appears on transfer of cells to acetate medium. This phenotype shows cytoplasmic (non-Mendelian) inheritance and the 20S RNA is associated with 23-kDa protein subunits as a 32S particle. We demonstrate that yeast 20S RNA is an independent replicon with no homology to host genomic, mitochondrial, or 2-μm plasmid DNA or to the L-A, L-BC, or M1 double-stranded RNA viruses of yeast. The circularity of the 20S RNA is shown by the apparent absence of 3′ and 5′ ends, by two-dimensional gel electrophoresis, and by electron microscopy. Replication of yeast 20S RNA proceeds through an RNA→RNA pathway, and a 10,000-fold amplification occurs on shift to acetate medium. The copy number of 20S RNA is also reduced severalfold by the S13 gene products, a host antiviral system that also lowers the copy numbers of yeast double-stranded RNA viruses. Yeast 20S RNA and the hepatitis δ virus show some similarities.

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**MATERIALS AND METHODS**

**Strains and Media.** Rich medium was YPAD, containing 1% yeast extract, 2% dextrose, 2% peptone, and 0.04% adenine sulfate. Potassium acetate (1%) at pH 7.0 was used to induce 20S RNA synthesis (16). Among the strains used were RE458 (MATα L-A-o 20S′), AN33 (MATα argl thr L-A-E 20S′), 4304-5C (MATα leu2 NUC1::LEU2 his3 PEP4::His3 ura3 L-A-HN M1), 4304-6B (MATα sk12-2 leu2 NUC1::LEU2 his3 PEP4::His3 ura3 L-A-HN M1), 2404 (MATα his4 karl-i-1 L-A-HNB 20S′), and 2507 (MATα argl sk12-2 L-A-o 20S′).

**Preparation of Yeast 20S RNA.** Cells were grown to logarithmic phase in YPAD medium, shifted into 1% potassium acetate (16), and harvested, and protoplasts were prepared by incubation with zymolase (Kirin Brewery, Japan) at 0.5 mg/ml (17). Protoplasts were disrupted in 0.2% SDS/0.3 M NaCl/0.1 M EDTA/2 mM dithiothreitol with bentonite (Sigma) at 1 mg/ml. The sample was extracted four times with phenol/chloroform and nucleic acids were precipitated with 2 volumes of ethanol. The 20S RNA was enriched from this total RNA preparation (from 6 g cells) by isopycnic density centrifugation in CsTFA (1.6 g/ml; Pharmacia) at 55,000 rpm for 12 hr at 4°C in a VTi 65 rotor (Beckman). The harvested RNA was then ethanol-precipitated, dissolved in 10 mM Tris Cl, pH 8.0/1 mM EDTA (TE), extracted sequentially with phenol/chloroform and chloroform, and again precipitated. The final purification was by gel filtration through a 50-ml Bio-Gel A-50 column (Bio-Rad) with 10 mM Hepes/5 mM EDTA buffer at pH 7.0. Pooled fractions were extracted with phenol/chloroform, then chloroform, and again precipitated with ethanol.

**Two-Dimensional Polyacrylamide Gel Electrophoresis.** Total yeast RNA was first separated through a nondenaturing agarose gel, with mobility of the RNA species dependent on length and native conformation. The different RNA species were then separated in a second dimension of denaturing polyacrylamide, such that the mobility of the RNA was dependent on mass and denatured conformation (18–21).

**Electron Microscopy.** Approximately 0.5 μg of the gradient-purified yeast 20S RNA was mixed with 1.2 μg of T4 gene 32 protein (ref. 22; provided by L. Harris and J. Griffith, Linburger Cancer Center, University of North Carolina, Chapel Hill, NC) in 20 μl containing 25 mM Hanes (pH 7.0) and 1 mM EDTA. After 5 min at 4°C and 15 min at 37°C, 1.3 μl of 10% glutaraldehyde (Ted Pella, Tustin, CA) was added. Following 5 min of crosslinking at 4°C, the solution was heated to 65°C for 5 min, followed by the further addition of 1.2 μg of gene 32 protein, with incubation and glutaraldehyde crosslinking as above. A 0.82-ml Sephacryl S200 (Pharmacia) minicolumn was prepared in 1-ml acid-washed glass pipette and equilibrated with 10 mM Tris Cl, pH 8/50 mM NaCl/1 mM EDTA. The RNA sample was diluted to 50 μl with the same buffer and loaded onto the Sephacryl S200 minicolumn, 3To whom reprint requests should be addressed: Building 8, Room 207, National Institutes of Health, Bethesda, MD 20892.
and drops 14–16 were collected. A spreading solution was prepared containing 40 µl of the Sephacryl S200 eluate, 100 mM Tris Cl (pH 8), 10 mM EDTA, 50% formamide (BRL), and 0.5 µg of cytochrome c (Sigma) and was spread over a hypophase containing 10 mM Tris Cl (pH 8), 1 mM EDTA, and 20% formamide. After 1.5 min the surface was picked up with a parlodion-coated grid prepared according to Fishel and Warner (23). Grids were shadowed at 10⁻² torr (1 torr = 133.3 Pa) with 0.8 cm of 0.008-in (1 in = 2.54 cm) diameter Pt/Pd wire (80%;20%, Ted Pella, Tustin, CA) in an Edwards 306A vacuum evaporator. Coated grids were examined at 60 kV in a Zeiss EM10 electron microscope and molecules were photographed on Kodak 4489 sheet film at magnifications calibrated with a replica plate grid (Ladd Research Industries, Burlington, VT; 28,600 lines per in. Molecule lengths were determined using a ScanJet Plus (Hewlett-Packard). The actual length was calculated by measurement of the molecules relative to the calibrated negative length divided by the magnification. Only molecules that appeared completely open were included in the contour-length calculations. High-magnification examination of the RNA/protein backbone revealed a smooth structure with no obvious abnormalities.

Isolation of cDNA Clones of Yeast 20S RNA. For isolation of clones of 20S RNA, the cDNA was generated by random priming (24) of gradient-purified 20S RNA. After 5 µg of RNA was treated with 10 mM methylmercuric hydroxide (25), the RNA was annealed with random primer (1:100 molar ratio) in 50 mM Tris Cl, pH 8.3/50 mM KCl in 25 µl at 42°C for 10 min. The components of the 250-µl first-strand reaction mixture included 50 mM Tris Cl (pH 8.3), 50 mM KCl, 4 mM dithiothreitol, 8 mM MgCl₂, 0.8 mM dNTPs, 25 ng of actinomycin D, and 30 units of X-L-grade avian myeloblastosis virus reverse transcriptase (Life Sciences, Saint Petersburg, FL) per µg of RNA. The reaction mixture was incubated at 42°C for 60 min. After the addition of 1 volume of TE, the solution was extracted with phenol/chloroform and residual primer was removed by Sephadex G-50 spin dialysis. Following ethanol precipitation, second-strand synthesis was performed (26). Size selection of cDNAs was performed by either Bio-Gel A-50 filtration or gel electrophoresis. The cDNAs were blunt-end-ligated into Bluescript vectors (Stratagene). The selection for cDNA clones of 20S RNA involved a preliminary colony filter hybridization using 5'-end-labeled, partially alkali-digested 20S RNA as probe, then verification using individual nick-translated cDNA clones as probes for Northern blots of total RNAs from strains with and without 20S RNA. A 1700-base clone (p20-9) was used as probe in the experiment shown in Fig. 3C.

RESULTS

Yeast 20S RNA Is Circular. We purified 20S RNA and attempted to label 5' ends with T4 polynucleotide 5'-kinase or 3' ends with T4 RNA ligase. Although minor amounts of contaminating 18S rRNA in the preparation were labeled (an unintended internal control), we could not detect labeling of intact 20S RNA (data not shown), suggesting a circular structure or blocked ends. Two-dimensional gel electrophoresis has been used to separate single-stranded RNAs with circular, linear, or other unusual topology from linear molecules (18–26). Examination of the migration of 20S RNA in comparison with 18S and 28S rRNAs in the same extract and with marker linear RNAs showed that the mobility of 20S RNA was distinct from that of linear RNAs, again suggesting a circular topology (Fig. 1). The electrophoretic migration of 20S RNA on the vertical axis (second dimension) was significantly less than that of the 18S and 28S rRNAs or the linear RNA markers, which migrated as points along an arc, showing that 20S RNA is not linear.

Limited nicking of yeast 20S RNA with RNase T1 followed by electrophoresis under denaturing conditions as described by Chen et al. (27) again indicated that 20S RNA is circular (data not shown).

Fig. 1. Nonlinearity of yeast 20S RNA shown by two-dimensional gel electrophoresis. (A) The predicted mobility paths of linear and nonlinear RNAs (23–26). The first dimension was agarose without denaturant, the second dimension was polyacrylamide with denaturant. (B) Analysis of total nucleic acid from yeast strains with (B1, strain RE458:MATa L-a-20S⁺) and without (B2, strain AN33:MATa arg1 thr1 L-a-20S⁻) 20S RNA. Analysis of RNA from the strains above supplemented with RNA markers of 9.49, 7.46, 4.4, 2.37, 1.35, and 0.24 kilobases (BRL) is shown in B3 and B4. Total nucleic acids from cells in which 20S RNA had been induced by growth in acetate was extracted. The first dimension was a 1.6% agarose gel with 50 mM Tris acetate, pH 7.2/10 mM EDTA (TAE), 1.5 mm thick, run at 15 V/cm. Individual lanes were excised, and the RNAs were denatured in a solution containing 50% (vol/vol) formamide, 10% (vol/vol) formaldehyde, and TAE; applied to the surface of the second-dimension gel (4.5% acrylamide/8 M urea in TAE); and overlaid with 2% agarose containing the formamide/formaldehyde buffer. After electrophoresis at 7.5 mA for 20 hr, the gel was stained with ethidium bromide (0.5 µg/ml) and photographed.

Fig. 2. Circularity of yeast 20S RNA shown by electron microscopy. A and B are different magnifications of the same material. The 20S RNA was refractory to normal published procedures for spreading single-stranded nucleic acid, presumably as a result of its secondary structure, thus requiring the modifications using the T4 gene 32 protein described in Materials and Methods.

The circularity of yeast 20S RNA was confirmed by electron microscopy (Fig. 2). We found that 20S RNA could not be adequately visualized in the electron microscope under conditions in which single-stranded nucleic acids are normally prepared (in this case, 90% formamide at 65°C; ref. 28). These results suggested that the 20S RNA has very tight secondary structure. Purified 20S RNA was then spread after reaction and crosslinking with T4 gene 32 protein and glutaraldehyde. A 1.5-fold increase in contour length occurs when single-stranded DNA is spread in the presence of T4 gene 32 protein (18). The average contour length of 130 molecules was 4635 ± 386; thus, the calculated size of 20S RNA is 3090 ± 257 bases. Approximately 35% of the molecules were either linear or uninterpretable and another 15% appeared incompletely opened by the spreading technique. However, since most of the molecules appeared circular, we conclude that the yeast 20S RNA is a single-stranded circular RNA.

The comigration of proteinase K-treated 20S RNA with an untreated control in a gel assay capable of distinguishing linear from circular RNA rules out the possibility of a protein-RNA linkage (data not shown). The inability to label the ends and the limited nicking experiment argue against a lariat or a panhandle structure. We conclude that the 20S RNA is a covalently closed circle, but it is impossible to completely rule out an unusual linkage of 3' and 5' ends that might block labeling.

Yeast 20S RNA Is an Autonomous Replicon. We isolated clones of 20S RNA by random priming (see legend of Fig. 3). Southern hybridization analysis using conditions sufficient to detect less than 0.01 copy per cell did not show DNA homologs of 20S RNA (Fig. 3). In particular, this proves that

20S RNA is not a form of rRNA as was previously suggested. The 20S RNA is thus not coded by nuclear, mitochondrial, or 2-μm DNA, and 20S RNA replication does not involve a DNA intermediate. No homology to the L-A or M1 dsRNA viruses of yeast was detected by Northern hybridization (data not shown), precluding the possibility that 20S RNA is a subgenomic fragment of these RNA replicons.

The predominant 20S RNA viral strand and the antiviral strand, present in lower copy number, were detected by Northern hybridization at high stringency using single-stranded probes (Fig. 4 A and B), indicating an RNA-RNA pathway for replication. The absence of 20S RNA in certain strains unable to induce its synthesis (15) was demonstrated by Northern hybridization (Fig. 4 C and D). The 20S RNA is thus an autonomous RNA replicon that is nonessential to the host.

Dual Control of Yeast 20S RNA Replication. Examination of replication control by Northern hybridization (Fig. 4 C and D) showed that during vegetative growth in rich medium, the copy number of 20S viral strand was maintained at 5–20 copies per cell. Following a shift to 1% potassium acetate medium (18 to 32 hrs), 10,000-fold amplification of the viral strand was observed, resulting in a copy number of 20,000–200,000 molecules per cell. Parallel amplification of the antiviral strand occurred, but with the copy number always 10-fold lower than that of the viral strand.

The products of the host SKI (super killer) genes of yeast, which lower the copy number of double-stranded RNA viruses (30), also lower the copy number of 20S RNA (ski2 and skib8 mutants were tested). In a cross heterozygous for ski2-2, high-copy number of 20S RNA and L-A double-stranded RNA cosegregated with ski- in the four tetrads examined (Fig. 4E and data not shown). Northern hybridization (Fig. 4 A and B) confirmed that the RNA species lowered in copy number by the SKI products was 20S RNA. The reduction of 20S RNA copy number mediated by the SKI

Fig. 3. Absence of 20S RNA sequences in cellular DNA. Total yeast DNA from strains AN33 (20S RNA -) and RE458 (20S RNA +) were analyzed by Southern blot hybridization utilizing nick-translated probes of the rDNA repeat (pRIB, an 11.8-kilobase clone containing the entire sequence for the 35S rRNA precursor, gift from J. R. Warner, Albert Einstein College of Medicine, Bronx, NY) in A, a single-copy gene (FR-2, 6.7-kilobase pyruvate kinase clone, gift from A. Hinnebusch, National Institutes of Health) in B, and a 20S RNA clone (p20-9, a 1.7-kilobase clone isolated as described in Materials and Methods) in C. DNA from strains AN33 and RE458 (6 μg) was subjected to electrophoresis without treatment (lanes 1 and 2), after digestion with Xba I (lanes 3 and 4), or after digestion with Xba I, HindIII, and Sst I (lanes 5 and 6). Southern transfer was to Nytran filters (Schleicher & Schuell) and prehybridization (6 hr) and hybridization (12 hr) were in 6× SSC/50% formamide/10× Denhardt's solution/1% SDS containing sonicated denatured calf thymus DNA at 100 μg/ml. The stringency wash was with 0.1× SSC/0.1% SDS at 65°C. Autoradiograms (24-hr exposure) are shown. (SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7; Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin.)
products is independent of the regulation of 20S RNA by metabolic conditions; ski− strains were derepressed by about the same factor as SKI+ strains on transfer to acetate (Fig. 4), and ski− strains had 3- to 5-fold more 20S RNA than SKI+ strains on acetate medium. Strains in which 20S RNA replication was doubly derepressed by both growth in acetate and by a ski− mutation had more 20 S RNA than 18S rRNA.

DISCUSSION

We show here that yeast 20S RNA is a circular RNA replicon. We present evidence against a protein linker, but the possibility of an unusual linkage between 3' and 5' ends, such as a 2'–5' linkage, has not been ruled out. We have also considered the possibility of a panhandle molecule, formed, as in the case of the linear single-stranded DNA adenovirus- associated viruses, as the result of an inverted terminal repeat too short to be visualized by electron microscopy (<50 bases). That both 3' and 5' ends are unavailable to labeling argues strongly against this possibility, as does the aberrant migration in the denaturing dimension on two-dimensional gel electrophoresis.

The 20S RNA sequences are completely absent (limit of detection, <0.01 copy per cell) in some strains, so 20S RNA is dispensable for the cell. The replication of 20S RNA is under dual control. The main control is by nutritional conditions, with a 10,000-fold derepression on transfer to acetate medium. The control by the chromosomal SKI genes is only 3- to 5-fold, but, because it is apparently independent of the nutritional control, makes it possible to produce cells in which the 20S RNA is even more abundant than 18S rRNA. The only essential function of SKI gene products is control of double-stranded RNA replication (31, 32). These genes may affect 20S copy number by an effect on a double-stranded RNA replication intermediate.

Garvik and Haber (15) showed that yeast 20S RNA could be introduced into strains apparently lacking it by introducing cytoplasm carrying the element. This was accomplished by both meiotic crosses (20S+ × 20S− → 4:0 segregation of 20S+) and by forming heterokaryons in a process called cytoduction. Our data show that the strains used by Garvik and Haber, such as AN33, actually lack yeast 20S RNA and are not simply unable to amplify its copy number. Taken together, these results show that 20S RNA can replicate in strains previously lacking it. Yeast 20S RNA is associated with protein subunits as a cytoplasmic particle (16), but whether this 23-kDa protein is encoded by 20S RNA is not known. These results suggest that the 20S RNA is an endogenous viral replicon whose replication is stringently regulated by the host. Other covalently closed circular RNA replicons include only the viroids, the viroid-like satellites, and the hepatitis delta virus (1–5). Yeast 20S RNA is similar to the hepatitis delta virus RNA in that both are larger (3100 and 1700 bases, respectively) than the viroids (about 350 bases), and both yeast 20S and hepatitis delta RNAs are also found associated with small proteins (23 kDa (16) and 24 kDa (10),...
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respectively]. Garvik and Haber (15) showed that 20S RNA was present in maki10 and maki3 strains, both of which are unable to maintain the L-A and M double-stranded RNA viruses (11), and our strains 2507 and RE458 lack both L-A and M but carry 20S RNA. Thus, 20S replication has no known dependence on these yeast viruses, although any possible dependence on L-BC double-stranded RNA or the retro elements Ty1 through Ty6 have not been tested. Similarly, hepatitis δ virus has been shown to replicate in cells in the absence of hepatitis B virus (9). The ease of genetic manipulation of S. cerevisiae should facilitate study of the replication of this element and its control.