ψ35 in the Branch Site Recognition Region of U2 Small Nuclear RNA Is Important for Pre-mRNA Splicing in Saccharomyces cerevisiae*

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Pseudouridine 35 (ψ35) in the branch site recognition region of yeast U2 small nuclear RNA is absolutely conserved in all eukaryotes examined. Pus7p catalyzes pseudouridylation at position 35 in Saccharomyces cerevisiae U2. The pus7 deletion strain, although viable in rich medium, is growth-disadvantaged under certain conditions. To clarify the function of U2 ψ35 in yeast, we used this pus7 deletion strain to screen a collection of mutant U2 small nuclear RNAs, each containing a point mutation near the branch site recognition sequence, for a synthetic growth defect phenotype. The screen identified two U2 mutants, one containing a U40→G40 substitution (U40G) and another having a U40 deletion (U40Δ). Yeast strains carrying either of these U2 mutations grew as well as the wild-type strain in the selection medium, but they exhibited a temperature-sensitive growth defect phenotype when coupled with the pus7 deletion (pus7Δ). A subsequent temperature shift assay and a conditional pus7 deletion (via GAL promoter shutoff) in the U2-U40 mutant genetic background caused pre-mRNA accumulation, suggesting that ψ35 is required for pre-mRNA splicing under certain conditions.

The five high abundance spliceosomal small nuclear RNAs (snRNAs),1 U1, U2, U4, U5, and U6, are integral to pre-mRNA splicing (1, 2). During spliceosome assembly, an intricate network of RNA-RNA interactions among the snRNAs and the pre-mRNA facilitates two specific trans-esterification reactions that remove the intron. The well characterized interactions include base pairing between the 5′-sequence of U1 and the 5′-splice site of the pre-mRNA (3), the base pairing between the U2 branch site recognition sequence and the branch site in the pre-mRNA (4, 5), base pairing between U2 and U6 snRNAs (U2-U6 helices I, II, and III) (6–10), and base pairing between the conserved ACAGAGA sequence in U6 and the 5′-splice site of the pre-mRNA (11–13). In addition, non-Watson-Crick base-pairing interactions between the conserved loop of U5 and the exon sequences at the 5′- and 3′-splice sites (13–17), as well as interactions between the 5′- and 3′-splice sites themselves (18, 19), have also been well documented. A notable feature shared by all five spliceosomal snRNAs is their extensive internal modifications, including pseudouridylation and 2′-O-methylation (20, 21). Significantly, these modifications appear to be mostly clustered in regions of demonstrated functional importance. For instance, both the 2′-O-methylated nucleotides and pseudouridines are concentrated in the sequences involved in the spliceosomal RNA-RNA interactions described above, suggesting a possible functional role in pre-mRNA splicing (21).

Among the five snRNAs, U2 contains the most modifications (20, 21). There are 10 2′-O-methylated residues and 13 pseudouridines in the vertebrate U2 snRNA, representing more than 10% of the total nucleotides. In particular, all 6 uridines in the single-stranded branch site recognition region (4, 22) are converted to pseudouridines after transcription (Fig. 1). Three of these pseudouridines are conserved in yeast U2 snRNA (21) at equivalent positions within the branch site recognition sequence (Fig. 1, ψ35 and the adjacent downstream sequence). Modifications in higher eukaryotic spliceosomal snRNAs are believed to be catalyzed by a RNA-guided mechanism, directed by Box H/ACA sno/scaRNPs for pseudouridylation and Box C/D sno/scaRNPs for 2′-O-methylation (23–25). In some instances, this mechanism has been experimentally verified (26–28). However, the formation of at least two pseudouridines in S. cerevisiae U2 (ψ35 and ψ44) is catalyzed by an RNA-independent mechanism. Specifically, ψ35 formation is catalyzed by Pus7p (29), and ψ44 is introduced by Pus1p (30). Whether the other modification sites in the yeast spliceosomal snRNAs are introduced by protein-only or RNA-guided mechanisms remains to be determined.

The functional importance of vertebrate U2 modifications has recently been established (31–33). Using a Xenopus oocyte reconstitution system, we have shown that the 6 pseudouridines in the branch site recognition region as well as the 9 modified nucleotides within the 5′-most 27 nucleotides of Xenopus U2, including 3 pseudouridines and 6 2′-O-methylated residues, are all required for snRNP assembly and pre-mRNA splicing (31, 32). Using NMR, the Greenbaum group (34, 35) has recently shown that the base-pairing interaction between ψ34 (ψ35 in yeast U2) in the branch site recognition region and the nucleotide next to the pre-mRNA branch point adenosine results in the branch point nucleotide being bulged out, a configuration known to be important for the first step of the splicing reaction. Consistent with this result, Valadkhan and

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‡ The abbreviations used are: snRNA, small nuclear RNA; CMC, N-cyclohexyl-N′-(2-morpholinoethyl)-carbodiimide; metho-p-tolulosufonate; RNP, ribonucleoprotein; FOA, 5-fluoroorotic acid; RT, reverse transcription; YPD, yeast extract, peptone, and dextrose; SD, synthetic dextrose.

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Manley (36) have demonstrated that the change of uridine to pseudouridine at the same position (Ψ35) greatly activates splicing-related catalysis via protein-free snRNAs (37).

In light of these data, it remains puzzling, however, that the 

coupled with U2 mutation/deletion at position 40 (Fig. 2C, 

pus7Δ + U2- 

Ψ35, in the 

yeast U2 snRNA is critical for yeast pre-mRNA splicing and cell growth under certain conditions.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Two haploid strains, DM2484 (Mat a 

ura3Δ his3 lys2 ade2–101 pus7:::KanMX snr20::LYS2 pU2URA-

2 lpRS16(URA3 CEN SNR20)) and DM2486 (Mat a 

ura3Δ his3 

leu2Δ lys2 ade2–101 pus7:::KanMX snr20::LYS2 pU2URA-

2 lpRS16(URA3 CEN SNR20)), were derived from the cross of the 
two parental strains, pus7Δ (Mat a pus7::KanMX his3 

leu2Δ met15Δ ura3Δ) (ATCC) and DM1001 (Mat a trp1 ura3–52 his3 

lys2 ade2–101 snr20::LYS2 pU2URA-2 lpRS16(URA3 CEN SNR20)). DM1001 is 

equivalent to YHM111 (9), except that it contains a plasmid encoding the full-length U2 snRNA. The wild-type PUS7 haploid strain (BY4741) (MATa his3Δleu2Δmet15Δura3Δ) was purchased from ATCC.

Plasmid pCAU2 (CEN ARS HIS3 SNR20) contains a full-length wild-type U2 gene (40) and contains a single base insertion at the 5'-end of the U2 gene to create a unique EcoRI site. All U2 mutant plasmids were derived from pCAU2 by replacing the EcoRI-EcoNI fragment containing the wild-type U2 gene with a PCR fragment containing a mutant U2 gene derived from in vitro T7 expression plasmids (38). All U2 mutant plasmids were then sequenced to verify the presence of the mutation. Plasmid pPUS7 (CEN LEU2 PUS7) was constructed by inserting the wild-type PUS7 gene between the BamHI and PstI sites of YEpLac11 vector (41). Plasmid pPUS7-Gal (CEN URA3 P:puc10::PUS7) was constructed by inserting the PUS7 gene between the BamHI and PstI sites of pAVA0040 (42).

All U2 mutant plasmids were used for transformation and selection with 5-fluoroorotic acid (5FOA, see below). pCAU2 and pPUS7 were used to rescue the growth phenotype of strains with the 

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\[ \text{Ψ35 in U2 snRNA Is Required for Yeast Pre-mRNA Splicing} \]

FIG. 1. U2 spliceosomal snRNA. The primary sequence and secondary structure of mammalian U2 is shown along with the 5'-part of yeast U2. There are a total of 13 pseudouridines and 10 2'-O-methylated residues in mammalian U2 and 3 pseudouridines in yeast U2 (the number of 2'-O-methylated residues in yeast U2 has not been established). All three pseudouridines in yeast U2 (Ψ35, Ψ42, and Ψ44) have counterparts in mammalian U2 (Ψ34, Ψ41, and Ψ43). The base-pairing interaction between the branch site recognition sequence in yeast U2 and the branch site sequence in yeast pre-mRNA (N is the nucleotide immediately upstream of the branch site) is also schematized. The branch site recognition sequences are boxed, and the Sm binding sites are shaded. The sequences involved in U2-U6 interactions, Helices I, II, III (Helix III is not established in yeast) are highlighted by thick lines.

\[ \text{Vertebrate U2} \]

\[ \text{Yeast U2} \]

\[ \text{3'} \text{Pre-mRNA} \]

\[ \text{5'} \]

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\[ \text{X. Ma and Y.-T. Yu, unpublished data.} \]

\[ \text{D. S. McPheeters, unpublished data.} \]
pU7-GAL was used for the promoter shutoff assay (Fig. 4, and see below).

Transformation and 5FOA Selection—Log phase DM2484 or DM2486 cells were collected and adjusted to 50 A_{600}/ml in transformation buffer (40% (v/v) polyethylene glycol-3350, 0.1 M lithium acetate). The cells (20 μl) were mixed with 1 μl of carrier DNA (salmon sperm or calf thymus, 2 mg/ml). The resultant cell mixture then received ~1 μg of one of the U2 mutant plasmids or pCAU2 as a control and was subsequently incubated at 45 °C for 30 min. The cell mixture was then immediately mixed with 150 μl of YPD and incubated at 30 °C for another hour before plating on solid medium lacking histidine.

A single colony from the above plates was transferred to liquid SD–His synthetic medium (0.71% SD–His, 2% dextrose, and 2% peptone) and grown at 18 °C until the A_{600} reached 1.0. At that point, the incubation temperature was changed to 37 °C. Cells were collected at various time points (0.5–0.5 pmol (10,000 cpm) of the 5′-32P-radiolabeled 5′-primer specific for the gene to be analyzed (see the primers below), and 2.5 units of Taq polymerase (Invitrogen). A total of 5 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) was used, with the final cycle being followed by a 10-min room temperature incubation. The cDNA was then amplified by PCR in two steps. In the first step, the reaction was performed for 1 h at 37 °C in 20 μl containing 5 μg of DNA-free total RNA, 1× RT buffer (Promega), 0.5 μM dNTPs, 10 μM dithiothreitol, 2.5 μM random DNA hexamer, and 10 units of reverse transcriptase M-MLV (Promega). The primer extension reaction contained 106 cpm of the 5′-32P-radiolabeled 5′-primer specific for the gene to be analyzed (see the primers below), and 2.5 units of Taq polymerase (Invitrogen). A total of 5 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) was used, with the final cycle being followed by a 10-min room temperature incubation. The cDNA was then amplified by PCR in two steps. In the first step, the reaction was performed for 1 h at 37 °C in 20 μl containing 5 μg of DNA-free total RNA, 1× RT buffer (Promega), 0.5 μM dNTPs, 10 μM dithiothreitol, 2.5 μM random DNA hexamer, and 10 units of reverse transcriptase M-MLV (Promega). The primer extension reaction contained 106 cpm of the 5′-32P-radiolabeled 5′-primer specific for the gene to be analyzed (see the primers below), and 2.5 units of Taq polymerase (Invitrogen). A total of 5 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) was used, with the final cycle being followed by a 10-min room temperature incubation. The cDNA was then amplified by PCR in two steps. In the first step, the reaction was performed for 1 h at 37 °C in 20 μl containing 5 μg of DNA-free total RNA, 1× RT buffer (Promega), 0.5 μM dNTPs, 10 μM dithiothreitol, 2.5 μM random DNA hexamer, and 10 units of reverse transcriptase M-MLV (Promega). The primer extension reaction contained 106 cpm of the 5′-32P-radiolabeled 5′-primer specific for the gene to be analyzed (see the primers below), and 2.5 units of Taq polymerase (Invitrogen). A total of 5 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) was used, with the final cycle being followed by a 10-min room temperature incubation.

RESULTS

pus7 Deletion (pus7Δ) Coupled with Point Mutations in U2 snRNA at Position 40 Yields Temperature-sensitive Yeast Strains—As we have previously demonstrated (29), when incubated in a rich medium (YPD), the yeast pus7Δ strain, in which U2 pseudouridylation is completely blocked at position 35, shows no obvious growth defect phenotype (Fig. 2B, compare rows 1 and 2). To investigate the functional role of PUS35, we took advantage of a large collection of yeast U2 snRNAs, each containing a single point mutation near the branch site recognition region. Each of these U2-bearing plasmids was able to substitute for the yeast chromosomal U2 gene without compromising the healthy growth phenotype in rich media. These U2 mutants were combined with pus7Δ and selected for a synthetic growth defect phenotype. Briefly, we first created a yeast strain in which both the pus7Δ and U2 (SNR20) genes were deleted, and U2 was supplied by a plasmid containing a wild-type U2 gene and a URA3 selection marker (see “Experimental Procedures”). We then introduced each of the mutant U2 plasmids into the strain and streaked the cells on 5-FOA plates to select against the wild-type U2 plasmid via the URA3 marker. The yeast cells growing on 5-FOA should solely depend on the yeast chromosomal U2 gene and a URA3 selection marker.

| Deletion | Description | Observations |
|----------|-------------|--------------|
| PUS35 Δ | deletion | temperature-sensitive growth |
| PUS35 Δ | point mutation | temperature-sensitive growth |
| PUS35 Δ | point mutation | temperature-sensitive growth |
| PUS35 Δ | point mutation | temperature-sensitive growth |

The yeast cells growing on 5-FOA should solely depend on the yeast chromosomal U2 gene and a URA3 selection marker.
To confirm that the growth defect phenotypes were specifically associated with *pus7Δ* and the U2 point mutations, we introduced a plasmid containing *PUS7* or a plasmid containing a wild-type U2 gene into the temperature-sensitive yeast strains and found that either plasmid rescued the temperature-sensitive growth phenotypes (Fig. 2B, compare rows 7 and 8 with row 6 and rows 11 and 12 with row 10). Taken together, these results suggest that *pus7Δ* coupled with a mutation/deletion at U40 in U2 yields temperature-sensitive synthetic lethality.

**Yeasts with *pus7Δ* and U2-U40Δ Are Defective in U3 Pre-RNA Splicing at 37°C—**Because U40 is located immediately downstream of the U2 branch site recognition sequence (Fig. 1), it is possible that mutation at this position, when combined with a blockage of pseudouridylation at position 35 (due to *pus7Δ*), results in a splicing defect, and thus the observed growth defect, at higher temperatures. To test this possibility, we grew the mutant strains under permissive conditions at 18 °C and then shifted them to the nonpermissive temperature (37 °C). At various time points after the temperature shift, total RNA was extracted and tested for pre-mRNA splicing by primer extension. Two radiolabeled primers were used. One was complementary to the exon2-intron junction of U3 pre-RNA and should specifically hybridize with two forms of U3 pre-RNA (pre-U3a and pre-U3b), but not with spliced U3 RNA. The other primer was specific for U5 and served as a gel loading control. U3 pre-RNAs did not accumulate in wild-type cells, *pus7*-deleted cells, or *pus7*-deleted cells complemented with a *PUS7*-containing plasmid (Fig. 3, lanes 1–3). When total RNA from U2-U40Δ cells was used, only a slightly elevated level of U3 pre-RNAs was detected (lane 4). In contrast, cells containing both U2-U40Δ and *pus7Δ* showed a clear accumulation of U3 pre-RNAs (lane 5). Importantly, the levels of U3 pre-RNAs were greatly reduced upon subsequent transformation of a *PUS7*-containing plasmid into the cells (lane 6). Together, these results suggest that the lack of pseudouridylation at position 35 in combination with the U40 deletion in the U2 snRNA specifically results in a U3 pre-RNA splicing defect.

**Conditional Depletion of Pus7p from Mutant U2 Strains (U2-U40Δ and U2-U40G) Leads to Pre-mRNA Accumulation and Cell Death—**Although our temperature shift experiments to measure U3 pre-RNA levels were informative, they were not conclusive because the results could also reflect cellular responses to a sudden temperature change (i.e., “heat shock”). For instance, in both the mutant and wild-type strains, the shift from 18 to 37 °C elevated the levels of some pre-mRNAs as well as mRNAs (data not shown). Therefore, we took advantage of the widely used conditional promoter shutoff technique to confirm the above splicing results. A plasmid containing the *PUS7* gene under the control of the GAL promoter was introduced into the temperature-sensitive yeast strains (*pus7Δ*+U2-U40G and
pus7Δ+U2-U40Δ). The resulting yeast cells were grown at 30 °C, first in galactose medium and then in dextrose medium, thereby shutting off the GAL promoter and hence halting PUS7 transcription. At different time points after switching the medium, total RNA was recovered, treated with DNase, and used to assess U2 pseudouridylation and pre-mRNA splicing.

All yeast strains (wild-type and mutant) grew normally in galactose medium at 30 °C (Fig. 4A). However, 18 h after being switched to dextrose medium, mutant yeast strains with chromosomal pus7Δ and a U2 mutation or deletion at position 40 (closed diamonds or squares, respectively) grew significantly slower compared with the wild-type strain (open circles) or the pus7Δ strain that had also been transformed with a plasmid containing the PUS7 gene under the control of the Pgal promoter (open triangles). When total RNA recovered from cells either before or 18 h after the medium switch was tested for U2 pseudouridylation, a clear reduction of W35 signal was observed (Fig. 4B, compare lane 5 with lane 4, and lane 12 with lane 11). In contrast, pseudouridylation at positions 42 and 44 was virtually unchanged (compare lane 5 with lane 4, and lane 12 with lane 11). Furthermore, reverse-transcription sequencing of U2 snRNA from the same strains showed the expected mutation/deletion at position 40 in U2 (lanes 6–11 and lanes 13–16). Interestingly, for reasons that are yet unclear, the U2-U40G mutation, but not the U2-U40Δ mutation, also abolished the formation of W42 (compare lanes 4 and 5 with lane 2). Collectively, these results confirm that the deletion of Pus7p in the U2 mutant strains results in a growth defect phenotype as well as a blockade of U2 pseudouridylation at position 35 (and at position 42 for the U2-U40G strain).

We next analyzed pre-mRNA splicing using quantitative RT-PCR. Reverse transcription was performed using random hexamer primers and DNA-free total RNA (as used for U2 pseudouridylation mapping) as a template. The resulting cDNA was then amplified by PCR using 4 pairs of DNA oligonucleotides targeting four randomly chosen genes, including three intron-containing genes, RPL37A, RPL13A, and RPL37B, and the intronless gene, HHF1. Both RPL37A and RPL13A pre-mRNA levels in pus7Δ+U2-U40G and pus7Δ+U2-U40Δ strains increased dramatically (>10-fold for RPL37A and >3-fold for RPL13A) 18 h after the switch of media (Fig. 4C, top and middle panels, compare lane 5 with lane 4 and lane 8 with lane 7). The levels of these pre-mRNAs were very low in control strains (lanes 1–3 and 6) and did not change after the medium was switched (data not shown). As expected, the levels of the intronless HHF1 gene remained constant in all strains (lanes 1–8) and did not change after the medium was switched (data not shown). Interestingly, however, the pre-mRNA levels of the other intron-containing gene, RPL37B, apparently were not enhanced (Fig. 4C, bottom panel). Taken together, our data indicate that the lack of pseudouridylation at position 35 in yeast U2, coupled with a mutation/deletion at position 40 (U40G or U40Δ), significantly impairs the ability of U2 to participate in the splicing of at least a subset of pre-mRNAs.

**DISCUSSION**

Using a synthetic growth defect screen, we have determined that Pus7p, which is responsible for W35 formation in yeast U2, is important for pre-mRNA splicing activity. Neither pus7Δ alone nor mutation/deletion at position 40 in U2 alone had a large impact on pre-mRNA splicing and cell growth. When the two

### Table 1

**U2 mutant plasmids used to screen synthetic growth defects**

U2 mutant plasmids (CEN ARS HIS3 SNR20) could each substitute for the chromosomal U2 gene in a strain without compromising its growth phenotype, except as noted.

| Plasmid sequential number | Position in yeast U2 | Mutants assayed |
|---------------------------|---------------------|-----------------|
| 1                        | 23                  | U23A            |
| 2                        | 23                  | U23C            |
| 3                        | 23                  | U23G            |
| 4                        | 24                  | U24A            |
| 5                        | 24                  | U24C            |
| 6                        | 24                  | U24G            |
| 7                        | 24                  | U24D            |
| 8                        | 28                  | U28A            |
| 9                        | 28                  | U28C            |
| 10                       | 28                  | U28G            |
| 11                       | 33                  | U33A            |
| 12                       | 33                  | U33C            |
| 13                       | 33                  | U33G            |
| 14                       | 33                  | U33D            |
| 15                       | 40                  | U40A            |
| 16                       | 40                  | U40D            |
| 17                       | 40                  | U40G            |
| 18                       | 42                  | U42A            |
| 19                       | 42                  | U42C            |
| 20                       | 42                  | U42G            |
| 21                       | 42                  | U42D            |
| 22                       | 45                  | U45A            |
| 23                       | 45                  | U45C            |
| 24                       | 45                  | U45G            |
| 25                       | 45                  | U45D            |
| 26                       | 47                  | U47A            |
| 27                       | 47                  | U47C            |
| 28                       | 47                  | U47G            |
| 29                       | 47                  | U47D            |
| 30                       | 48                  | U48A            |
| 31                       | 48                  | U48C            |
| 32                       | 48                  | U48G            |
| 33                       | 49                  | U49A            |
| 34                       | 49                  | U49C            |
| 35                       | 49                  | U49G            |

* Exhibits temperature-sensitive growth (fails to grow at 37 °C).

* Exhibits cold-sensitive growth (fails to grow at 16 °C).
mutations were combined, however, the cells exhibited a clear temperature-sensitive phenotype. Under nonpermissive conditions (37 °C, or even 30 °C), the splicing of most pre-mRNAs tested was greatly inhibited, thus providing a direct functional link between Ψ35 in U2 and pre-mRNA splicing.

We screened 10 nucleotide positions in U2 (a total of 35 different mutations/deletions; Table I). Our data showed that U40 is crucial for pre-mRNA splicing if pseudouridylation at position 35 within the branch site recognition sequence is blocked (Fig. 2). Because U40 is immediately downstream of the branch site recognition sequence (ΨGUA) in U2, it may help stabilize the interaction between this sequence and the

![Image of a graph showing growth curves for four different yeast strains. The open circles represent the wild-type yeast strain. The open triangles represent the pus7Δ strain that had been transformed with a plasmid containing the PUS7 gene under the control of the PGal promoter. The closed squares represent the pus7Δ+U2-U40Δ strain transformed with the plasmid containing the PUS7 gene under the control of the PGal promoter. The arrow indicates the time point at which the medium was switched from galactose to dextrose. B, U2 pseudouridylation was analyzed using three different strains, including the wild-type (lanes 1 and 2), pus7Δ+U2-U40Δ transformed with the Pus7-GUS7-containing plasmid (lanes 3–5), and pus7Δ+U2-U40Δ transformed with the Pus7-GUS7-containing plasmid (lanes 10–12). Total RNA was isolated from each of these strains before (lanes 3, 4, 10, and 11) and 18 h after (lanes 1, 2, 5, and 12) the medium was switched. The RNA was assayed for pseudouridylation by CMC modification followed by primer extension using a radiolabeled antisense U2 oligodeoxynucleotide (lanes 2, 4, 5, 11, and 12). Lanes 1, 3, and 10 are controls where CMC modification was omitted. The U2 RNA from pus7Δ+U2-U40Δ cells (lanes 6–9) or from pus7Δ+U2-U40Δ cells (lanes 13–16) was also sequenced and subjected to electrophoresis in parallel. The three pseudouridine stops/pauses are indicated by asterisks and arrows. Changes at position 40 in the sequencing lanes are boxed. C, pre-mRNA levels were measured by RT-PCR. Template total RNA used in the study was isolated from various yeast strains, including wild-type (WT) (lane 1), pus7Δ (lane 2), U2-U40Δ (lane 3), U2-U40Δ (lane 6), pus7Δ+U2-U40Δ transformed with the Pus7-GUS7-containing plasmid (lanes 4 and 5), and pus7Δ+U2-U40Δ transformed with the Pus7-GUS7 (lanes 7 and 8). For the latter two strains, cells were first grown in galactose medium to the mid-log phase and then switched to YPD (dextrose medium). Total RNA was isolated before (lanes 4 and 7) and 18 h after (lanes 5 and 8) the medium was switched. Reverse transcription was primed with random hexamers, and PCR was performed using a pair of oligodeoxynucleotides (only the forward primer was radiolabeled) designed according to the sequences of the three different pre-mRNAs we analyzed, including RPL37A pre-mRNA (top panel), RPL13A pre-mRNA (middle panel), and RPL37B pre-mRNA (bottom panel). The intronless gene, HHF1, served as a control that was included in each analysis. The positions of RT-PCR products derived from respective pre-mRNAs/mRNA are indicated.

![Image of RT-PCR results showing RPL37A, RPL13A, and HHF1 pre-mRNA and mRNA levels from various yeast strains.]

FIG. 4. Promoter shutoff experiments showing that pus7Δ coupled with U2-U40Δ/U40Δ mutations leads to the accumulation of some pre-mRNAs. A, growth curves for four different yeast strains are shown. The open circles represent the wild-type yeast strain. The open triangles represent the pus7Δ strain that had been transformed with a plasmid containing the PUS7 gene under the control of the PGal promoter. The closed squares represent the pus7Δ+U2-U40Δ strain transformed with the plasmid containing the PUS7 gene under the control of the PGal promoter. The closed diamonds represent the pus7Δ+U2-U40Δ strain transformed with the plasmid containing the PUS7 gene under the control of the PGal promoter. The arrow indicates the time point at which the medium was switched from galactose to dextrose. B, U2 pseudouridylation was analyzed using three different strains, including the wild-type (lanes 1 and 2), pus7Δ+U2-U40Δ transformed with the Pus7-GUS7-containing plasmid (lanes 3–5), and pus7Δ+U2-U40Δ transformed with the Pus7-GUS7-containing plasmid (lanes 10–12). Total RNA was isolated from each of these strains before (lanes 3, 4, 10, and 11) and 18 h after (lanes 1, 2, 5, and 12) the medium was switched. The RNA was assayed for pseudouridylation by CMC modification followed by primer extension using a radiolabeled antisense U2 oligodeoxynucleotide (lanes 2, 4, 5, 11, and 12). Lanes 1, 3, and 10 are controls where CMC modification was omitted. The U2 RNA from pus7Δ+U2-U40Δ cells (lanes 6–9) or from pus7Δ+U2-U40Δ cells (lanes 13–16) was also sequenced and subjected to electrophoresis in parallel. The three pseudouridine stops/pauses are indicated by asterisks and arrows. Changes at position 40 in the sequencing lanes are boxed. C, pre-mRNA levels were measured by RT-PCR. Template total RNA used in the study was isolated from various yeast strains, including wild-type (WT) (lane 1), pus7Δ (lane 2), U2-U40Δ (lane 3), U2-U40Δ (lane 6), pus7Δ+U2-U40Δ transformed with the Pus7-GUS7 (lanes 4 and 5), and pus7Δ+U2-U40Δ transformed with the Pus7-GUS7 (lanes 7 and 8). For the latter two strains, cells were first grown in galactose medium to the mid-log phase and then switched to YPD (dextrose medium). Total RNA was isolated before (lanes 4 and 7) and 18 h after (lanes 5 and 8) the medium was switched. Reverse transcription was primed with random hexamers, and PCR was performed using a pair of oligodeoxynucleotides (only the forward primer was radiolabeled) designed according to the sequences of the three different pre-mRNAs we analyzed, including RPL37A pre-mRNA (top panel), RPL13A pre-mRNA (middle panel), and RPL37B pre-mRNA (bottom panel). The intronless gene, HHF1, served as a control that was included in each analysis. The positions of RT-PCR products derived from respective pre-mRNAs/mRNA are indicated.
branch site in pre-mRNA. A recent NMR study has also shown that conversion of U35 to \( \Psi \)35 increases the stability of this RNA-RNA duplex (34, 35). It is therefore possible that U40 as well as the branch site recognition sequence, including \( \Psi \)35, work in concert to ensure stable binding of the pre-mRNA branch site by U2. Mutations at position 40 or inhibition of pseudouridylation at position 35 alone may not be sufficient to destabilize this base-pairing interaction, whereas changes at both positions may preclude the interaction. Alternatively, mutation at position 40 could also destabilize the U2-U6 Helix III, an interaction that, although not established in the yeast spliceosome (39), appears to be important for mammalian pre-mRNA splicing (10). The hypothesis, linking \( \Psi \)35 and U40 to RNA-RNA stability in the spliceosome, is consistent with the fact that the mutant strains \( \text{pus7} \Delta + U2-U40G \) and \( \text{pus7} \Delta + U2-U40\Delta \) are heat-sensitive for splicing as well as growth.

Our results also suggest that the role of U2-U40 during splicing is more complex than its possible involvement in a canonical Watson-Crick base-pairing interaction with its partner nucleotide in pre-mRNA. We have tested a total of four pre-mRNAs, two of which (RPL37A and RPL13A) have a purine (G and A, respectively) immediately upstream of their highly conserved branch site (UACUAAC) that could potentially base pair with U40 in U2 snRNA (G-U or A-U pair, respectively). The change of U40 to G40 in U2 would abolish this base-pairing interaction and therefore inhibit splicing. This could explain why these two pre-mRNAs accumulated in the \( \text{pus7} \Delta + U2-U40G \) strain. However, U3 pre-RNA, which has a pyrimidine (U) at the equivalent position and should improve pairing with U2-U40G mutant, was also accumulated in the \( \text{pus7} \Delta + U2-U40G \) strain (data not shown). Clearly, this result does not agree with the simple Watson-Crick base-pairing interaction involving U40 in U2 and its partner in pre-mRNA. Also, we observed that both U2-U40G and U2-U40\Delta, when coupled with \( \text{pus7} \Delta \), had the same inhibitory effect on the splicing of RPL37A and RPL13A pre-mRNAs (Fig. 4C). The base-pairing model would not predict this outcome because position 41 of U2 is C, a pyrimidine as well. In other words, deletion of U40 from U2 would place C41 at the original position 40, thus restoring the base pairing with the purine (especially G) in the pre-mRNA, thereby driving the splicing reaction. Furthermore, we also tested the U40A mutant in U2 (Table I), but this mutation did not yield a growth defect phenotype. Like U40G, the U to A change at position 40 would also abolish the base-pairing interaction with RPL37A or RPL13A pre-mRNA, which have a G or A, respectively, at the corresponding position. An alternate hypothesis would be that U40 contributes to protein binding. In this regard, it has been reported that U40 is within the sequence involved in binding with SF3a and SF3b subunits and Prp5p (the DEAD protein) (39). It is therefore possible that mutation/deletion at this position results in a less stable binding with SF3 subunits and/or Prp5p. This defect, when coupled with the lack of \( \Psi \)35 in the branch site recognition sequence (and thus a less stable interaction with the pre-mRNA branch site), may result in a splicing defect. Clearly, further work is needed to address how \( \Psi \)35 and U40 contribute to pre-mRNA splicing.

It should be noted that because Pus7p also catalyzes tRNA pseudouridylation at certain positions (45), our data cannot exclude the possibility that pseudouridylation on tRNAs contributes to the observed phenotype. However, we think this possibility unlikely for at least three reasons. First, we observed a splicing defect in the mutant strains, and there is no evidence that a deficiency in tRNA pseudouridylation or any alteration in tRNA affects pre-mRNA splicing. Second, a normal phenotype was observed in a yeast strain carrying the \( \text{pus7} \Delta \) genotype only, and the splicing/growth defect was only evident when an additional U40 mutation in U2 was introduced. Because both U40 and \( \Psi \)35 are in U2, not tRNA, it is likely that the U40 mutation along with the inhibition of pseudouridylation at position 35 abolishes U2 splicing function at nonpermissive temperatures. Third, we did not observe abnormal tRNA processing in these strains by Northern analysis (data not shown), although some other steps, such as transport, amino-acylation, protein translation, etc., may still have been affected.

It is also interesting that the U40G mutation abolishes U2 pseudouridylation at position 42. It is possible that the mutation completely changes the element necessary for the \( \Psi \)42-specific enzyme (protein only or sno/scareNP) to recognize and catalyze the reaction, which would thereby abolish \( \Psi \)42 formation. Curiously, however, the deletion of U40 from U2 had no effect on the formation of \( \Psi \)42. At present, the mechanism of \( \Psi \)42 formation is still an open question. On the other hand, because the U40G mutation inhibits \( \Psi \)42 formation, we cannot rule out the possibility that the splicing defect observed in this mutant strain is a direct result of the three alterations in U2: the U40G mutation and the lack of pseudouridylation at positions 42 and 35. However, for at least two reasons, we think that the lack of \( \Psi \)42 has a minimal, if any, impact on pre-mRNA splicing in the \( \text{pus7} \Delta + U2-U40G \) strain. First, the defects in both splicing and growth of both \( \text{pus7} \Delta + U2-U40G \) and \( \text{pus7} \Delta + U2-U40\Delta \) strains are virtually identical (Figs. 2 and 4), despite the fact that pseudouridylation at position 42 differs in these two strains. Second, none of the U2-U42 mutants assayed (Table I) shows a growth defect despite the lack of \( \Psi \)42, even combined with either \( \text{pus7} \Delta \) or U40G mutation, suggesting that position 42 is not as important as positions 35 and 40 in contributing to pre-mRNA splicing and cell growth. Further study is necessary to clarify this issue.

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