Global identification of noncoding RNAs in Saccharomyces cerevisiae by modulating an essential RNA processing pathway

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Noncoding RNAs (ncRNAs) perform essential cellular tasks and play key regulatory roles in all organisms. Although several new ncRNAs in yeast were recently discovered by individual studies, to our knowledge no comprehensive empirical search has been conducted. We demonstrate a powerful and versatile method for global identification of previously undescribed ncRNAs by modulating an essential RNA processing pathway through the depletion of a key ribonucleoprotein enzyme component, and monitoring differential transcriptional activities with genome tiling arrays during the time course of the ribonucleoprotein depletion. The entire Saccharomyces cerevisiae genome was scanned during cell growth decay regulated by promoter-mediated depletion of Rpp1, an essential and functionally conserved protein component of the RNase P enzyme. In addition to most verified genes and ncRNAs, expression was detected in 98 antisense and intergenic regions, 74 that were further confirmed to contain previously undescribed RNAs. A class of ncRNAs, located antisense to coding regions of verified protein-coding genes, is discussed in this article. One member, HRA1, is likely involved in 18S rRNA maturation.

Results and Discussion

Nearly 400,000 36-mer oligonucleotide probes, tiling the entire yeast genome including the mitochondrial chromosome with an average gap of 10 bases between two consecutive probes, were synthesized on glass slides by using a maskless array synthesizer (Fig. 1) (20). The arrays contained culture medium but suppressed its expression in galactose-containing medium and subsequently transferred and resuspended into glucose-containing medium. Eight arrays were hybridized with RNA extracted from the Rpp1-depleted cells at 0, 4, 7, 12, 16, 21, and 30 h, and the control cell at 30 h, after initial transfer to glucose-containing medium. The scanned data were normalized by using standard procedures (21).

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Abbreviation: ncRNA, noncoding RNA.

Data deposition: The sequences reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession nos. for series: GSE4275; for platform: GPL3471; and for samples: GSM97576, GSM97577, GSM97581, GSM97582, GSM97583, GSM97584, GSM97585, and GSM97586).

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protein-coding genes, all tRNA, rRNA, and other known ncRNA genes (22), as well as a large number of antisense and intergenic regions (discussed below).

The gradual depletion of Rpp1 over a defined time course led to inactivation of the RNase P enzyme, thus disrupting the processing of the precursor tRNAs into their mature forms. Because of the short lengths of the tRNAs, typically only one probe measured the total expression for either the precursor or mature tRNA, making it difficult to distinguish between the two. However, the total signal measured by such tRNA-related probes increased over time likely due to rapid accumulation of precursor tRNAs (Fig. 5, which is published as supporting information on the PNAS web site), in accordance with previous studies (14). Moreover, shortage of matured tRNAs was expected to reduce protein synthesis and thus lead to disruption of all cellular processes including transcription. Such transcriptional anomalies were clearly observable at the last time point (t = 30 h; Rpp1-depleted strain) at most transcribed regions of the genome. For protein-coding genes, the signatures of the observed anomaly were (i) reduced hybridization signals at the 5′ ends of the genes, possibly by the shortening of the mRNAs due to modulation of XRN1 expression and (ii) increased signals near the 3′ ends, possibly from modulation of exosome activity, by changing the expressions of RRP43 and RRP42. Observed degradation of mRNA could also arise from an absence of translation. No such defect in mRNA quality was seen in the control sample.

All of the known ncRNAs (22–27), encompassing a large spectrum of tRNA, rRNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), RNase P, RNase MRP, and signal recognition particle components, showed strong or time-varying RNA transcription signals (Fig. 6, which is published as supporting information on the PNAS web site). For some ncRNAs (SCR1 and SNR10) and protein-coding genes (PMP2), transcription extended well beyond the annotated 3′ ends of their matured forms (Fig. 2a). As a characteristic pattern, overexpression of RPP1 led to increased transcription of known ncRNAs at...
functions.

interactions with genes of nucleolar and such as Drs1, have affinity-based interactions or two-hybrid contrast, other known ribosomal maturation-related proteins, argues against Drs2 being involved in rRNA biogenesis. In ically lethal with genes of similar function, this observation alone essential genes are usually functionally redundant and synthet-

phenotype related to cell membrane trafficking. Because non-

biogenesis. Instead, all four genes are involved in a common

CNB1

rRNA synthesis or in the maturation pathway (22). Moreover,

DRS2

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below that the characterization of the

DRS2

phenotype, available from ref. 28, and sequencing of the

drs2 locus performed by this work suggest that the observed defect in 18S rRNA maturation may be because of a mutation in

HRA1

rather than DRS2. The earlier study found that a 2.2-kb EcoRI–BglIII fragment, including only 528 aa (38% of the coding region) from the 5′ end of DRS2, complemented the observed rRNA-related phenotype (Fig. 2A in ref. 28). The fragment extended upstream to the 5′ end of DRS2 and fully included HRA1 (data not shown). On the other hand, a BamHI–BamHI fragment that included the entire coding region of Drs2, but no additional bases from its 5′ upstream region, did not complement the drs2 phenotype. HRA1 was only partially included in the second fragment. Moreover, Northern blot analysis of the 2.2-kb EcoRI–BglIII fragment DNA, which was successful in complementing the rRNA-related phenotype, detected an additional 1-kb transcript (Fig. 2B in ref. 28) similar in length to HRA1, based on the array data. The abovementioned Northern blot analysis detected transcripts from both strands of the corresponding genomic DNA. Finally, this work sequenced the 2.2-kb EcoRI–BglIII fragment from the mutant drs2 strain. The sequencing result showed mutations in three consecutive bases that were located within the segment of DRS2 that contained HRA1. The bases 99,563–99,565 of chromosome 1 in yeast were mutated from ATC to TCG.

Reanalysis of the original data used to characterize the

drs2 phenotype, available from ref. 28, and sequencing of the

drs2 locus performed by this work suggest that the observed defect in 18S rRNA maturation may be because of a mutation in

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the first time point (t = 0; galactose-containing medium). Within 4 h of transfer to the glucose-containing medium, the same RNA signals adjusted to their basal levels, as determined from the control strain (Fig. 6). The above observation suggests that Rpp1, as a component of RNase P and RNase MRP complexes, not only takes part in the processing of precursor RNA but also affects the overall expression and/or stability of the RNAs. This observation, if confirmed in vitro by an RNase P cleavage, could be helpful for determination of putative RNase P substrates.

Apart from the annotated regions of the genome, 21.5% of the expressed probes matched antisense or intergenic segments (Fig. 1b), suggesting the possible existence of a large number of putative novel ncRNAs or short ORFs. This result is an unexpected observation because the S. cerevisiae genome has been most extensively annotated. Additional screening of the transcribed probes led to the identification of 98 high-confidence ncRNAs (Tables 2 and 3; Figs. 7–18, which are published as supporting information on the PNAS web site), of which 83 were selected for further confirmation by using the RT-PCR method. RNA samples from both t = 4 h and t = 30 h time points for the Rpp1-depleted cells were used for RT-PCR. By this experiment, 74 previously undescribed RNAs could be confirmed (Table 4, which is published as supporting information on the PNAS web site).

The above list of confirmed previously undescribed ncRNAs includes 21 members located antisense to the protein-coding ORFs. One of them, named HRA1 (hidden in reading-frame antisense), was located antisense to a previously annotated gene called DRS2. Drs2, a Golgi membrane-located transport protein (22), was also determined to be involved in the maturation of 18S rRNA based on a temperature-sensitive mutation-based study (28), but the exact mechanism remained unknown. We suggest below that the characterization of the

DRS2

phenotype is likely incorrect, and the observed rRNA processing defect is likely caused by the RNA-coding gene

HRA1

rather than the protein-coding gene

DRS2

Drs2 has no known interaction with any protein involved in rRNA synthesis or in the maturation pathway (22). Moreover, none of the four nonessential genes (BN1, KIP2, GIMS, and CNB1) synthetically lethal with

DRS2

is associated with rRNA biogenesis. Instead, all four genes are involved in a common phenotype related to cell membrane trafficking. Because non-essential genes are usually functionally redundant and synthetically lethal with genes of similar function, this observation alone argues against Drs2 being involved in rRNA biogenesis. In contrast, other known ribosomal maturation-related proteins, such as Drs1, have affinity-based interactions or two-hybrid interactions with genes of nucleolar and/or rRNA biogenesis functions.

Table 2. Summary information for the previously undescribed transcripts detected by this article

<table>
<thead>
<tr>
<th>Total count</th>
<th>Median length, bases</th>
<th>No. of RT-PCR performed</th>
<th>No. of RT-PCR confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>All transcripts</td>
<td>98</td>
<td>460</td>
<td>83</td>
</tr>
<tr>
<td>Antisense transcripts</td>
<td>21</td>
<td>800</td>
<td>8</td>
</tr>
<tr>
<td>Promoter-based transcripts</td>
<td>50</td>
<td>350</td>
<td>48</td>
</tr>
<tr>
<td>Intergenic transcripts</td>
<td>27</td>
<td>500</td>
<td>27</td>
</tr>
</tbody>
</table>

Depletion of Rpp1 induced 42 previously undescribed transcripts and repressed 9, and 47 remained unaffected. This information was decided by comparing expression at 16–21 h with expression at 4–12 h.

Table 3. Summary information for the previously undescribed transcript distributions among all yeast chromosomes

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of novel transcripts</td>
<td>3</td>
<td>9</td>
<td>2</td>
<td>16</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>5</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
expression levels of promoter-based RNA MAN3 and its immediate downstream gene PHO5 had a strong inverse correlation (Pearson’s correlation coefficient, −0.82; see Materials and Methods for details), suggesting that MAN3 is a negative regulator of PHO5. A phylogenetic comparison between four different yeast species was conducted to determine whether the promoter-based transcripts were functionally conserved. DNA sequences for promoter regions of four closely related Saccharomyces species were aligned (30), and the conserved regions were ranked as functionally conserved or neutrally conserved by chance based on a computational approach (31). Among the 50 promoter-based transcripts, 21 were functionally conserved between all four species (Z score >3; and Table 5, which is published as supporting information on the PNAS web site). The alignment results, presented in the Supporting Data Set, which is published as supporting information on the PNAS web site, can be useful in mutation-based validation studies and for deriving the secondary structures for the corresponding RNAs. We also note that the functional conservation rate of the promoter-based RNAs was not different from other regulatory promoters, confirming our initial premise that the alignment-based computational approaches are unable to identify ncRNAs in the conserved regions of the genome and highlighting the importance of whole genome tiling array measurements.

In summary, this work demonstrates a powerful and versatile method for identifying previously undescribed ncRNAs by modulating an essential RNA processing pathway through depletion of a key ribonucleoprotein enzyme component and monitoring the differential transcriptional activities during the time course of its depletion. The method is applicable to other higher eukaryotes including mammals. In S. cerevisiae, this work discovered a class of RNAs located antisense to ORFs. One member, HRA1, is likely involved in 18S rRNA maturation. In addition, this work identified and confirmed 73 other previously undescribed ncRNAs, the functions of which, when determined, will provide insights into eukaryotic biology.

Materials and Methods

Design of the Arrays. Eight identical glass-based high-density arrays were constructed by using a maskless array synthesizer (20). Each array contained 388,562 36-mer oligonucleotide probes. Among them, 384,636 tiling probes were chosen from all nuclear and mitochondrial chromosomes of S. cerevisiae and 3,926 additional probes were selected to cover the known RNAs at a higher density. Chromosome sequences and annotations used for probe design were downloaded from the Saccharomyces Genome Database (SGD) (21). Tiling probes were selected uniformly from both strands of the chromosomes with average gaps of 10 bases between the consecutive probes on the chromosome. Probes with undesirable features that might have caused difficulties in hybridization were excluded by using an algorithm described in refs. 3–5. All probe sequences and corresponding hybridization data are available from the National Center for Biotechnology Information (NCBI) gene expression omnibus (GEO) database in MIAME format (see data deposition footnote for additional information).

Hybridization Experiment. The arrays were hybridized with total RNA extracted from two S. cerevisiae strains. Strain and RNA sample preparation techniques were described in detail in ref. 14. Briefly, a conditional lethal allele was created (VS164, Table 1) by placing RPP1 under the control of GAL10 promoter in a plasmid. It allowed expression of RPP1 in galactose-containing culture medium but suppressed RPP1 expression in glucose-containing medium. A wild-type isogenic strain (VS165, Table 1), containing pYCp-GAL plasmid, was used as a control. Both strains were initially grown in galactose medium, and subsequently transferred and resuspended into glucose medium. Seven arrays were hybridized with RNA extracted from VS164 strain 0, 4, 7, 12, 16, 21, and 30 h after initial transfer to glucose medium. The remaining array was hybridized with RNA extracted from VS165 30 h after its transfer to glucose medium.

Sample Labeling. Using Gibco/BRL SuperScript Choice System, total RNA extracted from the yeast cells was converted to double-stranded cDNA. Subsequently, cDNA was labeled by using an oligo(dT) primer containing the T7 RNA polymerase promoter (5′-GGCCGATAATTTGATACGACTACATAGGGAGCCGG-3′). Briefly, 10 μg of total RNA was incubated with 1× first strand buffer, 10 mM DTT, 500 μM dNTPs, and 5 μM primer for 60 min at room temperature. The second strand was synthesized by incubation with 200 μM dNTPs, 0.07 units per μl DNA ligase, 0.27 units per μl DNA polymerase I, 0.013 units per μl RNase, 1× second strand buffer and 10 units T4 DNA polymerase for 2 h. Double-stranded cDNA was purified by using phenol-chloroform extraction and Eppendorf PhaseLock Gel tubes and ethanol precipitated, washed with 80% ethanol, and resuspended in 3 μl water. In vitro transcription (IVT) was used to produce biotin-labeled cRNA from the cDNA by using the Ambion (Austin, TX) Megascript T7 kit. Briefly, 1 μg double-stranded cDNA was incubated with 7.5 mM ATP and GTP, 5.6 mM UTP and CTP, and 1.9 mM bio-11-CTP and...
bio-16-UTP (Sigma-Aldrich) in 1× transcription buffer and 1× T7 enzyme mix for 5 h at 37°C. Before hybridization, cRNA was fragmented to an average size of 50–200 bp by incubation in 100 mM potassium acetate, 30 mM magnesium acetate, and 40 mM Tris-acetate for 35 min at 94°C. For quality control at all steps, including input RNA quality, first and second strand cDNA synthesis, in vitro transcription, and fragmentation, assay performance was monitored by running small sample aliquots on the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA).

**Hybridization and Washing.** High-density 36-mer tiling arrays were hybridized with 12 μg cRNA in 300 μl in the presence of 50 mM Mes, 0.5 M NaCl, 10 mM EDTA, and 0.005% (vol/vol) Tween-20 for 16 h at 45°C. Before application, samples were heated to 95°C for 5 min, then 45°C for 5 min, and then centrifuged at 12,000 × g for 5 min. Hybridization was performed in a hybridization oven with continuous mixing. After hybridization, arrays were washed in nonstringent (NS) buffer (6× saline-sodium phosphate-EDTA; 0.01% Tween-20) for 5 min at room temperature, followed by washing in stringent buffer (100 mM Mes, 0.01 M NaCl, and 0.01% Tween-20) for 30 min at 45°C. After washing, arrays were stained with streptavidin-Cy3 conjugate (Amersham Pharmacia) for 25 min at room temperature, followed by a 5-min wash in NS buffer, a 30-s rinse in final rinse buffer, and a blow-dry step using high-pressure grade 5 argon.

**Scanning and Normalization.** Arrays were scanned on an Axon 4000B scanner and features were extracted by using NimbleGen software (NimbleGen Systems, Madison, WI). A quantile normalization procedure was applied to normalize data from different arrays (22). In this method, probes from all arrays were mapped to a reference distribution, which itself was created by taking averages of the sorted raw data (log base 2) from all eight arrays and scaled to a median of one. A statistical analysis of genome-wide data sets was performed by plotting joint distributions of log-scaled raw data from each pair of data sets (available on request). Also, pair-wise correlation coefficients between the data sets were computed. All pairs showed strong correlations (Pearson coefficient, 0.82–0.97), except for those sets containing data measured 30 h after depletion of Rpp1 was initiated (SET7). Different normalization schemes (e.g., quantile normalization on only seven unaffected sets) were tested with SET7 to account for this unusual distribution, however, none resulted in significant differences.

**GC Content of the Probes.** Normalized probe signals were further adjusted to take into account biases due to probe GC contents. Median signals were computed for probes located within verified genes and with identical numbers of GC nucleotides. Signals for probes with higher GC contents were stronger on average. Log-normalized data of all probes were adjusted with a corrective factor, which took such GC variation into account.

**Reference Set of Unexpressed Probes.** A reference set of 14,830 probes was created by considering all promoter regions of verified genes that did not have overlaps with other annotated ORFs, RNA, or repeat regions. Promoter regions were considered to be 50–300 bases upstream from the 5′ ends of the verified ORFs. Before determining overlaps, all annotated features were extended by 50 bases at both ends to account for precursor transcripts being longer than the matured forms.

**Filtering of the Expressed Genes.** Two different procedures were used to derive probes with significant activities. They were both based on the null set of 14,830 probes derived from the promoters of verified genes.

(i) **Filtering based on absolute signal intensity.** A cutoff was chosen so that only 2.5% of the probes selected from the promoter regions had absolute signal above it. All probes that had at least one measurement above the cutoff were chosen as positive.

(ii) **Filtering based on differential activity.** The difference between the maximum and minimum signal from all time-points were computed and a cutoff was chosen following the same criteria as above. All probes that had variations greater than the cutoff were included. The same procedure was followed for the differences between the second highest and the second lowest numbers. In total, 61,020 probes were selected based on absolute expression, and only 11,613 additional probes were chosen solely based on differential activities.

**Cross-Hybridization.** To check whether some of the observed activities in the intergenic regions were because of cross-hybridization from the mismatched probes, two methods were used. The first method determined which probes aligned with multiple regions of the genome when differences of up to two bases were considered. The second method searched for smaller overlaps between segments of the probes and the entire genome by using a “frequency parameter” computed for all probes. The details on computation of frequency parameter have been discussed in refs. 3–5.

**High-Confidence Transcripts.** A subset (37,872) of probes was selected from the group of 72,633 expressed probes based on a more stringent cutoff that allowed inclusion of only 1% of the promoter-based probes. The list was further screened to discard any probes matching annotated or repeat regions. The remaining probes were combined into 1,391 longer transcribed regions and were manually screened based on the lengths of transcripts, signal intensities, and differential activities. A high-confidence list of 98 transcripts was created (Table 4 and Figs. 7–18).

**RT-PCR.** Eighty-three candidates were chosen from the high-confidence list for further verification via RT-PCR. Oligonucleotide primer pairs (20- to 25-mers) were designed from the RNA sample by using the Massachusetts Institute of Technology Primer3 online server (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3/primers.cgi) and are listed in Table 4. RNA samples from two time points (t = 4 h and t = 30 h; Rpp1-depleted cells) were converted to ssDNA, and the primers were used to amplify cDNA by using reverse transcriptase. Positive identification by RT-PCR meant that the largest ethidium bromide-stained band observed in an agarose gel corresponded to the correct size for the transcript.

**Correlation Analysis for MAN3.** To decide whether the expression of MAN3 and the downstream gene PHO5 (Fig. 7) were inversely correlated, the Pearson correlation coefficient was computed for the log-normalized average expression levels. For MAN3, the average was computed for the probes located between 431,350 and 431,650 on the Watson strand of chromosome 2. For PHO5, only the probes near its 3′ end, between bases 429,700 and 429,900 on the Crick strand of chromosome 2, were considered because those probes best represented the differential expression of the gene. The anomalous SET7 (t = 30 h) was excluded from the calculation.

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