A Novel Cloning Strategy Reveals the Gene for the Yeast Homologue to Escherichia coli Ribosomal Protein S12*

Lefa E. Alksne† and Jonathan R. Warner§
From the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

(Received for publication, November 16, 1992, and in revised form, February 5, 1993)

Using a novel technique designed to identify genes of Saccharomyces cerevisiae which carry introns, we have cloned two genes encoding ribosomal protein S28. Although the genes differ by 15 nucleotides within their coding regions, they are predicted to encode identical proteins of 145 amino acids. The predicted amino acid sequence of S28 contains significant homology to ribosomal protein S25 of Tetrahymena thermophila and to ribosomal protein S12 of several archaeabacteria, suggesting a relationship to S12 of Escherichia coli. Dot matrix analysis confirmed that regions of S12, especially those implicated in the accuracy of translation, have been conserved in S28 of S. cerevisiae. Either RPS28A or RPS28B alone can support growth, but heterozygous disruption of both genes abolishes the ability to sporulate. Haploids harboring a disruption of both genes cannot survive without an intact gene on a plasmid. RPS28A maps to the right arm of chromosome VII and RPS28B to the right arm of chromosome XVI.

The ribosome is an ancient organelle, remarkably conserved throughout evolution. We would like to make use of the wealth of information available about the Escherichia coli ribosome to learn about the structure and function of the eukaryotic ribosome. Although recent evidence has implicated the rRNA as having a direct role in peptide bond formation (1), many studies in E. coli have defined specific roles in translation for the ribosomal proteins, particularly in the maintenance of translational fidelity (2). The sophistication of the genetic and biochemical tools available in Saccharomyces cerevisiae will aid in our analysis of eukaryotic ribosomes.

Although cross-linking studies have begun to establish physical contacts among the ribosomal proteins and rRNA of S. cerevisiae (3), functional interactions between these molecules are virtually unexplored. More than half of the ribosomal protein genes of S. cerevisiae have been cloned, but it is imperative that more genes be cloned to establish homologies with the well characterized E. coli proteins. In an effort to do this, we devised a selective screen with which to clone ribosomal protein genes specifically. We took advantage of the fact that introns are rare in yeast genes, except for in the ribosomal protein genes (4). We purified intron RNA from a mutant strain in which intron lariat structures accumulate (5) and used it to probe a yeast genomic library. Several of the genes identified in this screen were shown to encode ribosomal proteins. Furthermore, one novel gene was shown to encode ribosomal protein S28 and to have significant homology to ribosomal protein S12 of E. coli, known to play an important role in the accuracy of translation.

To begin to understand the role of S28 in the yeast ribosome and its possible involvement in accuracy, we have characterized the protein and the two genes encoding it. A second gene encoding S28 was identified by genomic Southern analysis and was cloned from a λgt11 library. The two genes encode proteins of identical sequence, with nucleotide sequence divergence beginning almost immediately outside the coding region. S28 is essential for haploid viability; in a diploid, deletion of one copy of each of the genes apparently causes a specific inhibition of sporulation. Experiments are now under way to determine whether the function of this protein in translational accuracy has been conserved between prokaryotes and eukaryotes.

EXPERIMENTAL PROCEDURES

Strains, Media, Plasmids, and Libraries—Strain W303 (MAT a/α, or a, ade-2-1, leu-2-3, 112, his-3-11, 15, trp 1-1, ura 3-1, can 1-100) was a gift of Dr. R. Rothstein, Columbia University. The homozygous diploid strain L-1381 used in gene disruption (MAT a/α, met 8-1, leu-2-1, trp 1-1, lys 2-BB, his 3-52, ura 3-52) was constructed by and was a kind gift of Richard Anthony in the laboratory of Dr. S. Liebman at the University of Illinois at Chicago. tso45 (MAT a/α, ade-2-101, ura 3-52, his 30200, tyr1, pro26), obtained from Dr. John Abelson, accumulates intron lariat structures unconditionally (6). E. coli strain DH5 α (F, phi80d, lac zp M15, end A1, rec A1, sd R17 [rmt-5]) was used for the propagations of all plasmids. For propagation of a yeast genomic library, strain C600 (F, thi-1, leu26, lac Y1, ton A21, supE44) was used. DNA isolation was essentially as described by Sambrook et al. (6). Strain Y1090, (∆lac/lac168, proA, ∆[lnr], araD139, strA, supF (trp C22: Tn16, pMC9 (rk, mK9)) was used for λ maintenance and DNA preparation, and was a gift of Dr. M. Snyder.

pUC-based vectors used for sequencing and subcloning included pGEM-Blue, pSP72 (Promega), and pBluescript (Stratagene). Yeast shuttle vectors included YEp24 (7) and the pRS series (8). RPS28A was cloned from a yeast genomic library obtained from Dr. L. Neigeborn. The library contained genomic DNA partially digested with Sau3A1 and ligated into the BamHI site of YEP24. E. coli strain C600 colonies transformed with the library were transferred to nitrocellulose filters as described by Grunstein and Hogness (9) and probed with labeled lariat RNA, prepared as described below.

RPS28B was cloned from a λgt11 yeast genomic library received as a gift from Dr. M. Snyder. The library contained randomly sheared...
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genomic DNA from strain S28C ligated into λgt11 with EcoRI linkers.

Radioactive Labeling Methods—To create radioactively labeled DNA fragments 50-250 ng of the fragment of interest was nick translated with [α-32P]dCTP (specific activity 3,000 Ci/mmol, Du Pont-New England Nuclear) using DNA polymerase I Klenow fragment (10). 32P-Labeled RNA transcripts were generated from DNA fragments subcloned into PUC-based plasmids by in vitro transcription using T7, T3, or SP6 RNA polymerase primers (6) and [32P]UTP (800 Ci/mmol, Du Pont-New England Nuclear). Oligonucleotides were radioactively labeled at their 5' ends with 100 μCi of [γ-32P]ATP (specific activity 3,000 Ci/mmol, Amersham Corp.) and 10 units T4 polynucleotide kinase. Lariat RNA was also labeled in this manner after fragmentation for 10 min at 85 °C, pH 9.5 (11).

Manipulation and Analysis of DNA Fragments—Subcloning techniques were performed using protocols basically as described by Sambrook et al. (6). Plasmid or genomic DNA was digested with appropriate restriction enzymes and analyzed by Southern blot as originally described (12), using Nytran nylon membranes (Schleicher & Schuell). Hybridization and washing conditions varied according to the type of probe used.

Yeast Transformation and Genomic DNA Preparation—Transformation was performed as described by Ito et al. (13). A method described by Carlson and Botstein (14) was used to isolate total yeast DNA from stationary cells. Southern transfer was performed as described previously.

Yeast RNA Isolation and Northern Analysis—Total RNA was isolated from midexponentially growing cells in YPD or dropout medium by the lithium chloride/hot phenol method (15). Northern analysis followed the protocol of Pearson et al. (16). Lariat RNA was purified from total RNA of strain tsa45 as described under “Results.”

Two-dimensional Gel Analysis of in Vivo Labeled Total Yeast Protein—Exponentially growing cells in minimal medium were pulse labeled with 330 μCi/ml [3H]lysine (100 Ci/mmol, Amersham Corp.) for 1 min. Labeling was stopped by pouring the cells onto crushed ice. Total cell protein was extracted with glacial acetic acid by the method of Gorenstein and Warner (17). Pulse-labeled total protein was analyzed by a two-dimensional gel system specifically designed for ribosomal proteins (18).

Screening of a λgt11 Library for S28 Copy B—Differential hybridization of a λgt11 yeast genomic library was used to distinguish between RPS28A and RPS28B. Two probes, one of which could hybridize to both copies and one of which was RPS28A-specific, were applied to duplicate plaque lifts prepared as described by Vinson et al. (19) and Singh et al. (20). λ DNA was purified from large scale preparations by glycerol gradient centrifugation following a modified procedure of Sambrook et al. (6).

PCR to Determine Splice Site Junction—To confirm the predicted intron splice junctions in RPS28A and RPS28B, PCRs were performed using a cDNA template generated from polyadenylated yeast mRNA (21). The cDNA was prepared using avian reverse transcriptase and the downstream oligonucleotide.Opposing copy-specific oligonucleotides flanking the suspected splice site were used to amplify the spliced sequences. Resulting DNA fragments were purified, made blunt-ended with Klenow DNA polymerase, and ligated into pGEM-Blue vector digested with SmaI. The DNA inserts were sequenced using the upstream oligonucleotides.

RESULTS AND DISCUSSION

Purification of Lariat RNA—Only rarely do yeast genes have an intron; most of those that do are ribosomal protein genes (4). To isolate specifically genes that contain introns, we took advantage of a strain containing a mutant PRP26 gene, in which branched intron lariat structures accumulate (5). A scheme involving both centrifugal and chromatographic techniques to purify a heterogeneous mixture of these lariats was devised (Fig. 1, A–C), with the aim of using the preparation as a probe for intron containing genes in a S. cerevisiae library.

Total RNA was isolated from 1 liter of culture of strain tsa45 grown in YPD to midexponential phase. The RNA was loaded onto 10-25% w/w sucrose gradients (2.5 mg of RNA/10 ml) in 0.1 M sodium chloride, 10 mM Tris-Cl, pH 7.4, 10 mM lithium chloride, and 0.2% sodium dodecyl sulfate and centrifuged at 33,000 rpm in an SW 41 rotor for 13.5 h. This treatment separated the smaller lariat RNAs from the rRNA and most of the messenger RNAs. Fractions that were determined by Northern analysis (Fig. 1A) contained lariat RNA were pooled, extracted with phenol-chloroform-isooamyl alco-

1 G. Silver, personal communication.
2 The abbreviation used is: PCR, polymerase chain reaction.

FIG. 1. Purification of lariat RNA. Panel A, Northern analysis of total RNA from strain tsa45 separated by sucrose gradient centrifugation and probed with 32P-labeled RNA complementary to RPS10A RNA and intron RNA sequences. Each lane represents one fraction of the gradient. Panel B, nonpolyadenylated RNA separated by oligo(dT)-cellulose chromatography. Equal concentrations of nonpolyadenylated (lane 1) and polyadenylated (lane 2) RNA were analyzed by Northern blot and probed with 32P-labeled RNA complementary to RPS10A. 32P-labeled oligonucleotide complementary to tRNA, and another oligonucleotide complementary to 5S ribosomal RNA.

C

0.5

0.9 M NaCl

S10 (Lariat)

tRNA

5S
Among those that appeared in the screen were actin and the ribosomal protein genes RPL46, RPL30A, RPL30B, and RPS10B.

One novel gene was found to encode a yeast ribosomal protein. Cells carrying the gene on the multicopy vector YEp24 were labeled briefly with \(^{3}H\)lysine, and the total protein was isolated and electrophoresed in two-dimensional gels (Fig. 3). As is evident in the lower gel, there is overexpression of a protein spot corresponding to rp37 in the nomenclature of Warner and Gorenstein (18), now known as ribosomal protein S28 (22-24).

**RPS28 Is Encoded by Two Genes**—Many ribosomal proteins in *S. cerevisiae* are encoded by two genes. That this is true for S28 was shown by probing parallel Southern blots with a probe from the coding region (Fig. 4, right), expected to be common to the two genes, and with a probe from the intron (left), expected to have diverged. This analysis clearly demonstrated the existence of a second copy of the gene.

**Cloning of S28B—RPS28B** was cloned from a λgt11 bank (gift of Dr. M. Snyder) using differential hybridization with the probes described in Fig. 4. Six clones were identified which contained RPS28B. DNA from purified phage was

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**Fig. 2.** Representative Northern analysis of total RNA from W303a wild type and tsα45 strains, probed with a \(^{32}P\) nick-translated fragment of two genes cloned using the lariat probe. Lanes 1, clone containing a gene with an intron. Lanes 2, clone without an intron, presumably containing a tRNA gene.

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J. Blanchard and V. Stoll, personal communication.
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Fig. 4. S28 is encoded by two genes. Two parallel Southern blots of genomic DNA from W303a digested with EcoRV, SalI, EcoRI, or HindIII were prepared. The left blot is probed with a 32P-labeled oligonucleotide complementary to the intron of RPS28A. The right blot is probed with a nick-translated fragment of the open reading frame of RPS28A generated by PCR amplification. Labeled λ DNA digested with HindIII as a size marker is shown on the left.

prepared from four of the RPS28B plaques as described under "Experimental Procedures." Restriction analysis showed that one clone contained the entire gene. The gene was subcloned out of Agt11 as an approximately 2.5-kilobase ClaI fragment and also as several other overlapping subclones for sequencing purposes.

Sequence Analysis of S28A—The sequence of the RPS28A gene is shown in Fig. 5B. The gene has an open reading frame predicted to encode a 145-amino acid protein with a molecular mass of 16,019 Da. The protein contains 32 lysine and arginine residues and 11 glutamate and aspartate residues and has a predicted isoelectric point of 11.47.

The region upstream of the gene was analyzed for possible cis-acting transcription signals. Most yeast genes have upstream regions that are AT-rich. The upstream region of RPS28A is unusually GC-rich. Most yeast ribosomal protein genes contain two RPG consensus sequences, ACACCCA-TACATTT (with underlined nucleotides invariant), or a related sequence, UAST (25), and a T-rich region (26-29). In RPS28A there are two identical sequences with an 11/14 nucleotide match to the RPG consensus, beginning at nucleotides -387 and -70. No obvious T-rich region was discernible between these sites and the start site of transcription, although 11 consecutive Ts are present 52 nucleotides upstream of the putative RPG boxes. There are potential TATA boxes starting at nucleotides -119 and -54.

The coding region of the gene is interrupted with a putative 318 nucleotide intron beginning with the 5' splice site A/GTATGT, splitting the 22nd codon. The intron is predicted to end at the second AG following the TACTAAC branch site consensus sequence, 27 nucleotides downstream. These splice sites were confirmed by PCR analysis as described below.

Sequence Analysis of RPS28B and Comparison with RPS28A—The RPS28B gene (Fig. 5C) is predicted to encode a 145-amino acid protein identical to that of RPS28A. Fifteen
silent nucleotide changes exist between the two genes, all at the 3rd base position, except for a difference in codon from CTA to TTG, both of which encode leucine. Analysis of the upstream region revealed a 13/14 match to the RPG consensus sequence beginning at -310. Again, there is no long T-rich region. Potential TATA boxes are present beginning at nucleotides -85 and -50.

Comparison of the RPS28A and RPS28B genes showed that the two genes diverge completely outside the coding region except for AAAG, immediately preceding the ATG translation start site, and GCA, immediately downstream of the TAA stop codon.

The Predicted Splice Sites of RPS28A and RPS28B Confirmed by PCR Analysis—In yeast the 5' splice site is generally G/GUAUGU, with underlined nucleotides invariant (4). In RPS28 the sequence A/GUAUGU at nucleotide 64 in both RPS28A and RPS28B is nearest to the consensus. The intron was assumed to end at the second AG (nucleotide 383) following the consensus TACTAAC branch site in RPS28A, since the first AG immediately follows the branch site. In RPS28B, however, the first AG is not preceded by a pyrimidine, as is usual for yeast 3' splice sites, and results in an out of frame reading context. The second AG following the branch site corresponds to the reading frame deduced for RPS28A. These predictions were fortified by comparison with the amino acid sequence of S25 from Tetrahymena thermophila. To prove that these were in fact the splice sites used by the splicing machinery in vivo, we determined the sequence of a PCR product derived from the mRNA.

One oligonucleotide downstream of the 3' predicted splice site homologous to both genes was used for amplification along with one of two gene-specific oligonucleotides upstream of the suspected 5' splice site. Sequence obtained from the subcloned DNA fragments confirmed the predicted splice sites, as shown in Fig. 6 for RPS28A. No cDNA fragment containing a splice to the first AG following the branch site in RPS28B was detected.

Disruption of RPS28A and RPS28B—To determine whether the ribosomal protein S28 is essential for growth, both genes encoding the protein were disrupted by the one-step gene disruption method (30), as described in the legend of Fig. 7.

Southern analysis was performed on DNA isolated from transformed diploids containing disruptions of either the RPS28A or RPS28B gene to determine if the constructs had been integrated correctly. Surprisingly, 50% of the transformants selected to contain disruption of the RPS28A gene showed that integration had not occurred at the homologous site in the genome (data not shown). Southern analysis of tetrads obtained from diploids containing the correct integrations demonstrated that it segregated 2:2 along with prototrophy for tryptophan, as shown in Fig. 7A.

In the case of RPS28B, all of the transformants examined were the result of homologous integration into the RPS28B gene. On sporulation and dissection of tetrad, the disruption segregated 2:2 (Fig. 7B), and this corresponded to segregation of lysine prototrophy.

Northern analysis of RNA isolated from the wild type strain suggests that S28 is encoded by mRNAs of slightly different electrophoretic mobilities (Fig. 8). The two bands arise from transcription of the two genes. The mRNA of the RPS28A gene is slightly longer, presumably because of differences in noncoding region. In the wild type strain L-1381 the ratio of mRNA from RPS28A is roughly three times that derived from RPS28B. When the levels of messenger RNA in the disruptant strains are compared using a counterprobe (for U3 snRNA) to normalize lane loading, it appears that the levels of RNA derived from the remaining gene do not change, suggesting that there is no regulatory compensation between the genes.

A Diploid Containing ΔRPS28A and ΔRPS28B Cannot Sporulate—The ΔRPS28A and ΔRPS28B haploids were mated to determine if a strain containing both disruptions would be viable. The diploid, containing one intact and one disrupted copy of each gene, exhibits a growth rate only slightly slower than the wild type. However, when placed on sporulation medium the heterozygous diploid does not sporulate and instead dies rapidly. This was the case for several independently obtained diploid strains. Remarkably, this inability to sporulate was not relieved by the presence of a wild type RPS28A gene on either a centromere based or multicopy plasmid. It is unknown whether transcription of the plasmid is inhibited under these circumstances.

Clearly, disruption of the RPS28 genes causes severe disturbances during meiosis. A precedent for these effects is found in mutations affecting ribosomal fidelity in the fungus Podospora anserina, where mutations conferring hyperaccurate translation eliminate sporulation at discrete steps (31–33). Sporulation was restored by the addition of the antibiotic paromomycin or mutations which increased ribosomal suppression. This led the researchers to propose that a heightened level of ribosomal ambiguity might be required during specific stages of the life cycle. However, they further found that double mutants containing two or more ribosomal ambiguity mutations were also defective in sporulation. They suggested that a regulated increase in suppression was required during sexual differentiation (31).

RPS28 Is Essential for Viability—To determine whether
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Fig. 7. Genomic Southern analysis of RPS28A and RPS28B disruptions. Panel A, Southern blot of genomic DNA from strains harboring the RPS28A disruption. A 350-nucleotide SacI fragment of RPS28A including part of the upstream region, the first 21 codons, and part of the intron was deleted from the genome and replaced by a 1.5-kilobase EcoRI fragment containing TRP1 in the opposite orientation by integrative transformation. DNA from strains carrying this disruption or control strains was digested with EcoRV and SalI, and the blot was probed with a nick-translated fragment of DNA from strains carrying a genomic disruption or control strains was digested with ClaI and the blot was probed with a nick-translated DNA fragment hybridizing to the 5' upstream region of RPS28A.

Panel B, Southern blot of genomic DNA from strains harboring the RPS28B disruption. The entire coding region of RPS28B was replaced by a 1.5-kilobase EcoRI fragment containing TRP1. Lanes include DNA isolated from 1, diploid wild type L-1381; 2, the diploid strain carrying the disrupted RPS28A gene; and 3–6, four haploid segregants of the diploid carrying the disruption. Panel B, Southern blot of genomic DNA from strains harboring the RPS28B disruption. The entire coding region of RPS28B between an upstream SacI restriction site and a downstream HindIII site was deleted in the genome and replaced with a cassette carrying the LYS2 gene transcribed in the opposite orientation (43). DNA from strains containing the genomic disruption or from control strains was digested with ClaI and the blot was probed with a nick-translated DNA fragment hybridizing to the 5' upstream region of RPS28B. Deletion of the RPS28B coding region with insertion of the LYS2 gene results in a shift from approximately 2.5 to 0.675 kilobases in DNA digested with this enzyme because of a site added at the LYS2 junction. Lanes include DNA isolated from 1, diploid wild type strain L-1381; 2, the diploid strain harboring the RPS28B disruption; and 3–6, four haploid segregants of the diploid carrying the disruption.

double disruption of S28 would be lethal, a haploid strain carrying a genomic disruption of RPS28B was transformed with the wild type RPS28A gene on a multicopy plasmid carrying URA3, and the RPS28B gene was disrupted by integrative transformation as described above. We then asked whether this strain could lose the plasmid carrying the wild type gene. Dilutions of cells ranging from 10^2 to 10^6 were spread onto plates containing 5-fluoroorotic acid (34) and incubated for up to 5 days to select against plasmid maintenance. Although 10–25% of control cells containing a wild type genomic copy of RPS28B readily lost the plasmid, none of the cells lacking both genomic copies did so (Fig. 9).

In a parallel experiment, cells harboring deletions of both genomic genes and which carried the wild type gene on a plasmid were streaked successively on rich medium and then replica plated to Ura selection medium. Again, Ura^- cells did not appear. These experiments strongly suggest that S28 is essential for growth.

The S28 Protein Is Homologous to Several Previously Identified Ribosomal Proteins—The complete predicted amino acid sequence of S28 was compared with the GenBank data bank using the FASTA program (35). Although the most striking similarity was found with ribosomal protein S25 of T. thermophila (36), with 69% identity, S28 also showed homology to S12 of the archaeabacteria Halobacterium halobium, Halococcus morrhuae, Thermococcus celer, Sulfolobus acidocaldarius, and Methanococcus vannielii, all at approximately 55% identity, and to a lesser extent with tko, a mitochondrial ribosomal protein in Drosophila melanogaster.

T. thermophila S25 was shown using dot matrix analysis to contain significant homology to functional regions of S12 of E. coli, a protein known to be involved in translational accuracy (37, 38). A similar analysis (Fig. 10A) confirmed the homology between S28 of S. cerevisiae and S25 of T. thermophila, even at high stringency. In spite of the very limited homology between S28 and S12 of E. coli, Dot-Plot analysis suggests that two regions appear to be conserved (Fig. 10B). In particular, a region including a lysine residue specifically mutated in bacterial hyperaccurate mutants (39) was con-
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Acknowledgments—We are grateful to Susan Liebman and Ric Anthony for extensive practical and intellectual assistance, to V. Stoll for help with chromatography of the lariat RNA, to S. Hawley and L. Marsh for discussions, and to U. Vijayaraghavan and J. Abelson for the strain containing prp26.

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Fig. 10. Dot matrix comparison of ribosomal protein S28 from Saccharomyces cerevisiae with S28 of Thermus thermophilus or with E. coli. The Dot-Plot program of the GCG-Wisconsin computer package (42) was used to make the comparisons. Diagrams represent comparisons of S28 with ribosomal protein S28 of Thermus thermophilus, using a window of 30 and a stringency of 15 (panel A) and ribosomal protein S12 of E. coli, using a window of 30 and a stringency of 12 (panel B).