Pseudouridylation of yeast U2 snRNA is catalyzed by either an RNA-guided or RNA-independent mechanism

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Yeast U2 small nuclear RNA (snRNA) contains three pseudouridines (Ψ35, Ψ42, and Ψ44). Pus7p and Pus1p catalyze the formation of Ψ35 and Ψ44, respectively, but the mechanism of Ψ42 formation remains unclear. Using a U2 substrate containing a single ³²P radiolabel at position 42, we screened a GST-ORF library for pseudouridylation activity. Surprisingly, we found a Ψ42-specific pseudouridylation activity that coincided with Nhp2p, a protein component of a Box H/ACA sno/scaRNP (small nucleolar/Cajal body-specific ribonucleoprotein). When isolated by tandem affinity purification (TAP), the other protein components of the H/ACA sno/scaRNP also copurified with the pseudouridylation activity. Micrococcal nuclease-treated TAP preparations were devoid of pseudouridylation activity; however, activity was restored upon addition of RNAs from TAP preparations. Pseudouridylation reconstitution using RNAs from a Box H/ACA RNA library identified snR81, a snoRNA known to guide rRNA pseudouridylation, as the Ψ42-specific guide RNA. Using the snR81-deletion strain, Nhp2p- or Cbf5p-conditional depletion strain, and a *cbf5* mutation strain, we further demonstrated that the pseudouridylation activity is dependent on snR81 snoRNP *in vivo*. Our data indicate that snRNA pseudouridylation can be catalyzed by both RNA-dependent and RNA-independent mechanisms.

The EMBO Journal (2005) 24, 2403–2413. doi:10.1038/sj.emboj.7600718; Published online 16 June 2005

Subject Categories: RNA

Keywords: pseudouridylation reconstitution; RNA pseudouridylation; *S. cerevisiae*; snR81 snoRNP; U2 snRNA

Introduction

All five spliceosomal small nuclear RNAs (snRNAs) (U1, U2, U4, U5, and U6) contain a large number of post-transcriptional modifications, including a 5' cap and internal 2'-O-

methylation and pseudouridylation sites (Massenet *et al*, 1998). Remarkably, the internally modified nucleotides are often clustered in regions important for pre-mRNA splicing, suggesting their functional role in this process (Yu *et al*, 2005). Among all snRNAs, vertebrate U2 is the most extensively modified (Massenet *et al*, 1998). In fact, more than 10% of its total ~189 nucleotides are either 2'-O-methylated or pseudouridylated. Most noticeably, all six uridines in the branch site recognition region and its adjacent 3' region are converted to pseudouridines following transcription. Three of these pseudouridines (Ψ34, Ψ41, and Ψ43) have counterparts in *Saccharomyces cerevisiae* U2 snRNA (Ψ35, Ψ42, and Ψ44, as numbered in yeast U2) (Figure 1).

The functional importance of U2 modifications in pre-mRNA splicing and small nuclear ribonucleoprotein (snRNP) biogenesis was recently confirmed experimentally in vertebrates (Yu *et al*, 1998; Donmez *et al*, 2004; Zhao and Yu, 2004) and in *S. cerevisiae* (Yang *et al*, 2005). Consistently, the NMR study from the Greenbaum group (Newby and Greenbaum, 2002) also showed that, when paired with the pre-mRNA branch site, a pseudouridine in the U2 branch site recognition region (equivalent to Ψ35 of yeast U2) is favored over uridine for maintaining the bulge of the branch point nucleotide adenosine for nucleophilic attack in the first step of splicing reaction. The importance of the pseudouridine in splicing is further supported by the recent work of Valadkhan and Manley (2003), who showed that pseudouridylation at the same position greatly enhances the production of X-RNA, a product generated by a splicing-related reaction in a cell- and protein-free system (Valadkhan and Manley, 2003).

Experimental and bioinformatic data accumulated thus far suggest that vertebrate U2 snRNA and the other vertebrate spliceosomal snRNAs are modified by an RNA-guided mechanism (Yu *et al*, 2005). Specifically, pseudouridylation is catalyzed by a class of RNA-protein complexes called Box H/ACA sno/scaRNPs (small nucleolar/Cajal body-specific RNPs), each containing four core proteins and a single RNA that base-pairs with the target RNA, thereby directing pseudouridylation at specific sites (Ganot *et al*, 1997; Ni *et al*, 1997). Similarly, 2'-O-methylation is catalyzed by another class of RNA-protein complexes, named Box C/D sno/scaRNPs, in which a single RNA component serves as a guide for site-specific modification (Cavaille *et al*, 1996; Kiss-Laszlo *et al*, 1996).

By contrast, current experimental data indicate that modification of *S. cerevisiae* U2 snRNA is catalyzed by an RNA-independent mechanism (Massenet *et al*, 1999; Ma *et al*, 2003). It has been reported that pseudouridylation at positions 35 and 44 is catalyzed by Pus7p (Ma *et al*, 2003) and Pus1p (Massenet *et al*, 1999), respectively. Thus, two of the three pseudouridines in yeast U2 are introduced into the branch site recognition region via a protein-only mechanism. By analogy, it has thus far been assumed that the other

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Received: 25 April 2005; accepted: 25 May 2005; published online: 16 June 2005

pseudouridine (Ψ 42) in yeast U2 is also produced via an RNA-independent mechanism, although this has yet to be demonstrated. Consistent with this idea, extensive yeast database searches have failed to reveal any guide RNAs specific for spliceosomal snRNA modifications, although a near-complete set of sno/scaRNA guides have been identified for the modification of yeast ribosomal RNAs (Balakin *et al*, 1996; Lowe and Eddy, 1999; Samarsky and Fournier, 1999; Bachellerie *et al*, 2002; Edvardsson *et al*, 2003; Schattner *et al*, 2004).

Figure 1 Sequences of the branch site recognition region of vertebrate U2 and *S. cerevisiae* U2. The three pseudouridines numbered 34, 41, and 43 in vertebrate U2 are equivalent to the three pseudouridines numbered 35, 42, and 44 in yeast U2 (three arrows). Cm and Um in vertebrate U2 indicate 2'-O-methylated residues. The branch site recognition sequences are boxed.

To address the mechanism by which pseudouridylation is achieved at position 42 in yeast U2, we used U42-radiolabeled U2 snRNA in an *in vitro* system to screen a yeast GST-ORF fusion protein library and a Box H/ACA RNA library for the candidate pseudouridylyase. We show that snR81 snoRNP, a Box H/ACA snoRNP particle, is necessary for pseudouridylation at position 42, indicating that the formation of Ψ 42 in yeast U2 is catalyzed by an RNA-dependent mechanism that is completely different from that for the other two pseudouridines, Ψ 35 and Ψ 44 (Massenet *et al*, 1999; Ma *et al*, 2003). Thus, pseudouridylation within yeast U2 is catalyzed by two different mechanisms—an RNA-independent mechanism for Ψ 35 and Ψ 44 and an RNA-guided mechanism for Ψ 42.

Results

A pseudouridylation activity responsible for the formation of Ψ 42 coincides with the GST-Nhp2p fusion protein

To identify the enzymatic activity responsible for the formation of Ψ 42 in yeast U2 snRNA, we used a previously established *in vitro* system in which U2 snRNA, radiolabeled only at the 5' side of U42, was used to screen a yeast GST-ORF fusion protein library for the candidate pseudouridylyase (Ma *et al*, 2003). Briefly, a yeast GST-ORF genomic library, consisting of 6144 strains, each expressing a unique GST-ORF fusion protein under the control of the P_{CUP1} promoter, was grown in 64 pools of 96 strains each (Phizicky *et al*, 2002). After induction, the expressed GST-ORF fusion proteins in every pool were purified via glutathione agarose chromatography (Phizicky *et al*, 2002) and incubated under the pseudouridylation conditions with the U2 substrate containing 32 P-labeled U42 (see Materials and methods). U2 was then recovered, digested to completion with nuclease P1, and analyzed by thin-layer chromatography (TLC). As shown in Figure 2A, only pool 44 was tested positive for the

Figure 2 Ψ 42-specific pseudouridylyase activity coincides with Nhp2p. Yeast U2 snRNA containing a single 32 P label at the 5' side of U42 was used to screen a GST-ORF library (Phizicky *et al*, 2002) for pseudouridylyase activity. After the pseudouridylation reaction, U2 snRNA was digested with nuclease P1 and analyzed by TLC. (A) Of all 64 pools of GST-ORF fusion proteins, pool #44 was positive. (B) The 96 strains in pool #44 were arranged into 12 columns and eight rows, with each column and row representing a 'subpool'. The column #2 subpool and row A subpool were positive, implicating strain 2A as the source of the pseudouridylyase activity. (C) Individual strains were further screened to confirm that strain 2A, expressing GST-Nhp2p, was indeed positive. The migration of labeled 32 Pu and 32 P Ψ is indicated. Control reactions (Con.) contained either yeast cell extract (+) or water (—).

pseudouridylation activity. The 96 strains in pool 44 were then arranged individually into 12 columns (columns 1–12) and eight rows (rows A–H), and the combined strains in each column or row (i.e., ‘subpools’) were further assayed for pseudouridylation. Two of the subpools, column 2 and row A, tested positive for pseudouridylation, pinpointing strain 2A (column 2 and row A) as the source of the activity (Figure 2B). Indeed, the GST-ORF fusion protein purified from strain 2A was sufficient to catalyze the conversion of U42 to Ψ 42 (Figure 2C). Surprisingly, strain 2A was found to express a fusion protein containing Nhp2p, which is one of the four core protein components of Box H/ACA sno/scaRNPs (Henras *et al*, 1998; Watkins *et al*, 1998; Dragon *et al*, 2000; Pogacic *et al*, 2000; Watanabe and Gray, 2000; Rozhdestvensky *et al*, 2003; Wang and Meier, 2004). This result suggests that Nhp2p alone, or a Box H/ACA sno/scaRNP that copurifies with Nhp2p, is responsible for the pseudouridylation activity.

The Nhp2p-associated pseudouridylation activity specifically catalyzes Ψ 42 formation

Having identified a pseudouridylation activity, we then tested whether it was specific for the formation of Ψ 42. For this purpose, we prepared three synthetic U2 substrates, each containing a single 32 P label at the 5' side of U42 (as described above), U35, or U44 (all of these sites are naturally occurring pseudouridylation sites). The three U2 snRNAs were incubated separately with the Nhp2p-associated pseudouridylation prepared from strain 2A. As shown in Figure 3, the Nhp2p-associated pseudouridylation activity converted U42 to Ψ 42, and the extent of conversion correlated directly with the amount of pseudouridylation added to the reaction (lanes 2–4). In contrast, the Nhp2p-associated activity failed to catalyze the conversion of uridine into pseudouridine at position 35 or 44 (lanes 6–8 and 10–12). As controls, yeast cell extracts were able to pseudouridylate U2 snRNA at all three positions (lanes 5 and 9, and see Figure 2, control lanes). Taken together, these results indicate that the Nhp2p-associated pseudouridylation activity is specific for position 42.

Figure 3 Nhp2p-associated pseudouridylation activity is specific for the formation of Ψ 42 in yeast U2 snRNA. Yeast U2 snRNA containing a 32 P label at the 5' side of either U42 (lanes 1–4), U35 (lanes 5–8), or U44 (lanes 9–12) was used in pseudouridylation assays (see Materials and methods, and Figure 2 legend). The final concentration of the GST-Nhp2p preparation (purified from strain 2A) in the reactions was ~15 ng/ml (lanes 2, 6, and 10), ~75 ng/ml (lanes 3, 7, and 11), or ~375 ng/ml (lanes 4, 8, and 12). As controls, the wild-type yeast cell extract (lanes 5 and 9) or water (lane 1) was used in place of the GST-Nhp2p preparation.

Tandem affinity purification, targeting either Nhp2p, Cbf5p, or Gar1p, copurifies the pseudouridylation activity for Ψ 42 formation

Since the pseudouridylation activity coincided with GST-Nhp2p, it is possible that Nhp2p is a modifying enzyme. To test this possibility, GST-Nhp2p or His-Nhp2p was expressed in *Escherichia coli* and purified by affinity chromatography. The tagged Nhp2p proteins were subsequently assayed for pseudouridylation activity using the same procedure described above. However, no pseudouridylation activity was detected (data not shown), suggesting that Nhp2p alone does not have pseudouridylation activity.

Another possibility is that GST-Nhp2p expressed in yeast copurified a Box H/ACA sno/scaRNP complex, and it is this complex that comprises the Ψ 42-specific pseudouridylation activity. According to published data (Henras *et al*, 1998; Watkins *et al*, 1998; Dragon *et al*, 2000; Pogacic *et al*, 2000; Watanabe and Gray, 2000; Rozhdestvensky *et al*, 2003; Wang and Meier, 2004) that Box H/ACA sno/scaRNP contains four core proteins, Nhp2p, Cbf5p, Gar1p, and Nop10p, we used the tandem affinity purification (TAP) procedure (Rigaut *et al*, 1999) to insert a TAP tag at the 3' end of each of these four genes in yeast chromosomes. Selection and sequencing revealed successful TAP tagging of all but the *NOP10* gene, as no live cells were selected after transformation with a *NOP10*-specific DNA tag. After TAP purification, we assessed the pseudouridylation activity and protein composition.

The pseudouridylation assay showed that each of the three TAP-tagged proteins copurified the pseudouridylation activity that effectively converted U42 to Ψ 42 (Figure 4A, lanes 3–5). Importantly, when compared with yeast cell extract (lane 2), the TAP preparations were greatly enriched for the pseudouridylation activity (compare lanes 3–5 with lane 2, and see Discussion). As a control, TAP-tagged Mak5p, an unrelated protein, did not copurify the pseudouridylation activity (lane 6). Figure 4B presents an SDS-PAGE gel stained with Coomassie blue. Each of the three TAP-tagged proteins (Gar1p, Cbf5p, and Nhp2p) coselected the same set of four proteins (compare lanes 3–5; the band migrating below 42 kDa is Protease 3C, which was used during the TAP procedure). Based on previously published data and the sizes of the proteins (Wang and Meier, 2004), we inferred that they were the four core proteins, Cbf5p, Gar1p, Nhp2p, and Nop10p, of Box H/ACA sno/scaRNPs (Figure 4B). In fact, we confirmed the identity of each protein by protein sequencing (data not shown). Because the proteins used for selection contained a C-terminal tag, the tagged protein (Gar1p-TAP, Cbf5p-TAP, or Nhp2p-TAP) in each of the three lanes (lanes 3–5) migrated slower than its native form. As expected, TAP-tagged Mak5p, as a control, did not coselect any of the four core proteins (lane 6). Taken together, these results, coupled with our current knowledge of Box H/ACA sno/scaRNPs, suggest that each of the four core proteins contributes to the pseudouridylation activity.

Ψ 42 formation is RNA dependent

Given that a complete Box H/ACA sno/scaRNP contains an RNA guide as well as protein components, our results suggested that yeast U2 pseudouridylation at position 42 is catalyzed by an RNA-guided or RNA-dependent mechanism. To test this hypothesis experimentally, we treated the *GAR1*-TAP

Figure 4 TAP-purified Box H/ACA sno/scaRNPs catalyze Ψ 42 formation. (A) Yeast U2 snRNA containing a single ^{32}P label at the 5' side of U42 was used to assess the pseudouridylation activity of TAP preparations targeting Gar1p (lane 3), Cbf5p (lane 4), Nhp2p (lane 5), or Mak5p (as a control, lane 6). The final protein concentration of the TAP preparations in the pseudouridylation reaction (see Materials and methods, and previous figure legends) was ~ 75 ng/ml. As controls, water (lane 1) or the wild-type yeast cell extract (lane 2) was used in place of the TAP preparations. (B) The protein compositions of TAP preparations were analyzed on an SDS-PAGE gel stained with Coomassie blue. Lanes 3, 4, and 5 contain samples of Gar1p-TAP, Cbf5p-TAP, and Nhp2p-TAP preparations, respectively. Lane 6 is a control containing the sample of Mak5p-TAP preparation. The positions of the Box H/ACA sno/scaRNP core proteins, Cbf5p, Gar1p, Nhp2p, and Nop10p (both TAP-tagged and native forms), are indicated on the left. The TAP-tagged proteins migrated more slowly than their native forms. The label 3C indicates protease 3C, which was used during TAP purification. Lane M is a standard protein marker lane.

preparation with micrococcal nuclease in the presence of Ca^{2+} . After inactivation of the nuclease by chelating Ca^{2+} with EGTA, we performed the pseudouridylation assay using the U2 substrate containing a single ^{32}P label at the 5' side of U42. As shown in Figure 5A, the mock-treated TAP preparation was highly active in converting U42 into Ψ 42 (lane 2). However, upon micrococcal nuclease treatment, the TAP preparation completely lost its pseudouridylation activity (lane 3). Importantly, when supplemented with total Box H/ACA sno/scaRNA extracted from any of the three TAP preparations (GAR1-TAP, NHP2-TAP, or CBF5-TAP), the micrococcal nuclease-treated TAP preparation regained pseudouridylation activity—at least a substantial fraction of U42 was converted into Ψ 42 (lane 4). As a control, a reconstitution reaction was carried out in parallel using a yeast 25S rRNA fragment containing a single ^{32}P label at the 5' side of U989, a naturally occurring pseudouridylation site known to be modified by a Box H/ACA snoRNP (Ofengand and Fournier, 1998). As

Figure 5 The formation of Ψ 42 in yeast U2 requires an RNA component. (A) Pseudouridylation assay was performed using yeast U2 containing a single ^{32}P label at the 5' side of U42 (see Materials and methods, and previous figure legends). (B) Pseudouridylation assay was performed using a fragment of yeast 25S rRNA containing a single ^{32}P label at the 5' side of U989. In lane 2 (in panels A and B), the Gar1p-TAP preparation (~ 75 ng/ml) was present in the reaction. In lane 3 (A, B), micrococcal nuclease (MN)-treated Gar1p-TAP preparation was used. The reaction in lane 4 (A, B) was identical to that in lane 3 except that it contained total RNA prepared from the Gar1p-TAP preparation (not treated with micrococcal nuclease). Lane 1 in each case shows a control in which no TAP preparation was added.

expected, the mock-treated GAR1-TAP preparation effectively converted U989 into Ψ 989 (Figure 5B, lane 2), whereas the micrococcal nuclease-treated GAR1-TAP preparation did not catalyze this pseudouridylation reaction (Figure 5B, lane 3). However, upon addition of total RNA extracted from the TAP preparations, the pseudouridylation activity of the micrococcal nuclease-treated GAR1-TAP preparation was regenerated (Figure 5B, lane 4). Taken together, these results demonstrate that a complete Box H/ACA sno/scaRNP, including both protein and RNA components, constitutes the pseudouridylation activity that catalyzes the formation of Ψ 42.

SnR81 is the guide RNA responsible for the formation of Ψ 42

To identify the guide RNA responsible for Ψ 42 formation, we took advantage of our powerful reconstitution system

discussed above (see Figure 5). Briefly, total Box H/ACA sno/scaRNA was isolated from the *GAR1*-TAP preparation, and subsequently fractionated on a denaturing polyacrylamide gel. The gel (entire lane) was then sliced, from top to bottom (based on size), into 11 fractions (Figure 6A), and RNAs were recovered from each fraction and added to the micrococcal nuclease-treated *GAR1*-TAP preparation to test their ability to reconstitute U2 pseudouridylation at position 42. As shown in Figure 6B, fraction E (ranging from ~180 to ~200 nucleotides) was able to reconstitute the pseudouridylation activity (lane 9), suggesting strongly that the guide RNA responsible for Ψ 42 formation was present in fraction E and is about 180–200 nucleotides long. However, it appeared that fraction E contained a number of Box H/ACA RNAs, as a smeared signal ranging from ~180 to 200 nucleotides in size was observed when the total RNA extracted from this fraction was 3' radiolabeled with 32 Pcp (data not shown).

To pinpoint which RNA in fraction E is responsible for the activity, a Box H/ACA RNA library was constructed. Briefly, RNAs recovered from fraction E were 3' polyadenylated by poly(A) polymerase. The resulting RNAs were then used as templates for oligo(dT)-primed reverse transcription, generating complementary DNAs (cDNAs) that contained a 5'

poly(dT) tail. The cDNAs were further 3' tailed with a stretch of guanines using terminal deoxynucleotidyl transferase (TDT), and were subsequently amplified by PCR using an oligo(dC) and an oligo(dT) as primers. The amplified DNAs were cloned and transformed into an *E. coli* strain, resulting in a large number of colonies on a solid medium. Plasmids purified from single colonies were used as templates for *in vitro* RNA transcription. The transcribed RNAs were then tested for their ability to reconstitute U2 pseudouridylation at position 42 in the micrococcal nuclease-treated *GAR1*-TAP preparation.

As shown in Figure 6C, one functional RNA (lane 11) was identified among 25 RNAs tested. Sequencing of its corresponding plasmid indicated that the RNA was snR81, a recently identified Box H/ACA snoRNA responsible for 25S rRNA pseudouridylation at position 1051 (Schattner *et al*, 2004). SnR81 has two hairpins, each containing a pseudouridylation pocket. According to Schattner *et al* (2004), the 3' pocket is specific for Ψ 1051 formation in 25S rRNA, whereas the 5' pocket has no known target. Remarkably, sequence comparison indicated a perfect match between the guide sequence in the 5' pocket and the sequence surrounding U42 of yeast U2 snRNA, positioning U42 perfectly at the pocket for pseudouridylation (see Figure 6D).

Figure 6 snR81 snoRNA directs the formation of Ψ 42 in yeast U2. (A) Total Box H/ACA RNA isolated from the *GAR1*-TAP preparation was fractionated on an 8% polyacrylamide–8M urea gel. The entire lane (lane 2) was sliced into 11 fractions (A–K). RNAs were eluted from each fraction for reconstitution analysis. Lane 1 is a size marker of *MspI*-digested pBR322 DNA. (B) Fractionated RNAs (A–K, lanes 5–15) were added to micrococcal nuclease-treated *GAR1*-TAP preparation (lanes 3–15) to reconstitute U2 pseudouridylation at position 42. Lanes 3 and 4 are controls in which no RNA (lane 3) or unfractionated total Box H/ACA RNA (lane 4) was added to the reaction. Lane 1 is an unreacted singly radiolabeled U2 snRNA. In lane 2, U2 pseudouridylation was carried out using mock-treated *GAR1*-TAP preparation. (C) Reconstitution of Ψ 42 formation was carried out using micrococcal nuclease-treated *GAR1*-TAP preparation (lanes 2–15) and individual RNAs (lanes 4–15) derived from a Box H/ACA RNA library (see Materials and methods). Lanes 2 and 3 are controls where the reaction was supplemented with no RNA (lane 3) or total RNA isolated from fraction E (see panel B) (lane 2). In lane 1, mock-treated *GAR1*-TAP preparation was used. Sequencing data indicated that RNA 8 in lane 11 was snR81 snoRNA. (D) Primary sequence and secondary structure of snR81 snoRNA. As indicated, the 3' pseudouridylation pocket guides 25S rRNA pseudouridylation at position 1051 (Schattner *et al*, 2004), whereas the 5' pseudouridylation pocket directs U2 snRNA pseudouridylation at position 42 (this work). The conserved H and ACA boxes are shaded.

Depletion of either *Nhp2p* or *Cbf5p*, or deletion of *snR81* precludes $\Psi 42$ formation *in vivo*

To confirm that the snR81 Box H/ACA sno/scaRNP is the natural pseudouridylase for $\Psi 42$, we next analyzed U2 pseudouridylation *in vivo* under conditions in which one of the Box H/ACA sno/scaRNP core proteins was depleted. Here, we used a conditional depletion approach because all four core proteins of the sno/scaRNP complex are essential for cell growth. Specifically, we constructed two strains in which the transcription of either *NHP2* or *CBF5* was under the control of the P_{GAL1} promoter. When grown in galactose-

containing medium, the two mutant strains grew as well as the wild-type strain. However, when the galactose medium was replaced by glucose-containing medium, the Gal promoter was shut-off, and *Nhp2p* or *Cbf5p* was gradually depleted in the mutant cells. After about four generations (~ 10 h after the medium switch), the mutant cells grew significantly slower compared with the wild-type cells (Figure 7A). Both mutant and wild-type cells were collected at three different time points (6, 16, and 48 h) after the medium switch. Consistent with previous report (Lafontaine *et al*, 1998), depletion for 48 h resulted in almost complete loss of Box

Figure 7 Depletion of either *Nhp2p* or *Cbf5p* specifically abolishes $\Psi 42$ formation in yeast U2 snRNA *in vivo*. (A) Growth curves for three yeast strains after the switch from galactose medium to glucose medium (YPD). Closed circles represent the *nhp2*-depletion strain in which chromosomal *nhp2* was replaced by a plasmid containing a wild-type *NHP2* gene under the control of the Gal promoter. Closed triangles represent the strain in which chromosomal *cbf5* was replaced by a plasmid containing a wild-type *CBF5* gene under the control of the Gal promoter. Closed squares represent the wild-type strain. (B) The U2 pseudouridylation assay (CMC modification followed by primer extension) was performed using total RNA isolated either from the wild-type strain that had been incubated in YPD for 6 h (lanes 1 and 2), from the *nhp2*-depletion strain that had been incubated in YPD for 6 h (lanes 3 and 4), 16 h (lane 5), or 48 h (lane 6), or from the *cbf5*-depletion strain that had been incubated in YPD for 6 h (lanes 7 and 8), 16 h (lane 9), or 48 h (lane 10). In lanes 1, 3, and 7, CMC modification was omitted. The lanes labeled U, C, G, and A represent a yeast primer-extension sequencing ladder. The lane labeled H₂O contained no ddNTP. The three primer-extension stops/pauses representing the three pseudouridines ($\Psi 35$, $\Psi 42$, and $\Psi 44$) in U2 snRNA are indicated. (C) A pseudouridylation assay for yeast 25S rRNA was performed exactly as in panel B except that the primer-extension primer was complementary to yeast 25S rRNA (see Materials and methods). The naturally occurring pseudouridylation sites are indicated.

H/ACA sno/scaRNP proteins (data not shown). Total RNA was then extracted from the cells, and pseudouridylation of endogenous U2 snRNA was analyzed by CMC (*N*-cyclohexyl-*N'*-(2-morpholinoethyl)-carbodiimid metho-*p*-toluolsulfonate) modification followed by primer extension (Bakin and Ofengand, 1993).

The pseudouridylation results are consistent with the growth data. As shown in Figure 7B, when RNA was isolated from cells that had been incubated for only 6 h after the medium switch (at this time point, no growth defect was observed for the mutant strains; see Figure 7A), primer extension clearly detected all three U2 pseudouridine stops/pauses, regardless of which strain was used (compare lanes 2, 4, and 8). This was not the case, however, when we isolated RNA from *nhp2* (lanes 5 and 6) or *cbf5* (lanes 9 and 10) depletion strains that had grown for 16 h (lanes 5 and 9) or 48 h (lanes 6 and 10) after the medium switch (at these time points, the mutant cells clearly showed growth defects; see Figure 7A). Using either of these strains, U2 pseudouridylation at position 42 was greatly diminished, whereas Ψ 35 and Ψ 44 formation was essentially unchanged (Figure 7B).

We also assessed 25S rRNA pseudouridylation, which is catalyzed by Box H/ACA snoRNPs (Ganot *et al*, 1997; Ni *et al*, 1997). As shown in Figure 7C, all pseudouridine signals were dramatically reduced in *nhp2* or *cbf5* depletion strains that had been incubated for 16 or 48 h after the medium switch (lanes 5, 6, 9, and 10). However, for the mutant cells that had grown for only 6 h after the switch, all pseudouridines were clearly detected and the level of pseudouridylation was equivalent to that of the wild-type strain (compare lanes 4 and 8 with lane 2).

To further confirm that a Box H/ACA sno/scaRNP catalyzes the formation of Ψ 42 *in vivo*, we took advantage of the availability of a slow growth mutant strain in which a point mutation was introduced into the *cbf5* gene, which is believed to encode the catalytic component of the complex (Zebarjadian *et al*, 1999). It has been reported that this single point mutation (D95 changed to A95), presumably at the catalytic center of the protein, greatly diminishes rRNA pseudouridylation *in vivo* (Zebarjadian *et al*, 1999; see Figure 8A, compare lane 2 with 4). When the RNA isolated from this strain was used in the U2 pseudouridylation assay, we found that the Ψ 42 signal was indeed greatly decreased compared with the wild-type control (Figure 8B, compare lane 4 with 2). In contrast, signals corresponding to Ψ 35 and Ψ 44 were essentially unaffected (Figure 8B, lanes 2 and 4). Collectively, these results suggest that a Ψ 42-specific Box H/ACA snoRNP, not a protein enzyme alone, catalyzes the pseudouridylation reaction *in vivo*.

Finally, we tested whether the formation of Ψ 42 in U2 snRNA is dependent on snR81 RNA *in vivo*. An snR81-deletion strain (with no growth defect phenotype) was created, total RNA was isolated from the deletion strain, and pseudouridylation of U2 snRNA and 25S rRNA was analyzed by CMC modification followed by primer extension. As shown in Figure 9, the formation of both Ψ 42 in U2 (panel U2, lane 8) and Ψ 1051 in 25S rRNA (panel 25S, lane 8) was abolished in the snR81-deletion strain (compare lane 8 with 6). In contrast, pseudouridylation at the other positions was not affected. To confirm this observation, a plasmid containing a wild-type snR81 gene under the

Figure 8 The *cbf5D95A* mutant abolishes the formation of Ψ 42 in yeast U2 snRNA. (A) The yeast 25S rRNA pseudouridylation assay was conducted exactly as in Figure 7C except that total RNA was isolated from the *cbf5D95A* strain (see Figure 7B and C legends). (B) The yeast U2 snRNA pseudouridylation assay was conducted exactly as in Figure 7B except that total RNA was isolated from the *cbf5D95A* strain (see Figure 7B legend).

control of P_{GAL1} promoter was introduced into the snR81-deletion strain. When the cells were grown in galactose medium, both Ψ 42 formation in U2 (panel U2, lane 10) and Ψ 1051 formation in 25S rRNA (panel 25S, lane 10) were rescued. However, essentially no rescue was observed when the cells were grown in glucose medium (YPD) (lane 12). These results demonstrate that snR81 is indeed the guide RNA for the formation of both Ψ 42 in U2 and Ψ 1051 in 25S rRNA.

Figure 9 The formation of $\Psi 42$ in U2 and $\Psi 1051$ in 25S rRNA is dependent on snR81 RNA. The pseudouridylation assay for both U2 snRNA and 25S rRNA was carried out exactly as in Figure 7B and C except that total RNA was isolated from different yeast strains grown in different media (see Materials and methods, and Figure 7B and C legends). Specifically, the wild-type yeast strain (lanes 5 and 6) and snR81 Δ strain (lanes 7 and 8) were grown in YPD medium. The snR81 Δ strain that had been transformed with a plasmid containing a wild-type snR81 gene under the control of P_{GAL1} promoter (lanes 9–12) was grown either in YPD (lanes 11 and 12) or YPGal (lanes 9 and 10) medium. Total RNA recovered from each of these strains was subjected to pseudouridylation assay (see Materials and methods, and Figure 7B and C legends).

Discussion

We have demonstrated that the pseudouridylation of yeast U2 snRNA at position 42 is catalyzed by snR81 Box H/ACA sno/scaRNP, and therefore the reaction is RNA dependent. Using an *in vitro* screening system, we identified a $\Psi 42$ -specific pseudouridylase activity (Figures 2 and 3). Unexpectedly, this activity was associated with Nhp2p, one of the four core protein components of Box H/ACA sno/scaRNPs, suggesting that a protein–RNA complex rather than a protein enzyme alone is responsible for $\Psi 42$ formation. Consistently, the TAP experiments showed that not only Nhp2p, but also the other Box H/ACA sno/scaRNP proteins, copurified the pseudouridylase activity (Figure 4). Using micrococcal nuclease digestion followed by subsequent reconstitution with fractionated Box H/ACA RNAs or RNAs from a Box H/ACA RNA library, we further demonstrated that the pseudouridylase activity is in fact dependent on snR81 Box H/ACA RNA. We also constructed the *snr81*-deletion strain, *nhp2* or *cbf5* conditional depletion strains, or used a *cbf5* point mutation strain to demonstrate that the pseudouridylase activity is dependent on snR81 RNA and the Box H/ACA sno/scaRNP proteins *in vivo* (Figures 7–9). Our results indicate that the conversion of U42 to $\Psi 42$ in yeast U2 is indeed catalyzed by a sno/scaRNP via an RNA-guided mechanism, which is completely different from previously identified protein-only mechanism catalyzing yeast U2 pseudouridylation at the other two positions, $\Psi 35$ (Ma *et al*, 2003) and $\Psi 44$ (Massenet *et al*, 1999).

Functional Box H/ACA sno/scaRNP complex

Since the Box H/ACA sno/scaRNP complex is involved in the pseudouridylation process, it seems puzzling that we identified only Nhp2p but not the other protein components of the complex during the initial screening of the yeast GST-ORF fusion protein library. There are at least four possible explanations. First, it is entirely possible that the other protein components of the complex are not expressed, or are expressed at a low level, in the GST-ORF fusion library (Phizicky *et al*, 2002). Second, it is also possible that the GST fusion to the N termini of other Box H/ACA sno/scaRNP protein components results in nonfunctional products (Phizicky *et al*, 2002). Third, the GST-Nhp2p is perhaps the only fusion core protein that can readily exchange with its native form (Nhp2p) present in the Box H/ACA RNPs. Fourth, nonstoichiometric expression of the protein components of the Box H/ACA sno/scaRNP may have contributed to the low recovery of pseudouridylase activity. Since the GST-ORFs were under the control of the P_{CUP1} promoter, a unique GST-ORF was presumably overexpressed in a particular strain in response to induction. In contrast, the expression of the other protein components of the complex should be much lower in the same strain. If the GST fusion protein has more than one binding partner in the complex (Wang and Meier, 2004), then partial binding or assembly would occur, giving rise to nonfunctional complexes. The TAP procedure, on the other hand, is completely different. Here, the TAP tag was introduced into the 3' end of the gene (C terminus of the protein) and the production of the fusion protein was entirely under the control of its own natural promoter. Thus, an optimal (stoichiometric) amount of the components of the complex should be produced; hence, an optimal level of complex should be assembled in which one of the proteins is TAP tagged. In fact, our TAP procedure recovered high $\Psi 42$ -specific pseudouridylase activity for each of the three Box H/ACA sno/scaRNP proteins targeted by the procedure (see Figures 4–6).

In the current work, we developed a pseudouridylation reconstitution system in which a mixture of at least four Box H/ACA sno/scaRNP core proteins (micrococcal nuclease-treated TAP preparations) when incubated with total guide RNA catalyzed the pseudouridylation of yeast U2 at position 42 and yeast rRNAs at various positions (Figure 5, and data not shown). The fact that we observed only four core proteins in the pseudouridylase complex suggests that we might be able to reconstitute the activity by expressing and purifying these four core proteins from *E. coli*. However, such attempts have thus far failed (data not shown), suggesting that one or more specific protein component(s) is required for a specific pseudouridylase activity. In fact, besides the four core proteins, a number of low-abundance proteins can be visualized on the SDS–PAGE gel stained with Coomassie blue (Figure 4B). While it is possible that they are contaminants, it is equally possible that they are integral proteins of specific Box H/ACA sno/scaRNP complexes that are required for function. In this regard, Wang and Meier (2004) recently showed that the four *in vitro*-translated core proteins indeed form a complex with a guide RNA, yet the complex had no pseudouridylase activity. Only when the assembled complex was incubated with a cytoplasmic extract was it able to catalyze pseudouridylation. One explanation is that one or more specific proteins in the extract became incorporated

into the complex, thereby converting the nonfunctional complex into a competent pseudouridylyase. An alternative explanation for the failure to detect pseudouridylyase activity with bacterially expressed versions of the four core proteins is that one or more of these proteins might require post-translational modification for pseudouridylyase activity. Further study is necessary to clarify this issue.

RNA-independent versus RNA-dependent mechanisms

Computational and experimental data accumulated thus far indicate that pseudouridylation of higher eukaryotic spliceosomal snRNAs is catalyzed by Box H/ACA sno/scaRNPs (i.e., an RNA-dependent mechanism) (Yu *et al*, 2005). In fact, a relatively large fraction of spliceosomal snRNA-specific Box H/ACA guide RNAs has been identified in vertebrates (Yu *et al*, 2005). In contrast, prior to this work, two proteins, Pus1p and Pus7p, were identified as pseudouridine synthases responsible for the pseudouridylation of yeast U2 snRNA at positions 44 and 35, respectively (Massenet *et al*, 1999; Ma *et al*, 2003). These results, in combination with our current data demonstrating that snR81 sno/scaRNP is responsible for the formation of Ψ 42, indicate that the pseudouridylation of yeast U2 snRNA (and perhaps the other yeast spliceosomal snRNAs as well) is catalyzed by an RNA-dependent mechanism as well as an RNA-independent mechanism.

While it is not clear why yeast cells use two completely different mechanisms to modify a single spliceosomal snRNA, the coexistence of the two mechanisms is rather interesting from an evolutionary point of view. In light of the postulation that the RNA-dependent mechanism evolved from the protein-only (RNA-independent) mechanism (Lafontaine and Tollervey, 1998), our results suggest that the Box H/ACA sno/scaRNP responsible for Ψ 42 formation in U2 snRNA has in fact evolved in both higher eukaryotes and *S. cerevisiae*. Interestingly, however, the RNA-dependent modifying enzymes for the other sites in *S. cerevisiae* U2 snRNA (i.e., positions 35 and 44) may never have evolved (or evolved but were subsequently lost from the genome; e.g., via chromosomal deletion). Conversely, it has recently been suggested that the RNA-guided mechanism is probably the most ancient mechanism, given that archaeal Box H/ACA RNP and Box C/D RNP share a common core protein with ribosomes (Tran *et al*, 2004). This suggests a possibility that the RNA-guided mechanism evolved from the protein translational apparatus. The preservation of the putatively ancient RNA-dependent (or RNA-independent) modifying mechanism throughout evolution implies that such modifications are functionally important. In this regard, it has been reported that two different 2'-O-methylation mechanisms (RNA-dependent and RNA-independent) act on the same large subunit rRNA in *S. cerevisiae*. Specifically, Spb1p, a protein enzyme, catalyzes rRNA 2'-O-methylation at position 2922 (Bonnerot *et al*, 2003; Lapeyre and Purushothaman, 2004), whereas all other 2'-O-methylation sites appear to be modified by an RNA-guided mechanism (Lowe and Eddy, 1999; Samarsky and Fournier, 1999). Although it is still unclear as to how important the other methylated residues are, 2'-O-methylation at position 2922 (Gm₂₉₂₂) is required for ribosome biogenesis and protein translation (Lapeyre and Purushothaman, 2004), suggesting that the ancient RNA-independent mechanism is indeed crucial for cell viability. It is yet to be determined whether each of the highly

conserved pseudouridines in yeast U2 snRNA—introduced by either an RNA-independent or RNA-dependent mechanism—functions during pre-mRNA splicing and cell growth.

Where within the nucleus are *S. cerevisiae* spliceosomal snRNAs modified?

Our data demonstrate that snR81 snoRNA guides U2 pseudouridylation at position 42 in *S. cerevisiae*. Interestingly, this snoRNA was recently identified to direct 25S rRNA pseudouridylation at position 1051 (Schattner *et al*, 2004). Thus, snR81 functions as a pseudouridylation guide for two different types of RNA—spliceosomal snRNA (U2) and rRNA (25S). Specifically, its 5' side pseudouridylation pocket directs the formation of Ψ 42 in U2 snRNA, and its 3' side pocket is for 25S rRNA pseudouridylation at position 1051 (see Figure 6D). The fact that the same guide RNA directs the modification of two different types of RNA raises an interesting question as to where within the nucleus yeast U2 pseudouridylation occurs. It has been reported that in higher eukaryotic cells, spliceosomal snRNA (U1, U2, U4, or U5)-specific Box H/ACA RNPs are localized to Cajal bodies, suggesting that maturation of the Box H/ACA RNPs and possibly pseudouridylation of their target RNAs (higher eukaryotic spliceosomal snRNAs) occur in Cajal bodies (Darzacq *et al*, 2002). In the current work, we have detected snR81 in the nucleolus (data not shown), but it is still unclear whether the RNA is also present in the nucleolar body, a subnuclear organelle similar to Cajal body in higher eukaryotic cells. If snR81 is present in the nucleolar body as well, a likely scenario would be that pseudouridylation of U2 snRNA and rRNA may occur in the separate subnuclear organelles, in a manner similar to pseudouridylation of spliceosomal snRNA and rRNA in higher eukaryotic cells (Darzacq *et al*, 2002). However, it is possible that snR81 does not reside in the nucleolar body. This would suggest that U2 pseudouridylation and rRNA pseudouridylation might both occur in the nucleolus. This hypothesis would require that yeast U2 snRNA transits through the nucleolus during its biogenesis. In this regard, it has been demonstrated that spliceosomal snRNAs in higher eukaryotic cells indeed travel through the nucleolus (Sleeman *et al*, 1998; Yu *et al*, 2001). A detailed localization analysis is necessary to clarify this issue.

Besides U2 snRNA, the other *S. cerevisiae* spliceosomal snRNAs, including U1 and U5, are post-transcriptionally pseudouridylylated as well (Massenet *et al*, 1998). It will be of interest to characterize the mechanism by which these yeast spliceosomal snRNAs are modified. If the modification is in fact catalyzed by Box H/ACA sno/scaRNPs, the advent of our reconstitution system (Figures 5 and 6) affords an excellent opportunity to identify the guide RNAs.

Materials and methods

Yeast GST-ORF library

Pools of yeast GST-ORF fusion proteins and the original plate corresponding to pool 44 were kindly provided by E Phizicky and E Grayhack (Phizicky *et al*, 2002). Subpools of GST-ORF fusion proteins from pool 44 were prepared from a 250 ml culture, exactly as described previously (Phizicky *et al*, 2002). Purified GST-ORF fusion proteins (~150–350 µg/ml) were of ~50–70% purity, as judged by visual inspection of Coomassie blue-stained SDS-PAGE gels.

Pseudouridylation assays

The *in vitro* pseudouridylation assay was performed exactly as described previously (Ma *et al*, 2003). CMC modification followed by primer extension (Bakin and Ofengand, 1993) was used to analyze pseudouridylation of endogenous U2 snRNA and 25S rRNA (as a control) from wild-type and mutant yeast strains. The assay was performed exactly as described previously (Ma *et al*, 2003).

TAP preparation

The TAP tagging plasmid was originally conceived by B Seraphin's group (Rigaut *et al*, 1999) and subsequently modified by Alexandrov *et al* (2005). The modification removed the original CBP-TEV-Protein A tag (Rigaut *et al*, 1999) from the pBS1539 vector and replaced it with a 6xHis-HA-3C-Protein A tag (Alexandrov *et al*, 2005). The TAP purification procedure was performed as described (Rigaut *et al*, 1999), except that we used 3C protease instead of TEV protease and Talon resin (Clontech) instead of Calmodulin Sepharose.

Micrococcal nuclease digestion and reconstitution of pseudouridylation

A 200 µl aliquot of purified TAP-tagging preparation (purified through either Nhp2p-TAP, Cbf5p-TAP, or Gar1p-TAP) was mixed with 10 µl of 44 mM CaCl₂ and 10 µl of 30 U/µl micrococcal nuclease and incubated at 30°C for 20 min. The reaction was then stopped by adding 10 µl of 92 mM EGTA. RNA was recovered by PCA extraction and ethanol precipitation. No RNA larger than ~30 nucleotides was detected in micrococcal nuclease-treated TAP-tagging preparations, as assessed by 3'-end labeling with ³²PCp or 5'-end labeling by dephosphorylation followed by rephosphorylation with [γ-³²P]ATP and polynucleotide kinase.

Micrococcal nuclease-treated TAP-tagging preparations were then used to reconstitute pseudouridylation *in vitro* with various RNAs (see below). Detailed procedure is described in Supplementary data.

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Fractionation of Box H/ACA RNAs and construction of a Box H/ACA RNA library

A 10 µg portion of total Box H/ACA RNA isolated from the TAP-tagging preparation (either Nhp2p-TAP, Cbf5p-TAP, or Gar1p-TAP) by PCA extraction and ethanol precipitation was resolved on an 8% polyacrylamide–8 M urea gel. The entire lane was then sliced from top to bottom into 11 fractions (fractions A–K; see Figure 6A). Thereafter, 10% of RNAs eluted from each fraction were then added back to the micrococcal nuclease-treated TAP-tagging preparations for reconstitution analysis.

RNAs eluted from fraction E were used for the construction of a Box H/ACA RNA library (see Supplementary data). Individual Box H/ACA RNAs derived from the library were subsequently used for pseudouridylation reconstitution assay (see Supplementary data).

Construction of yeast deletion and conditional-depletion strains

To analyze whether snR81 snoRNP is required for Ψ42 formation, an snR81-deletion strain, and Nhp2p- and Cbf5p-conditional depletion strains were constructed (see Supplementary data).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to J Carbon, M Baum, and MJ Fournier for providing *cbf5D95A* strain and advice for related experiments. We thank EM Phizicky for providing the *S. cerevisiae* GST fusion protein library. We also thank members of the Yu laboratory for discussion and inspiration. This work was supported by grant GM62937 (to Y-T Yu) from the National Institutes of Health.

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