Cwc24p, a Novel Saccharomyces cerevisiae Nuclear Ring Finger Protein, Affects Pre-snoRNA U3 Splicing*

Received for publication, September 20, 2007, and in revised form, October 31, 2007. Published, JBC Papers in Press, November 1, 2007. DOI 10.1074/jbc.M707885200

Mauricio B. Goldfeder1 and Carla C. Oliveira2

From the Department of Biochemistry, Chemistry Institute, University of São Paulo, 748 Av. Prof. Lineu Prestes, São Paulo, SP, 05508-900, Brazil

U3 snoRNA is transcribed from two intron-containing genes in yeast, snR17A and snR17B. Although the assembly of the U3 snoRNP has not been precisely determined, at least some of the core box C/D proteins are known to bind pre-U3 co-transcriptionally, thereby affecting splicing and 3′-end processing of this snoRNA. We identified the interaction between the box C/D assembly factor Nop1p and Cwc24p, a novel yeast RING finger protein that had been previously isolated in a complex with the splicing factor Cef1p. Here we show that, consistent with the protein interaction data, Cwc24p localizes to the cell nucleus, and its depletion leads to the accumulation of both U3 pre-snoRNAs. U3 snoRNA is involved in the early cleavages of 35 S pre-rRNA, and the defective splicing of pre-U3 detected in cells depleted of Cwc24p causes the accumulation of the 35 S precursor rRNA. These results led us to the conclusion that Cwc24p is involved in pre-U3 snoRNA splicing, indirectly affecting pre-rRNA processing.

In eukaryotes, three of the rRNAs (18, 5.8, and 25 S in the yeast Saccharomyces cerevisiae) are transcribed by RNA pol II and contain introns but are not polyadenylated. Instead, the U3 snoRNA 3′-end is formed after Rnt1p endonucleolytic cleavage (23).

U3 snoRNA has been reported to associate with about 30 proteins (3, 24), and the final assembly of this snoRNP seems to occur during 35 S pre-rRNA processing (25). The C/D box snoRNP core proteins, however, associate with the precursor U3 snoRNA at a very early stage of the U3 biogenesis pathway (26, 27). Snu13p binds U3 snoRNA early in processing and is important for recruitment of the other C/D box snoRNP core proteins (28). Interestingly, depletion of Snu13p has been shown to result in the accumulation of unspliced U3 snoRNA precursor (29). Binding of the human Snu13p ortholog 15.5 K to box C/D snoRNAs is essential for the subsequent association of Nop56p, Nop58p, and fibrillarin as well as the assembly factors Tip48 and Tip49 (30). Although it is thought to be recruited by Snu13p, Nop1p has been shown to associate specifically with actively transcribed box C/D snoRNA coding genes (31) and also to be associated with U18 precursor snoRNA (32).

In addition to being one of the box C/D core subunits, Snu13p also associates with U4 snoRNA (33), participating in two different RNA processing pathways, rRNA processing and splicing. In addition to the five small nuclear RNPs (U1, U2, U4, U5, and U6), other protein factors participate in splicing, among them the NTC complex, which is involved in stabilization of U6 snRNA and pre-mRNA base pairing and release of Lsm proteins (34, 35). Cef1p binds Prp19p and is part of the NTC complex (36, 37). Cwc24p has been previously iso-

* This work 7888888/* was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo Grant 05/56493-9 (to C. C. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior fellowship.

2 To whom correspondence should be addressed. Tel.: 55-11-3091-3810 (ext. 208); Fax: 55-11-3815-5579; E-mail: ccoliv@iq.usp.br.

3 The abbreviations used are: pol, polymerase; GST, glutathione S-transferase; GFP, green fluorescent protein; RNP, ribonucleoprotein.

4 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
lated in a complex with Cef1p, indicating that this novel yeast protein may be involved in splicing (38). Here we show that Cwc24p also interacts with Nop17p, which is involved in assembly of box C/D snoRNP (39), and that Cwc24p affects splicing of U3 pre-snoRNA, indirectly influencing pre-rRNA processing.

EXPERIMENTAL PROCEDURES

DNA Manipulation and Plasmid Construction—Plasmids used in this study, described in Table 1, were constructed according to the cloning techniques described by Sambrook et al. (43) and sequenced by the Big Dye method (PerkinElmer Life Sciences). Cloning strategies were as follows. CWC24 gene, encoded by the YLR323C open reading frame, was PCR-amplified from *S. cerevisiae* genomic DNA using primers 5’-CAAAAGCTCCAGGAATTCATG-3’ and 5’-GTTGATATGAGGATCTTCCAT-3’. For the two-hybrid assays, the PCR product was digested with EcoRI and BamHI and cloned into pBTM116 (40) and pGAD-C2 (41) digested with the same enzymes, generating pBTM-CWC24 and pGAD-CWC24 (which code for the fusions BD-Cwc24 and AD-Cwc24, respectively, where BD refers to the LexA DNA binding domain and AD refers to the Gal4p transcription activation domain). Plasmid pACT-CWC24 was isolated from a cDNA library obtained from the American Type Culture Collection (ATCC 87002) and consists of a GAL4-Cwc24p-(6–260) fusion (the numbers in parentheses refer to Cwc24p amino acid residues encoded by the plasmid). For heterologous expression in *Escherichia coli*, pGEX-CWC24 (which expresses GST-Cwc24 fusion protein) was generated through the insertion of an EcoRI-BamHI fragment into pGEX (GE Healthcare). Vector pET28a-CEF1 (which expresses His-Cef1p fusion protein) was generated by the insertion of a PCR-amplified BamHI-Sall DNA fragment containing the *CEF1* gene into pET28a (Novagen), digested with BamHI and XhoI. YCp111GAL-CWC24, which carries CWC24 under the control of the GAL1 promoter, was obtained by inserting a fragment from pBTM-CWC24 digested with EcoRI (following filling in with T4 DNA polymerase) and PstI into YCp111-GAL digested with NdeI (following T4 DNA polymerase 3’ overhang removal) and PstI. To determine the subcellular localization of Cwc24p by fluorescence microscopy, the plasmid pGFP-N-FUS-CWC24 was constructed by first cloning CWC24 into pBS, after digestion of the plasmid with EcoRI and Smal, and inserting a Smal-Sall fragment from the latter vector into pGFP-N-FUS (42), digested with the same enzymes.

**Yeast Maintenance, Transformation, and Sporulation—**Yeast genetic techniques were conducted as described by Sherman et al. (44). Strains described in Table 2 were maintained in yeast extract-peptone medium (YP) or synthetic medium (YNB) with 2% (w/v) galactose or glucose as carbon source as indicated and supplemented with amino acids when required. Yeast cells were transformed using a lithium acetate method (44). A ∆cwc24 diploid strain (2n, CWC24/cwc24) obtained from Euroscarf was transformed with YCP111GAL-CWC24, induced to sporulation (growth in 1% potassium acetate, 0.005% zinc acetate medium), and spore separation was performed as described elsewhere (44). Strains CWC24 and ∆cwc24/GAL1::CWC24 were grown in galactose-containing medium to stationary phase, and cell suspension was 10-fold concentrated and plated in glucose containing medium in a 10-fold serial dilution (Fig. S3A).

For the growth curve in liquid medium (Fig. 3B), conditional strain ∆cwc24/GAL1::CWC24 was transformed with plasmid pGAD-CWC24 (which constitutively expresses Cwc4 under the control of *ADH1* promoter) or pGAD as a control, resulting in strains ∆cwc24/GAL1::CWC24 and ∆cwc24/GAL1::CWC24,

### Table 1

| Plasmid | Relevant characteristics | Source or reference |
|---------|-------------------------|---------------------|
| pBTM116 | lexA DNA binding domain, TRP1, 2 μm | Ref. 40 |
| pBTM-CWC24 | lexA::CWC24, TRP1, 2 μm | This study |
| pBTM-CWC24 [X]| lexA::cwc24 [X], TRP1, 2 μm | This study |
| pGAD-C2 | GAL4 activation domain, LEU2, 2 μm | Ref. 41 |
| pACT-cwc24 [6–260] | GAL4::cwc24 [6–260], LEU2, 2 μm | This study |
| pGAD-CWC24 | GAL4::CWC24, LEU2, 2 μm | This study |
| YCp111GAL-CWC24 | GAL1::CWC24, LEU2, CEN4 | This study |
| YCp33GAL-CWC24 | GAL1::CWC24, URA3, CEN4 | This study |
| pGFP-N-FUS | MET25::GFP, CEN6, URA3 | This study |
| pGEX-CWC24 | GST::CWC24, AmpR | This study |
| pET-His-NOP17 | His::NOP17, AmpR | Ref. 39 |
| pET-His-CEF1 | His::CEF1, AmpR | This study |

* X corresponds to Cwc24p amino acid residues encoded by the plasmids.

### Table 2

| Strain | Relevant features | Reference |
|--------|------------------|-----------|
| L40 | MATα his3Δ200 trp1-901 leu2-3,112 ade2-1 lys2-801am pir1-lacZ | Ref. 45 |
| L40–1 | MATα his3Δ200 trp1-901 leu2-3,112 ade2-1 lys2-801am pir1-lacZ | 46 |
| YFG-131 | L40, pBTM-NOP17, pACT-NOP8 | 39 |
| YFG-221 | L40, pBTM-NOP17, pACT-cwc24 [6–260] | Gonzales and Oliveira, unpublished observations |
| YMG-222 | L40, pGAD, pBTM-CWC24 | This study |
| YMG-223 | L40, pBTM-NOP17, pACT-cwc24 [X]| This study |
| CWC24 | MATα his31, leu2Δ3; lys2Δ3; ura3Δ0; CWC24 | Research Genetics |
| CWC24/cwc24-kanα | MATα his3Δ1, his3Δ1, leu2Δ2; lys2Δ0/LYS2; ura3Δ0/ura3Δ0; CWC24/cwc24-kanα | Euroscarf |
| ∆cwc24/GAL1::CWC24 | MATα his3Δ1, leu2Δ3; lys2Δ0/LYS2; ura3Δ0/ura3Δ0; CWC24/cwc24-kanα | This study |
| ∆cwc24/GAL1::CWC24, AD | ∆cwc24/GAL1::CWC24, YCp33GAL-CWC24, pGAD | This study |
| ∆cwc24/GAL1::CWC24, AD-CWC24 | ∆cwc24/GAL1::CWC24, YCp33GAL-CWC24, pGAD-CWC24 | This study |
| W303 | MATα Tn10, ura3Δ2; ade2-1/ade2-1/leck1-112/leck1-112; his3-11/his3-11/ade2-1/ade2-1/leck1-112/leck1-112 | This study |
| YMG-224 | W303, pGFP-N-FUS | This study |
| YMG-225 | W303, pGFP-N-FUS-CWC24 | This study |

* X corresponds to Cwc24p amino acid residues encoded by the plasmids.
AD-Cwc24, respectively. Wild type CWC24 strain was also used as a control. Cells were grown in galactose-containing medium overnight until stationary phase and then shifted to glucose medium. Yeast Two-hybrid Assays—Fusion proteins with either LexA DNA binding domain (BD-protein) or Gal4p transcription activation domain (AD-protein) were expressed in the host strain L40 (45), which has two reporter genes for two-hybrid interactions integrated into the genome: yeast HIS3 and E. coli lacZ. Transformants were plated in minimal medium lacking histidine as a first selection, and viable clones were further tested for β-galactosidase activity, as described below. Exponentially growing cultures in minimal medium (supplemented with histidine) were 10-fold concentrated and either transferred to nitrocellulose membranes and incubated overnight at 30 °C for β-galactosidase activity assay (45) or plated in His− medium in a 10-fold serial dilution. β-Galactosidase activity in cell extracts generated in buffer Z was quantitated using ortho-nitrophenylacetate as a positive control, and strain YMG-222 was used as negative control for two-hybrid interaction (Table 2).

Immunofluorescence Analysis—In order to determine its subcellular localization, Cwc24p was expressed in the strain YMG-224 with an NH2-terminal green fluorescent protein (GFP) tag, encoded by the plasmid pGFP-N-FUS-CWC24. DNA in the cell nucleus was stained using Hoechst.

Protein Pull-down and Immunoblot Analysis—In the pull-down assay, cellular extracts generated in phosphate-buffered saline of E. coli cells expressing either GST or GST-Cwc24p (TE1; see Fig. 1) were incubated for 1 h at 4 °C with 250 μl of glutathione-Sepharose beads (GE Healthcare), and the unbound (FT1) material was washed. Beads where then incubated with cellular extract containing His-Nop17p or His-Cef1p (TE2; not shown), flow-through was collected (FT2), and beads where once again washed with phosphate-buffered saline. Bound proteins were eluted (B) with 50 mM Tris, pH 8.0, 10 mM reduced glutathione, resolved on SDS-PAGE, and transferred to polyvinylidine difluoride membranes (Bio-Rad). Membranes were incubated with anti-polyhistidine antibody (GE Healthcare), anti-GST antiseraum (Sigma), anti-Cwc24p antiseraum, or anti-Nop17p antiseraum, and the immunoblots were developed using the ECL system (GE Healthcare). To analyze the presence of Cwc24p in the strain Δcwc24/GAL1::CWC24 grown at the restrictive condition, yeast cells were collected at various times after medium shift and broken with glass beads. Immunoblot of the cellular extracts was conducted as described above, and the membrane was additionally incubated with anti-Nip7 as an internal control.

RNA Analysis—Exponentially growing cultures of yeast strains were shifted from galactose to glucose medium. At various times, samples were collected and quickly frozen in a dry ice-ethanol bath. Total RNA was isolated from yeast cells by a modified hot phenol method (39). RNAs were separated by electrophoresis (20 μg of total RNA was loaded in each lane) on 1.3% agarose gels, followed denaturation with glyoxal (43), and transferred to Hybond nylon membranes (GE Healthcare). Membranes were probed with 32P-labeled oligonucleotides (Table 3) complementary to specific regions of the 35 S pre-rRNA or with a 32P-labeled DNA fragment corresponding to scR1 RNA, using the hybridization conditions described previously (47) and analyzed in a PhosphorImager (Amersham Biosciences).

Microarray Analysis of Splicing—Total RNA was extracted from yeast strains CWC24 and Δcwc24/GAL1::CWC24 grown in glucose medium for different periods of time and used as templates for cDNA synthesis. cDNA labeling and microarray hybridization and analysis were performed as described (51).

Primer Extension Analysis—Total RNA extracted as described above was used for primer extension analysis. Reactions were performed by annealing 1 pmol of 32P-labeled oligonucleotide to 5 μg of total RNA. Following annealing, extension was performed with 100 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and dNTPs (0.5 mM) for 30 min at 37 °C. cDNA products were precipitated, resuspended in H2O, treated with RNase A, denatured, and analyzed on 6% denaturing polyacrylamide gels. Gels were dried and analyzed in a PhosphorImager. Oligonucleotides used in primer extension analyses are listed in Table 3.

RESULTS

Cwc24p is an essential yeast protein that had previously been isolated in a complex with Cef1p (38), but its function had not been addressed. Cwc24p is a 30-kDa protein containing a zinc finger and a RING domain. In a two-hybrid screen with Nop17p, we identified the interaction between BD-Nop17p and AD-Cwc24p (6–260) (39).4 We subsequently tested the interaction between these two proteins in the two-hybrid sys-

---

4 F. A. Gonzales and C. C. Oliveira, unpublished results.
tem by switching the tags, and the results show that the interaction is independent of the tag, although it is stronger when Nop17p is fused to the DNA binding domain (BD-Nop17p; Fig. 1, A and B). In order to determine the Nop17p-interacting domain of Cwc24p, deletion mutants of the latter were created. The mutants were deleted of either the zinc finger, the RING domain, or the amino-terminal region of the protein (Fig. 1A). The results show that the central portion of Cwc24p, containing the zinc finger domain, BD-Cwc24-(86–164), is sufficient for interaction with Nop17p (Fig. 1B). A Cwc24p amino-terminal fragment, not containing the zinc finger domain, BD-Cwc24-(1–85), however, still interacts with Nop17p, although less strongly (Fig. 1B). The interaction between these two proteins was confirmed through GST pull-down assays of the recombinant proteins His-Nop17p and GST-Cwc24p (Fig. 1C). In these experiments, GST or GST-Cwc24p was immobilized on a glutathione-Sepharose resin, after which total extract of E. coli cells expressing His-Nop17p was incubated with the resin. After washing and elution of bound proteins, fractions were analyzed through immunoblot with various antisera, anti-His, and anti-Nop17 to detect His-Nop17p (Fig. 1C, top) or anti-GST and anti-Cwc24 to detect GST-Cwc24p (Fig. 1C, bottom). Both protein fusions, GST-Cwc24p and His-Nop17p, seem to be unstable under the electrophoresis and immunoblot conditions used, since breakdown products are detected. Anti-GST antiserum, however, detects only one band corresponding to GST-Cwc24p fusion (Fig. 1C).

Although Cwc24p had been identified in a complex with Cef1p (38), it was not known whether the interaction between these two proteins was direct or whether it depended on another NTC subunit. In order to address that question, the recombinant proteins GST-Cwc24p and His-Cef1p were used in GST pull-down assays, as described above. The results show that GST-Cwc24p pulls down His-Cef1p, leading us to the conclusion that these proteins interact directly with each other (Fig. 1D).

The interaction of Cwc24p with the NTC complex subunit Cef1p and with Nop17p indicates a nuclear subcellular localization for the protein. In order to confirm that, a GFP-Cwc24p fusion protein was used to monitor its localization through fluorescence microscopy (Fig. 2). In accordance with the interaction data, GFP-Cwc24p localizes to the nucleus (Fig. 2). Despite
Cwc24p Affects Pre-snoRNA U3 Splicing by Interacting with Cef1p

FIGURE 3. CWC24 is an essential S. cerevisiae gene. A, 10-fold serial dilution of CWC24 and Δcwc24/GAL1::CWC24 strains growing on glucose-containing plates. B, growth curve of CWC24, Δcwc24/GAL1::CWC24, and Δcwc24/GAL1::CWC24, AD-Cwc24 strains in glucose medium. C, Western blot analysis of GAL1::CWC24 expression in Δcwc24/GAL1::CWC24 cells in glucose medium. Cwc24p was detected with an anti-Cwc24p serum, and Nip7p, used as an internal control, was detected with an anti-Nip7p serum. Endogenous Cwc24p could not be detected due to low expression level in the wild type strain.

The interaction between Cwc24p and Nop17p, however, Cwc24p is not concentrated in the nucleolus but rather is distributed throughout the nucleus.

In order to characterize Cwc24p function, we first had to obtain a conditional mutant strain containing the CWC24 gene under the control of an inducible promoter. A heterozygous diploid deletion strain (CWC24/cwc24::kan⁶) was obtained from Euroscarf and transformed with a plasmid containing the CWC24 gene under control of the GAL1 promoter. Sporulation of this strain generated the haploid conditional strain Δcwc24/GAL1::CWC24, which grows on galactose-containing medium, but not on glucose medium (Fig. 3). Growth analysis of the conditional strain shows that Δcwc24/GAL1::CWC24 starts to grow more slowly after 12 h in glucose medium (Fig. 3B). This conditional strain was then transformed with a plasmid encoding the fusion protein AD-Cwc24p (AD corresponds to Gal4p transcription activation domain) under control of the constitutive promoter ADH1 (Δcwc24/GAL1::CWC24, AD-Cwc24) and tested for growth in glucose medium. The results show that AD-Cwc24p rescues the growth in glucose (Fig. 3B). Immunoblot assays to monitor Cwc24p expression in the conditional strain show that the Cwc24p band can only be detected up to 12 h in glucose, corresponding to the time when the strain starts to grow more slowly (Fig. 3C). These results indicate that Cwc24p is a very stable protein, being detected up to 12 h after the shift to glucose medium (repression of GAL1::CWC24; Fig. 3C).

Since Cwc24p interacts with Nop17p, a factor that affects assembly of box C/D snoRNP and, consequently, pre-rRNA processing (39), we next investigated the possible involvement of Cwc24p in this process. Δcwc24/GAL1::CWC24 cells and isogenic haploid wild type cells were incubated in glucose-containing medium for 30 h and subsequently subjected to RNA pulse-chase labeling experiments with [³H]URacil. rRNA analysis from these cells shows that the 35 S pre-rRNA processing is much slower in the strain Δcwc24/GAL1::CWC24 than in the control CWC24 strain, since the precursor rRNA is still visible after 30 min of chase in the conditional strain, whereas it is no longer visible after 3 min of chase in the wild type strain (supplemental Fig. S1). It also appears that since processing is less efficient after depletion of Cwc24p, transcription by RNA pol I is being inhibited, since the portion of the film containing the conditional strain RNAs had to be exposed for a longer period than the CWC24 samples. Surprisingly, despite the slower pre-rRNA 35 S processing in the strain Δcwc24/GAL1::CWC24, only minor alterations could be detected in the kinetics of mature rRNA formation, which indicates that depletion of Cwc24p confers a mild phenotype to the cells, so that pre-35 S pre-rRNA processing is not blocked but rather slowed down.

Interestingly, similar effects have been reported for NOP1 and NOP56 mutants, two box C/D core subunits (14, 52).

rRNA processing was also analyzed through Northern blot hybridizations. The results confirm the pulse-chase labeling experiments, also showing the accumulation of the precursor rRNA 35 S (2.2-fold accumulation) and the intermediates 23 S (1.3-fold), 20 S (1.3-fold), and 27 S (3.0-fold) in the conditional strain over time in glucose medium (Fig. 4). Quantitation of the bands also show a 30% decrease on the mature rRNAs 25 and 18 S levels. From these data, it can be inferred that Cwc24p affects the initial steps of pre-rRNA processing, which involve cleavage at A₀, A₁, and A₂ sites. In order to analyze the cleavage efficiency at these sites in the Δcwc24/GAL1::CWC24 strain, we performed primer extension experiments, using primers that anneal to the pre-rRNA downstream of these cleavage sites. Interestingly, the results show a higher concentration of longer products in the conditional strain, due to the higher levels of nonprocessed pre-rRNAs, although the main cleavage reactions are still detected (Fig. S2). A similar effect was observed for mutants of the box C/D core protein Nop58p, which have also been shown to have little decrease in the efficiency of cleavage at A₉ site (11, 22). The primer extension data presented here corroborate the Northern hybridizations and pulse-chase labeling results, which show higher levels of pre-rRNAs in the conditional strain but only a small change in mature rRNAs concentration. Similar effects on rRNA processing have been reported for temperature-sensitive mutants of NOP1p (53).

The interaction of Cwc24p with Cef1p and the nuclear localization of the protein indicated that Cwc24p could also be involved in splicing. Therefore, we decided to analyze the efficiency of splicing in Δcwc24/GAL1::CWC24 cells. In a global
Cwc24p Affects Pre-snoRNA U3 Splicing by Interacting with Cef1p

FIGURE 4. Northern blot analysis of pre-rRNA processing. A, 20 µg of total RNA extracted from cells incubated in glucose medium for different periods of time and hybridized against specific oligonucleotide probes. The relative positions of the probes on the 35 S pre-rRNA are indicated in B. Bands corresponding to the major intermediates and to the mature rRNAs are indicated on the right. The lower panels show hybridizations with probes against the 5 S rRNA and the scr1 RNA, used as internal controls. B, structure of the 35 S pre-rRNA and major intermediates of the rRNA processing pathway in S. cerevisiae. The positions of the probes used for Northern blot hybridizations are indicated below the 35 S pre-rRNA. Processing of 35 S pre-rRNA starts with endonucleolytic cleavages at sites A₂ and A₁, in the 5′-ETS, generating 32 S pre-rRNA. The subsequent cleavage at site A₁ in ITS1, generates the 20 S and 27 S pre-rRNAs (dotted arrows indicate a possible pathway including the aberrant intermediate 23 S). The 20 S pre-rRNA is then processed at site D to the mature 18 S rRNA. The major processing pathway of the 27 S pre-rRNA involves cleavage at site A₂, producing 27 SA₂, which is digested quickly by exonucleases to generate the 27 SBs (27 SB short) pre-rRNA. The subsequent processing step occurs at site B₂ at the 3′-end of the mature 25 S rRNA. Processing at sites C₁ and C₂ separates the mature 25 S rRNA from the 7 S pre-rRNA. This pre-rRNA is subsequently processed exonucleolytically to generate the mature 5.8 S, rRNAs. A fraction of the 27 SA₂ pre-rRNA is processed at the 5′-end by a different mechanism and, following processing at the remaining sites, gives rise to the 5.8 S (5.8 S long) rRNA, which is 6–8 nucleotides longer than the 5.8 S rRNA at the 5′-end.

approach (51), splicing of all yeast genes was analyzed by microarray by comparing the conditional and wild type strains growing in galactose or glucose media. The results showed that very few pre-mRNAs had their splicing affected in the mutant strain (Fig. 5). However, splicing of both U3 pre-snoRNAs, coded by snr17A and snr17B, were among the most affected in the cell after depletion of Cwc24p. Splicing of TEF4 and IMD4 pre-mRNAs is also affected in the conditional strain (Fig. 5), and interestingly, the box C/D snoRNAs snr38 and snr54 are coded in the introns of these pre-mRNAs, respectively. RPL7A and ASC1 pre-mRNAs also host C/D box snoRNAs (snr39 and snr24, respectively) and are slightly affected upon depletion of Cwc24p.

The microarray results were confirmed through Northern blot hybridization and primer extension experiments, which show an accumulation of pre-U3 snoRNA upon depletion of Cwc24p (Fig. 6). Interestingly, both pre-U3 snoRNAs, encoded by snr17A and snr17B, accumulate in the nonpermissive condition (Fig. 6B). The oligonucleotides used in the primer extension experiments are complementary to the U3 second exon (P10) or to pre-U3A or pre-U3B introns, P11 and P12, respectively. Some cross-hybridizations are detected, since pre-U3A oligonucleotide (P11) also hybridizes with pre-U3B intron, although less efficiently (Fig. 6C, P11). Similarly, pre-U3B oligonucleotide (P12) hybridizes also with pre-U3A intron (Fig. 6C, P12). Northern blot hybridization with primer P10 confirms the accumulation of pre-U3 snoRNAs upon depletion of Cwc24p (Fig. 6D). The results obtained from various RNA preparations and three different methods show that the depletion of Cwc24p leads to the accumulation of pre-U3 snoRNAs, although the levels of mature U3 are not strongly affected. These results indicate that Cwc24p is a U3 splicing efficiency factor. Interestingly, depletion of the core C/D box protein and splicing factor Snu13p has also been shown to result in the accumulation of unspliced U3 snoRNA precursor without altering much the levels of mature U3 snoRNA (29).

Cwc24p has two domains potentially involved in protein-protein or protein-nucleic acid interactions, a zinc finger and a RING finger. As shown above, a portion of Cwc24p containing the zinc finger is involved in the interaction with Nop17p (Fig. 1). In order to determine whether the role of Cwc24p in splicing of pre-snoRNA U3 is due to direct protein-RNA interaction, RNA co-immunoprecipitation was performed, using a ProtA-Cwc24p fusion. The results showed that Cwc24p does not co-precipitate RNAs, indicating that Cwc24p is not involved in stable RNA interactions (Fig. S3, A and B). Interestingly, however, recombinant Cwc24p binds DNA in vitro, although nonspecifically and with very low affinity (Fig. S3C). These results indicated that Cwc24p could affect splicing of pre-U3 snoRNA co-translationally. In order to address that question, chromatin immunoprecipitation experiments were performed in which snr17A and -B promoter and coding regions were probed. Surprisingly, Cwc24p does not co-precipitate chromatin (data not shown), leading us to the conclusion that Cwc24p participates in pre-U3 splicing through protein-protein interactions. Accordingly, a truncated Cwc24p mutant, containing only the zinc finger and RING domains, complements growth of the conditional mutant in glucose medium (Fig. S4). Moreover, a single amino acid substitution in the zinc finger domain results in no complementation of growth of Δcwc24/GAL1::CWC24 under conditions in which CWC24 expression is repressed.
DISCUSSION

Cwc24p is an essential yeast protein previously identified by affinity isolation in Cef1p complexes (38), although its function had not yet been addressed. In this study, we identified the direct interactions between Cwc24p and Cef1p and a box C/D snoRNP assembly factor, Nop17p (39), and showed that Cwc24p is involved in pre-U3 splicing, indirectly affecting pre-35S rRNA processing.

Analysis of Cwc24p sequence shows conserved domains, a zinc finger and a RING domain. These domains are conserved in other eukaryotic proteins, and sequence alignments indicate that there may be Cwc24p orthologs in other organisms, although it remains to be determined. Mapping of Cwc24p regions involved in Nop17p interaction showed that the central portion of the protein, including the zinc finger, is responsible for the interaction. Although zinc fingers are generally considered as DNA binding domains, they are also responsible for protein-RNA and protein-protein interactions (54). Interestingly, Cef1p also contains a zinc finger domain, and Prp19p contains a RING finger-like domain, both of which are considered to be involved in protein-protein interactions (36, 55).
ther indicating that Cwc24p zinc finger and RING domains are involved in protein interaction, a truncated Cwc24p mutant containing these domains complements growth of the conditional mutant strain \( \Delta cwc24/GAL1::CWC24 \) in glucose, whereas the insertion of a single amino acid mutation in the Cwc24p zinc finger no longer complements growth in glucose. These results indicate that protein interaction is responsible for Cwc24p molecular function.

Depletion of Cwc24p leads to the accumulation of 35 S pre-rRNA, although the levels of the mature rRNAs are only slightly affected. Interestingly, a similar phenotype has been reported for a temperature-sensitive mutant of Nop1p, a box C/D core subunit (53). Analysis of the 35 S pre-rRNA cleavage sites that are affected in the \( \Delta cwc24/GAL1::CWC24 \) strain by primer extension showed that although bands corresponding to precursor rRNAs are detected, the cleavages at \( A_1 \) and \( A_2 \) are little affected, the strongest inhibition being at site \( A_0 \). It is important to note that some mutants of box C/D core proteins Nop58p and Snu13p also show little inhibition of cleavage at \( A_1 \) (11, 33).

Since Cwc24p depletion leads to the accumulation of the 35 S precursor, mainly affecting the early cleavage reactions, directed by the snRNPs U3, U14, snR10, and snR30 (56, 57). However, despite its interaction with Nop17p, a nucleolar protein involved in assembly of box C/D snoRNPs, Cwc24p localizes to the nucleus rather than being concentrated in the nucleolus. That observation indicated that the effects of the Cwc24p depletion on 35 S pre-rRNA might be indirect. Among the snoRNPs involved in the early 35 S pre-rRNA cleavages, U3 and U14 are of box C/D and therefore associate with Nop1p. Because Cwc24p also binds Cef1p, it was hypothesized that it could affect splicing, and coincidentally, U3 is encoded by two intron-containing genes in yeast, snR17A and snR17B. As shown here, Cwc24p actually affects splicing of U3 pre-snoRNAs. Cwc24p might influence specifically pre-U3 splicing by protein-protein interaction, since recombinant Cwc24p did not bind RNA directly \textit{in vitro}; nor did ProtA-Cwc24p co-immunoprecipitate RNA from total yeast extracts. The data shown here lead to the conclusion that Cwc24p is a splicing efficiency factor, whose depletion causes accumulation of pre-snoRNA U3 but does not affect significantly assembly or stability of mature U3 snoRNAs. Similar effects on U3 snoRNA splicing and stability have been reported for the box C/D core subunits Nop1p, Nop56p, and Snu13p (14, 29, 52, 53).

A large number of proteins have been shown to be part of the mature U3 snoRNP (3), although it remains unclear whether some of those factors may bind pre-U3 snoRNA co-transcriptionally, thereby influencing its splicing. U3 snoRNA 3’-end processing involves Rnt1p cleavage and 3’-5’ trimming by the exosome and probably also the Rex complex (23). Lhp1p binds a U3 processing intermediate at a 3’-end poly(U) tract, inhibiting further 3’-5’ trimming. After splicing has taken place, binding of box C/D core proteins Nop1p and Nop58p displaces Lhp1p, allowing the exosome to form the mature 3’-end of U3 snoRNA (23). Interestingly, Nop17p interacts with Nop58p, the exosome subunit Rrp43p (39), and with Cwc24p. Lsm complexes have also been shown to be involved in processing of U3 snoRNA, binding internal poly(U) tracts at the 3’-end of pre-U3 (58). Although Lsm proteins and Lhp1p are important for the

![Schematic representation of a model for Cwc24p function.](image)
Cwc24p Affects Pre-snoRNA U3 Splicing by Interacting with Cef1p

site sequence and that the 5′-end of the intron base pairs with exon 1 (26). These observations may explain why Cwc24p is required for efficient splicing of these pre-snoRNAs.

There is increasing evidence of the coordinated regulation of rRNA processing and splicing. Prp43p, a DEK/H-box helicase involved in the late steps of splicing (62, 63), has recently been shown to localize to the nucleolus and also to influence rRNA processing (64). Prp43p has also been identified in a complex with Cwc23p (65), which, as Cwc24p, is complexed with Cef1p (38). The presence of these proteins in different subcomplexes may allow the regulation of the processes they are involved in. Interestingly, the ribosome protein genes form the major group of genes containing introns in yeast (51, 66). It has been shown that proteins associated with promoters of ribosome protein genes may also be involved in RNA pol I transcription and rRNA processing (67) and that the CURI complex may regulate both the transcription of ribosome protein genes and the early steps of rRNA processing directed by U3 snoRNP (68). The coordinated regulation of ribosome formation seems, therefore, to occur at various levels, including RNA pol I and pol II transcription, rRNA processing, and splicing of both ribosome protein mRNAs and pre-U3 snoRNA.

Acknowledgments—We are indebted to Jeffrey A. Pleiss and Christine Guthrie for providing technical expertise and reagents for microarray experiments. We also thank Juliana S. Luz and Nilson I. T. Zanchin for anti-Cwc24p and anti-Nip7p antisera, respectively, and Tereza C. Lima Silva and Zildene G. Correa for DNA sequencing.

REFERENCES

1. Fatica, A., and Tollervey, D. (2002) Curr. Opin. Cell Biol. 14, 313–318
2. Grandi, P., Rybin, V., Bassler, J., Petfalski, E., Strauss, D., Marzioch, M., Schäfer, T., Kuster, B., Tschochner, H., Tollervey, D., Gavin, A. C., and Hurt, E. (2002) Mol. Cell. 10, 105–115
3. Dragon, F., Gallagher, J. E. G., Compagnone-Post, P. A., Mitchell, B. M., Granneman, S., Gallagher, J. E. G., Vogelzangs, J., Horstman, W., Venrooij, W. J., Baserga, S. J., and Pogacic, V. (2002) Curr. Opin. Cell Biol. 14, 319–327
4. Bassler, J., Grandi, P., Rybin, V., Petfalski, E., Tollervey, D., Lechner, J., and Hurt, E. (2002) Mol. Cell. 10, 517–529
5. Maxwell, E. S., and Fournier, M. J. (1995) Methods Enzymol. 219, 5539–5547
6. Baserga, S. J., Walz, T., and Gould, K. (2005) Mol. Cell. Biol. 25, 7053–7065
7. Tollervey, D., Lehtonen, H., Carmo-Fonseca, M., and Hurt, E. C. (1991) EMBO J. 10, 337–342
8. Warner, J. R. (2001) Cell 105, 133–136
9. Schimmang, T., Tollervey, D., Kern, H., Frank, R., and Hurt, E. C. (1989) EMBO J. 8, 4015–4024
10. Tollervey, D., and Aris, J. P. (1997) Chromosoma 105, 515–522
11. Hughes, J. M., and Ares, M., Jr. (1991) J. Biol. Chem. 266, 16453–16463
12. Bhatt, R., and Tollervey, D. (2000) Mol. Cell. Biol. 20, 5415–5424
13. Kufel, J., Allmang, C., Chanfreau, G., Petfalski, E., Lafontaine, D. L. J., and Tollervey, D. (2000) Mol. Cell. Biol. 20, 5415–5424
14. Bleichert, F., Granneman, S., Osheim, Y. N., Beyer, A. L., and Baserga, S. J. (2006) Proc. Natl. Acad. Sci. U. S. A., 103, 9464–9469
15. Granneman, S., Gallagher, J. E. G., Vogelzangs, J., Horstman, W., Venrooij, W. J., Baserga, S. J., and Pujol, G. M. (2003) Nucleic Acids Res. 31, 1877–1887
16. Mougin, A., Grémory, A., Banroques, J., Ségault, V., Fournier, R., Brulé, F., Chevrier-Miller, M., and Branlant, C. (1996) RNA 2, 1079–1093
17. Watkins, N. J., Emm, I., Ingellinger, D., Schneider, C., Hosbach, M., Uzlhaub, H., and Lührmann, R. (2004) Mol. Cell. 16, 789–798
18. Cléry, A., Senty-Ségault, V., Leclerc, F., Raué, H. A., and Branlant, C. (2007) Mol. Cell. Biol. 27, 1191–1206
19. Stevens, S. W., Barta, I., Ge, H. Y., Moore, R. E., Young, M. K., Lee, T. D., and Abelson, J. (2001) RNA 7, 1543–1553
20. Watkins, N. I., Dickmanns, A., and Lührmann, R. (2002) Mol. Cell. Biol. 22, 8342–8352
21. Morlando, M., Ballarino, M., Greco, P., Caffarelli, E., Dichtl, B., and Bozzi, Zanchi, N. I. T., Luz, J. S., and Oliveira, C. C. (2005) Mol. Biol. 346, 437–455
22.荻野, 二股, 和 Gould, K. (2002) RNA 8, 798–815
23. Ohi, M. D., and Shuler, S. J. (2002) Methods Enzymol. 245, 241–263
24. James, P., Halladay, J., and Craig, E. A. (1996) RNA 2, 337–342
25. Ohi, M. D., Link, A. J., Ren, L., Jennings, I. L., McDonald, W. H., and Gould, K. L. (2002) Mol. Cell. Biol. 22, 2011–2024
26. Schimmang, T., Tollervey, D., Kern, H., Frank, R., and Hurt, E. C. (1989) EMBO J. 8, 4015–4024
27. Niedenthal, R. K., Riles, L., Johnston, M., and Hegemann, J. H. (1996) FEBS Lett. 379, 773–786
28. Sabbrook, I., Maniatis, T., and Fritsch, E. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
29. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
30. Zanchin, N. I. T., Luz, J. S., and Oliveira, C. C. (2005) J. Mol. Biol. 346, 437–455
31. Bartel, P. L., and Fields, S. (1995) Methods Enzymol. 254, 241–263
32. Voger, W. J., and Lee, T. D., and Abelson, J. (2001) Mol. Cell. Biol. 21, 5011–5015
33. Fatica, A., Cronshaw, A. D., Dlakic, M., and Tollervey, D. (2002) Mol. Cell. 9, 341–351
34. Oliveira, C. C., Gonzales, F. A., and Zanchin, N. I. T. (2002) Nucleic Acids Res. 30, 4186–4198
35. Bolet, C., Nolli-Dacry, C., Caizergues-Ferrer, M., and Henry, Y. (2002) Mol. Cell. Biol. 22, 7053–7065
36. Pleiss, J. A., Whittworth, G. B., Bergkessel, M., and Guthrie, C. (2007) PLoS Biol. 5, e90
37. Tollervey, D., Leighton, H., Carnero-Fonseca, M., and Hurt, E. C. (1991) EMBO J. 10, 573–583
38. Tollervey, D., Leighton, H., Jansen, R., Kern, H., and Hurt, E. C. (1993) Cell 72, 443–457
39. Rispens, J., Liew, C. K., Loughlin, F. E., Crossley, M., and Mackay, J. P. (2006) Trends Biochem. Sci. 32, 63–70
40. Ohi, M. D., Kooi, C. W. V., Rosenberg, J. A., Ren, L., Hirsch, J. P., Chazin, W. J., Walz, T., and Gould, K. (2005) Mol. Cell. Biol. 25, 451–460
41. Venema, J., and Tollervey, D. (1999) Annu. Rev. Genet. 33, 261–311
42. Nazar, R. N. (2004) J. Cell Biol. 170, 103–116
Cwc24p Affects Pre-snoRNA U3 Splicing by Interacting with Cef1p

58. Kufel, J., Allmang, C., Petfalski, E., Beggs, J., and Tollervey, D. (2003) J. Biol. Chem. 278, 2147–2156
59. Kufel, J., Allmang, C., Verdone, L., Beggs, J., and Tollervey, D. (2003) Nucleic Acids Res. 31, 6788–6797
60. Preti, M., Guffanti, E., Valinuto, E., and Dieci, G. (2006) Biochem. Biophys. Res. Commun. 351, 468–473
61. Chan, S. P., Kao, D. I., Tsai, W. Y., and Cheng, S. C. (2003) Science 302, 279–282
62. Arenas, J. E., and Abelson, J. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11798–11802
63. Martin, A., Schneider, S., and Schwer, B. (2002) J. Biol. Chem. 277, 17743–17750
64. Combs, D. J., Nagel, R. J., Ares, M., Jr., and Stevens, S. W. (2006) Mol. Cell. Biol. 26, 523–534
65. Gavin, A. C., Boeshe, M., Krause, R., Grandi, P., Marzioch, M., Bauer, A., and Superti-Furga, G. (2002) Nature 415, 141–147
66. Clark, T. A., Sugnet, S. W., and Ares, M., Jr. (2002) Science 296, 907–910
67. Hall, D. B., Wade, J. T., and Struhl, K. (2006) Mol. Cell. Biol. 26, 3672–3679
68. Rudra, D., Mallick, J., Zhao, Y., and Warner, J. R. (2007) Mol. Cell. Biol., 27, 4815–4824