A novel mitochondrial protein, Tar1p, is encoded on the antisense strand of the nuclear 25S rDNA

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In eukaryotes, it is widely assumed that genes coding for proteins and structural RNAs do not overlap. Using a transposon-tagging strategy to globally analyze the Saccharomyces cerevisiae genome for expressed genes, we identified multiple insertions in an open reading frame that is contained fully within and transcribed antisense to the 25S rRNA gene in the nuclear rDNA repeat region on Chromosome XII. Expression of this gene, TAR1 (Transcript Antisense to Ribosomal RNA), can be detected at the RNA and protein levels, and the primary sequence of the corresponding 124-amino-acid protein is conserved in several yeast species. Tar1p was found to localize to mitochondria, and overexpression of the protein suppresses the respiration-deficient petite phenotype of the R129D mutation in the mitochondrial RNA polymerase Rpo41p. In total, the discovery of TAR1 shatters the notion that structural RNAs and protein-coding sequences cannot overlap and holds significant implications in the evolution of these genes and the coordinate control of ribosome biogenesis and mitochondrial function.

Results and Discussion

In the course of a large-scale analysis of the yeast genome by transposon tagging with a lacZ reporter that lacks both promoter sequences and an initiator ATG codon, we identified a number of transposon insertions in previously nonannotated open reading frames (NORFs; Ross-Macdonald et al. 1999; Kumar et al. 2002). In most cases, these insertions represent in-frame fusions of the NORF with the lacZ bacterial gene present in the transposon insertion construct. Intriguingly, we found ~100 in-frame insertions in an NORF in the rDNA locus in S. cerevisiae Chromosome XII, a region previously known to contain protein-coding sequences (Fig. 1a; Johnston et al. 1997). This NORF (hereafter called TAR1) is encoded on the opposite strand of that encoding the 25S rRNA gene (Fig. 1a; Philippson et al. 1978; Petes 1979; Veldman et al. 1981). The TAR1 gene is 124 codons in length, encoding a protein with a predicted molecular mass of 14.34 kD. All of the insertion strains produce β-galactosidase activity as detected by filter assays (Fig. 1b), which is a strong indicator that TAR1 is transcribed and translated into protein. The high frequency with which the short TAR1 ORF was identified in the transposon screen (>50 times higher than the average yeast gene, size 1500 bp) suggests that many, if not all TAR1 units in the rDNA are transcribed. The Tar1p protein sequence is highly conserved in hemiascomycetous species (Fig. 1c; Souciet et al. 2000), but does not have any obvious motif or domain conserved in other proteins in the databases.

To determine if the TAR1 gene is transcribed as RNA, we analyzed TAR1 expression levels by means of strand-specific, dot-blot analysis. Labeled poly[A] RNA was hybridized to a 60-base oligonucleotide complementary to the TAR1 coding sequence; identical assays were performed with oligonucleotides derived from known genes and noncoding segments of the yeast genome. As shown in Figure 1d, significant levels of TAR1 RNA transcript are observed relative to signals generated with oligonucleotides derived from noncoding regions of the yeast genome.

To determine if the TAR1 ORF is normally expressed as a protein, we tagged the C terminus of Tar1p with 13

[Keywords: rDNA, mitochondria, gene annotation, mitochondrial RNA polymerase, transposon]

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copies of the myc epitope or with three copies of the haemagglutinin [HA] epitope (Tar1p–13myc and Tar1–3HA, respectively). As shown in Figure 2A (lane 2), two bands were detected by immunoblot analysis of total protein extracts probed with anti-myc antibodies. The protein with the slower mobility had an apparent molecular weight of ~41 kD, and the protein of higher mobility had an apparent molecular weight ~35 kD. The molecular weights of these proteins are similar to the predicted sizes of protein products derived from the first and second methionines of TAR1, respectively [Fig. 1c]. Two bands were also detected when the TAR1 gene was tagged with three copies of the HA epitope (data not shown). Based on these findings and the fact that strains containing lacZ fusions throughout the TAR1 ORF express β-galactosidase activity [Fig. 1b], we conclude that TAR1 is a protein-coding gene encoded on the antisense strand of the 25S rDNA gene.

To learn more about the TAR1 protein we determined the subcellular localization of Tar1p, using indirect immunofluorescence and subcellular fractionation experiments. We detected a faint fluorescence signal in mitochondria of cells carrying a single chromosomal copy of the myc-tagged TAR1 gene. This mitochondrial localization was even more evident in cells that overexpressed Tar1p. A centromeric plasmid containing the 13Xmyc-tagged TAR1 gene under control of the inducible GAL1 promoter overproduces both forms of the protein when cells are grown on galactose [Fig. 2c, lane 2]. Using indirect immunofluorescence of cells grown in medium containing galactose, we found that the tagged Tar1p localizes to the mitochondrial network [Fig. 3a, rows I, II, III]. Coelho et al.

colocalization was observed with both mtDNA [DAPI staining] and proteins present in the outer mitochondrial membrane [αOM]. Staining was not observed in cells grown in raffinose medium (data not shown) or in cells containing only the vector (Fig. 3a, row IV; see below), and in cells carrying the TAR1 gene on a high-copy 2μ plasmid, under its endogenous promoter (Fig. 3a, row V).

The subcellular localization of Tar1p was also examined using subcellular fractionation experiments. Total extracts of yeast cells expressing myc-tagged Tar1p from a single-copy chromosomal allele were prepared; mitochondria and other organelles were fractionated by differential centrifugation, and protein from the different fractions was analyzed by immunoblotting. Myc-tagged Tar1p cofractionated with porin, a mitochondrial protein, whereas the cytosolic protein, PGK, preferentially cofractionated with the soluble fraction [Fig. 3b, lanes 2, 3]. Altogether, these results demonstrate that Tar1p is a mitochondrial protein.

In an independent series of experiments, we also identified TAR1 in a random genetic screen for genes that when overexpressed suppress the phenotype of a point mutation that affects the mitochondrial RNA polymerase (Rpo41p; Greenleaf et al. 1986; Masters et al. 1987). This mutation changes arginine to aspartic acid at amino acid position 129 (R129D) in the N-terminal domain of the enzyme and causes a mitochondrial petite phenotype (impaired growth on YPG medium) at both 30°C and
37°C (Fig. 4; Rodeheffer et al. 2001). Initially, two separate low-copy centromeric plasmids, each containing a partial rDNA repeat unit (missing the SS rRNA gene and large portions of the 18S and 25S rRNA genes), were found to suppress the rpo41-R129D mutation [Fig. 4a]. Because the entire TAR1 ORF is present in both plasmids and Tar1p is localized to mitochondria, we predicted that TAR1 might be responsible for this multi-copy suppressor activity. To test this idea, we transformed the R129D DNA polymerase mutant strain (rpo41-R129D) with a plasmid bearing TAR1 under control of the galactose-inducible GAL1 promoter. Tar1p expressed from this plasmid suppressed the rpo41-R129D growth defect manifested in glycerol medium at 37°C. Likewise, overexpression of TAR1 by the addition of galactose (0.2%) partially rescued the severe growth phenotype of the rpo41-R129D mutant at 37°C (Fig. 4b). These data indicate that the TAR1 gene encodes a mitochondrial protein that can rescue a defect in mitochondrial gene expression.

TAR1 is readily expressed when the coding sequence plus 500 bp upstream of the ATG are inserted into a high-copy plasmid (Fig. 2c, Fig. 3, row VI), suggesting that it forms its own transcription unit in the rDNA. Some genes inserted into the rDNA locus are repressed by the silencing activity of Sir2p [Smith and Boeke 1997]. To determine if TAR1 expression is affected by the activity of Sir2p, we compared the protein levels of the single copy, chromosomally encoded myc-tagged Tar1p in an SIR2 null strain with that of a wild-type SIR2 strain. A 2.5-fold increase in the amount of Tar1p–13myc was observed in the SIR2 deletion strain (Fig. 2b, lane 3). In addition, we readily detected Tar1p–13myc by indirect immunofluorescence in an SIR2 null strain carrying a single copy of the construct in the chromosome, whereas the protein was difficult to detect in SIR2 wild-type cells (Fig. 3a, row IV).

Our results demonstrate for the first time that the rDNA locus contains overlapping genes; the 35S rRNA is produced from one strand (as part of a larger 35S rRNA precursor), and Tar1p coding information is located on the opposite strand. Although the RNA is transcribed by Pol I, it is likely that TAR1 is transcribed by Pol II because (1) the RNA is present in polyA+ RNA; (2) the RNA encodes protein; and (3) expression is elevated in sir2 mutant strains—all features normally ascribed to RNA polymerase II-transcribed messages. Additional experiments will be necessary to directly test this hypothesis.

After our paper was submitted, Kermechiev and Ivanova [2001] reported the identification of a protein, ribin, whose cDNA sequence is derived from sequence complementary to the large rRNA in mouse, and from a different region than the Tar1 protein. The ribin cDNA sequence was not identical to the antisense strand of the rRNA, and the 3′ untranslated region does not contain RNA sequences; therefore, the ribin transcript may not be encoded at the rDNA locus. The ribin protein is involved in rRNA transcription [presumably in the nucleolus], rather than mitochondrial function, raising the possibility that both RNA complementary sequences are involved in gene expression, albeit in different cellular compartments.

The presence of overlapping genes in the rDNA locus, one encoding a protein and the other encoding a structural (and probably catalytic) RNA, raises interesting issues regarding the origin and conservation of such genes. It is interesting to speculate that the RNA gene evolved first in the RNA world, and that the advent of protein-coding RNAs arose from the exact same gene, albeit on the other strand [Joyce and Orgel 1993]. Regardless of mechanism, these genes are expected to be tightly linked in their evolution; presumably it will be difficult to mutate one gene without affecting the function of the other, and thus the sequence of this region is expected to be highly conserved. Such constraints might be reflected in the observation that the TAR1 ORF is highly conserved among very diverse yeast species.

The function of Tar1p is not known. However, the N-terminal domain of mRNA polymerase is involved in mDNA stability [Wang and Shadel 1999] and interacts with Nam1p [Rodeheffer et al. 2001] and Sls1p [Bryan et al. 2002], two proteins involved in posttranscriptional gene expression in mitochondria. The presence of Tar1p in mitochondria and its ability to suppress the petite phenotype of a point mutation in this domain suggest that this protein increases the efficiency of oxidative phosphorylation directly or indirectly, by influencing mDNA stability or mitochondrial gene expression at the posttranscriptional level. The fact that this gene is not highly expressed during vegetative growth, but is elevated in a sir2 null mutant strain, might indicate that Tar1p expression and rDNA transcription are inversely
regulated. This type of regulation could provide a means to coordinate rDNA transcription and mitochondrial function in response to changing cellular needs or energy demands, for example, under different types of growth conditions, during mitosis, or in aging cells. Additional studies are likely to further elucidate the function of Tar1p and its role in coordinating nuclear and mitochondrial functions.

Materials and methods

Strains and plasmid construction

Growth media and genetic manipulation were as described (Guthrie and Fink 1991). All strains are derivatives of the diploid strain Y270, a descendant of S228C, except for the siz2 and its isogenic wild-type strains, which are derived from the deletion collection available from Research Genetics [Winzeler et al. 1999]. The epitope-tagged strains were grown at 30°C in YPAD medium (liquid media supplemented with adenine, Sherman et al. 1986). Plasmid-bearing strains were grown in synthetic complete medium. Cells bearing the GAL1-promoter plasmids were induced by adding galactose to the liquid medium to a final concentration of 2%.

The strain containing TAR1–13myc in the chromosome was constructed using the PCR epitope-tagging method of Longtine et al. [1998]. The 13-myc epitope followed by the ADH1 terminator was integrated at the C terminus of the coding region [before the termination codon], Oligonucleotides BW1 [5'-CCAAATTACATCGGGACACTGGATAC CAGATTTCAAAATTCGGATCCCCGGTTTAAATTTAA-3'] and BW2 [5'- CCGGATATCTCTATAGCAAGCGCTATTTAAC-3'] were used. Correct integration of the myc tag was confirmed by PCR analysis. The tags constructed appears to be present at a single or low copy in the rDNA as determined by PCR analysis.

Figure 3. Tar1p mitochondrial localization. [a] Micrographs of cells showing indirect immunolocalization of TAR1–13myc to the mitochondria using anti-myc antibody. The mitochondria were visualized by using an antibody against proteins present in the outer mitochondrial membrane (α-OM). The nucleus and mtDNA were visualized by staining the cells with DAPI. [Rows I–IV] Two examples of galactose-induced cells carrying a centromeric vector containing TAR1 under the control of the galactose-inducible GAL1 promoter [pGAL1–TAR1–13myc]. [Row III] A galactose-induced cell containing an empty pGAL1 vector as a control. [Row IV] An siz2 cell harboring a 13myc-tagged TAR1 as a single copy in the chromosome. [Row V] A cell carrying a multiplicity vector containing TAR1 under the control of its own promoter [p2A–TAR1–13myc]. [Row VI] A cell containing a multiplicity [p2A] empty vector as a control. [b] Fractionation of total cell extract by differential centrifugation. The samples derived from cells carrying a 13 myc-tagged TAR1 in the chromosome were prepared and analyzed by SDS-PAGE and immunoblotting. The blots were probed with an anti-myc antibody and anti-porin (mitochondrial marker) antibodies. The blot was stripped and probed again with an antibody against phosphoglycerokinase (PGK). T, total extract [lane 1]; M, medium-speed mitochondrial-enriched pellet [lane 3]. The supernatant of the medium-speed centrifugation was subjected to an ultracentrifugation, giving rise to a high-speed supernatant (S4, lane 2) and a high-speed pellet (P4, lane 4).

Figure 4. Suppression of a mitochondrial RNA polymerase N-terminal-domain point mutation by a partial nuclear rDNA repeat containing TAR1. [a] Two independent plasmids harboring a portion of a single nuclear rDNA repeat [denoted rDNA suppressors 1 and 2] were isolated as described [Bryan et al. 2002] through their ability to rescue the mitochondrial petite growth phenotype of the rpo41-R129D point mutation in mtRNA polymerase [Rpo41p] at 35°C. The genomic DNA insert contained in each plasmid is delineated in brackets below a diagram of a 9.1-kb rDNA repeat unit. The 18S, 5.8S, 25S, and 5S rRNA genes are depicted as boxes with the direction of transcription of the 35S rRNA precursor shown as a bent arrow. The TAR1 ORF embedded in the 25S rRNA is depicted as a solid black arrow, with the direction of the arrow indicating that it is transcribed antisense to the 25S rRNA gene. [b] Tar1p expressed from a GAL1 promoter suppresses the rpo41-R129D mutant phenotype. A plasmid that expresses TAR1 from the GAL1 promoter [pRS316-TAR1] was introduced into the rpo41-R129D strain [labeled R129D] as the empty vector [pRS316] as a control. Under glycolysis growth conditions [YPG medium + 0.2% galactose], the GAL1 promoter is derepressed, allowing elevated expression of Tar1p from the plasmid. At both 30°C [left column] and 37°C [right column] on YPG, the rpo41-R129D strain exhibits a severe growth defect compared with the wild-type strain, G122. Expression of Tar1p from the GAL1 promoter [pRS316-TAR1] substantially rescues this petite phenotype at both temperatures.
A protein-coding gene in the rDNA locus

Acknowledgments

We thank M. Smith and D. Gelperin for critical comments on the manuscript, and M. Smith and L. Pon for the anti-porin antibodies. This research was supported by grants from the NIH, to M.S. and G.S.S. P.S.R.C. was supported by an FAPESP (São Paulo, Brazil) postdoctoral fellowship and by a grant from the Burroughs Wellcome foundation. A.K. was supported by a postdoctoral fellowship from the American Cancer Society.

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References

Bryan, A.C., Rodeheffer, M.S., Wearn, C.M., and Shadel, G.S. 2002. Ssh1p is a membrane-bound regulator of transcription-coupled processes involved in <i>Saccharomyces cerevisiae</i> mitochondrial gene expression. <i>Genetics</i> 160: 75–82.

Chernoff, Y.O., Vincent, A., and Liebman, S.W. 1994. Mutations in eukaryotic 18S ribosomal RNA affect translational fidelity and resistance to anminoglycoside antibiotics. <i>EMBO J</i> 13: 906–913.

Glick, B.S. and Pon, L.A. 1995. Isolation of highly purified mitochondria from <i>Saccharomyces cerevisiae</i>. <i>Methods Enzymol.</i> 266: 213–223.

Kermekchiev, M. and Ivanova, L. 2001. Ribin, a protein encoded by a message complementary to rRNA, modules ribosomal transcription and cell proliferation. <i>Mol. Cell. Biol.</i> 21: 8255–8263.

Pringle, J., Adams, A.E., Drubin, D.G., and Haarer, B.K. 1991. Immunofluorescence analysis of subcellular fractionation. In <i>Methods in enzymology</i> (ed. J.N.A.S.M. Abelson), pp. 1–933. Academic Press, San Diego.

Rodeheffer, M.S., Boone, B.E., Bryan, A.C., and Shadel, G.S. 2001. The RNA world. In <i>The RNA world</i> (eds. R.F.A. Gesteland and J.F. Atkins), pp. 1–25. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ross-MacDonald, P., Coelho, P.S.R., Roemer, T., Agarwal, S., Kumar, A., Janacek, B., Cheung, K.H., and Shadel, G.S. 2001. Namlp, a protein involved in RNA processing and translation, is coupled to transcription through an interaction with yeast mitochondrial RNA polymerase. <i>J. Biol. Chem.</i> 276: 8616–8622.

Wash, A., Bracht, A., Philipsen, P., and Pringle, J.R. 1998. Additional modules for versatile and economical PCR-based gene deletion and modification in <i>Saccharomyces cerevisiae</i>. <i>Yeast</i> 14: 953–961.

Masters, B.S., Stohl, L.L., and Clayton, D.A. 1987. Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. <i>Cell</i> 51: 89–99.

Petes, T.D. 1979. Yeast ribosomal DNA genes are located on chromosome XII. <i>Proc. Natl. Acad. Sci.</i> 76: 410–414.
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Sherman, F., Fink, G., and Hicks, J. 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.

Smith, J.S. and Boeke, J.D. 1997. An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes & Dev. 11: 241-254.

Souciet, J., Aigle, M., Artiguenave, F., Blandin, G., Bolotin-Fukuhara, M., Bon, E., Brotsier, P., Casaregola, S., de Montigny, J., Dujon, B., et al. 2000. Genomic exploration of the hemiascomycetous yeasts: 1. A set of yeast species for molecular evolution studies. FEBs Lett. 487: 3–12.

Stein, L. 2001. Genome annotation: From sequence to biology. Nat. Rev. Genet. 2: 493–503.

Veldman, G.M., Kloorwik, J., de Regt, V.C., Planta, R.J., Branlant, C., Krol, A., and Ebel, J.P. 1981. The primary and secondary structure of yeast 26S rRNA. Nucleic Acids Res. 9: 6935–6952.

Wang, Y. and Shadel, G.S. 1999. Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase. Proc. Natl. Acad. Sci. 96: 8046–8051.

Wintzler, E.A., Shoemaker, D.D., Astronomo, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J.D., Bussey, H., et al. 1999. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285: 901–906.