REPORT

Multiple functions for the invariant AGC triad of U6 snRNA

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ABSTRACT
The invariant AGC triad of U6 snRNA plays an essential, unknown role in splicing. The triad has been implicated in base-pairing with residues in U2, U4, and U6. Through a genetic analysis in S. cerevisiae, we found that most AGC mutants are suppressed both by restoring pairing with U2, supporting the significance of U2/U6 helix Ib, and by destabilizing U2 stem I, indicating that this stem regulates helix Ib formation. Intriguingly, one of the helix Ib base pairs is required specifically for exon ligation, raising the possibility that the entirety of helix Ib is required only for exon ligation. We also found that U4 mutations that reduce complementarity in U4 stem I enhance U2-mediated suppression of an AGC mutant, suggesting that U4 stem I competes with the AGC-containing U4/U6 stem I. Implicating an additional, essential function for the triad, three triad mutants are refractory to suppression—even by simultaneous restoration of pairing with U2, U4, and U6. An absolute requirement for a purine at the central position of the triad parallels an equivalent requirement in a catalytically important AGC triad in group II introns, consistent with a role for the AGC triad of U6 in catalysis.

Keywords: U2; U4; U6; snRNA; splicing; spliceosome

INTRODUCTION
Pre-mRNA splicing, in which introns are excised from pre-mRNA to yield mRNA, is essential for the expression of most eukaryotic genes. The spliceosome, a ribonucleoprotein complex composed of five small nuclear RNAs (snRNAs)—U1, U2, U4, U5, and U6—and over 96 proteins (Staley and Guthrie 1998; Jurica and Moore 2003), catalyzes splicing. An intron recruits the spliceosome through conserved sequences at the 5′ and 3′ splice sites and the branch site. The spliceosome excises an intron by catalyzing two transesterification reactions. In the first reaction, the 2′ hydroxyl of an intronic adenosine, which forms the branch site, attacks the 5′ splice site, cleaving the 5′ exon from the intron and generating a branched lariat intermediate. In the second reaction, the 3′ hydroxyl of the freed 5′ exon attacks the 3′ splice site, excising the intron and ligation the exons. As the chemistry of pre-mRNA splicing is indistinguishable from group II intron self-splicing (Moore et al. 1993), the RNAs of the spliceosome may perform a critical function in catalysis (Villa et al. 2002).

Consistent with this hypothesis, in a catalytically active spliceosome U2 snRNA interacts with the branch site sequence, and U6 snRNA interacts with the 5′ splice site sequence (Staley and Guthrie 1998). Furthermore, base-pairing between U2 and U6 could serve to juxtapose the branch site adenosine with the 5′ splice site (Madhani and Guthrie 1992). Significantly, before the spliceosome recognizes an intron, U6 base pairs with U4, rather than U2, in a mutually exclusive manner (Brow and Guthrie 1988). Intron recognition triggers unwinding of U4/U6 and formation of U2/U6, activating the spliceosome for catalysis (Staley and Guthrie 1998).

Of the five snRNAs, U6 is the most attractive candidate for an snRNA that functions in catalyzing splicing. First, U6 has two highly conserved motifs—the ACAGAG box, which binds the 5′ splice site sequence (Kandels-Lewis and Séraphin 1993; Lesser and Guthrie 1993), and the AGC triad. Many mutations in these motifs are lethal, and many are deleterious for splicing in vitro. Phosphorothioate substitutions in the backbone of these motifs can also abolish splicing (Fabrizio and Abelson 1992; Yu et al. 1995). Second, a phosphate in the 3′ stem of U6 binds a key magnesium at a catalytic stage of splicing (Yean et al. 2000). Third, the catalytic domain V of group II introns shares structural similarities with U6 (for review, see Villa et al. 2002), including an AGC triad that is critical for catalysis (Boulanger et al. 1995; Peebles et al. 1995). Finally, base-paired U2/U6,
in the absence of protein, promotes a reaction that shares similarities with splicing (Valadkhan and Manley 2001). In contrast to U6, the snRNAs U1 and U4 are not required for the chemistry of splicing (Yean and Lin 1991, 1996), and the region of U5 that interacts with pre-mRNA does not play an essential role in splicing in vitro (O’Keefe et al. 1996; Segault et al. 1999).

The AGC triad of U6 performs an essential function in splicing. Mutations at the first two positions of the AGC triad confer lethality (Madhani and Guthrie 1992; McPeethers 1996), whereas mutations at the final position cause a strong growth defect (McPeethers 1996). Mutations at all three positions are defective for splicing in vitro (Fabrizio and Abelson 1990; Wolff et al. 1994). In mammals, mutations at all three positions result in a defect in 5’ splice site cleavage (Wolff et al. 1994). In budding yeast, whereas substitutions of the G and C similarly result in a defect in 5’ splice site cleavage, base and phosphate substitutions of the A (Fabrizio and Abelson 1990, 1992) result in a defect in exon ligation. Yet, despite its significance, the essential function for the AGC triad of U6 remains to be determined.

The AGC triad may interact with the snRNAs in one or more mutually exclusive structures (Fig. 1). The AGC triad has been implicated in interactions with U2 to form helix Ib (Fig. 1A; Madhani and Guthrie 1992) and with U6 to extend the 3’ stem (Fig. 1B; Sun and Manley 1995). The AGC triad could also extend the 3’ stem in free U6. In addition, the triad is complementary to U4 in U4/U6 stem I (Fig. 1C; Brow and Guthrie 1988). The AGC triad may also bind an additional component, forming a novel interaction.

![Diagram](https://example.com/diagram.png)

**FIGURE 1.** The AGC triad is complementary to sequences within U2, U4, and U6. (A) U2/U6 helix Ib, in which the AGC triad is complementary to U2 (Madhani and Guthrie 1992). Helix Ib is mutually exclusive with U4/U6 stem I and U2 stem I. Helix II is also mutually exclusive with U2 stem I. (B) U6 3’ stem extension, in which the AGC triad is complementary to downstream residues in U6. The extension could form in base-paired U2/U6, as suggested by Sun and Manley (1995), or in free U6. (C) U4/U6 stem I, in which the AGC triad is complementary to U4 (Brow and Guthrie 1988). U4/U6 stem I is mutually exclusive with U4 stem I (Fig. 3C). (D) U2 stem I. Throughout, structures containing the AGC triad are boxed.

To investigate the role of the AGC triad in *Saccharomyces cerevisiae*, we performed a systematic compensatory analysis of the putative AGC-containing structures. We found genetic evidence for the entirety of U2/U6 helix Ib. In contrast, we found no significant evidence for base-pairing between the AGC triad and downstream U6 sequences. U4/U6 stem I is necessary, although not sufficient, for wild-type growth. Unexpectedly, mutations that destabilize U2 stem I (Fig. 1D) suppress AGC triad mutants, suggesting that U2 stem I competes with helix Ib. Similarly, U4 stem I appears to compete with U4/U6 stem I. Significantly, three AGC mutants remain lethal even after simultaneous restoration of all three AGC-containing structures, suggesting that the AGC triad plays a critical, yet undiscovered, role beyond simply Watson–Crick base-pairing. Supporting a role for the AGC triad in catalysis, the central position of the triad must be a purine, paralleling an equivalent requirement in the AGC triad of the catalytic domain of group II introns.

**RESULTS AND DISCUSSION**

All but three mutations in the AGC triad of U6 are suppressed by reformation of U2/U6 helix Ib

Madhani and Guthrie (1992) provided genetic evidence for helix Ib by showing that U6 mutants -A59C and -A59G can be suppressed by compensatory mutations at U2-U23. Reciprocal suppression at this base pair and suppression at the other base pairs has not been reported, however, and in mammals the AGC triad has been implicated instead in base-pairing with downstream residues in U6 (Fig. 1B). We now report evidence confirming the significance of all three base pairs in helix Ib.

Providing further evidence for the first base pair of helix Ib, we found that U2-U23 mutants can be suppressed reciprocally by U6-A59 mutations. Although U2-U23 mutants grow like wild-type yeast at or above 25°C (Madhani and Guthrie 1992), we found that these mutants, in a background lacking the large, nonessential, fungal-specific domain of U2 (U2ΔFD; Shuster and Guthrie 1988), grow poorly at 15°C. Furthermore, the growth defects of U2ΔFD-U23C and -U23G are suppressed by U6-A59 mutations that restore helix Ib (Fig. 2B). As the U2-U23 mutants in full-length U2 are not similarly cold-sensitive (data not shown), these data also indicate that the fungal domain deletion enhances helix Ib mutants, suggesting a role for the fungal domain in stabilizing helix Ib. The suppression of U2ΔFD-U23 mutants by U6-A59 mutations is the first evidence that formation of helix Ib is a functionally important role of U2.

Base-pairing between U6-A59 and U2-U23 is not sufficient, however, as one Watson–Crick combination fails to support growth. Specifically, although Madhani and Guthrie (1994) showed that U6 mutants -A59C and -A59G are suppressed by compensatory mutations at U2-U23, suppression of U6-A59U by U2-U23A was not observed (cf.
The failure to suppress U6-A59U could result from misfolding or from a failure to satisfy an additional role for U6-A59. We have found no evidence that U6-A59U base pairs aberrantly with the bulge in U2/U6 helix I or that U6-A59U misfolds or from a failure to satisfy an additional role for helix Ib formation. Providing evidence for the third helix Ib base pair, we found that all three U6-C61 mutations are suppressed by U2 compensatory mutations that restore helix Ib formation. For instance, when stem I is maintained, severe, yet viable, mutations of the AGC triad do not affect U6 snRNA levels (data not shown). Just as the ad- enine of the AGC triad appears to function beyond helix Ib formation, these allelic constraints suggest that the gua- nine of the AGC triad serves an additional, purine-dependent role in splicing that is distinct from its role in helix Ib formation. Providing evidence for the second base pair of helix Ib, we found that a pyrimidine substitution at U6-G60, the triad residue least tolerant to mutation (Madhani and Guthrie 1992), is suppressed by reformation of helix Ib. Specifically, U2-C22U suppresses the lethality of U6-G60A (Fig. 2C, upper left). To maintain the integrity of U2 stem I and U2/U6 helix II (Fig. 1D and Fig. 1A, respectively) in this double mutant, we included mutations U2-G13A and U6-C92U. Suppression of U6-G60A requires the maintenance of helix II (data not shown), indicating that helix Ib is sensitive to the stability of helix II (cf. Field and Friesen 1996). In contrast to U6-G60A, pyrimidine mutations at G60 cannot be suppressed by restoration of U2/U6 helix Ib (Fig. 2C). Although we cannot rule out that these lethal AGC mutants are not suppressible for trivial reasons such as reduced levels of U6, we do find that severe, yet viable, mutations of the AGC triad do not affect U6 snRNA levels (data not shown). Just as the ad- enine of the AGC triad appears to function beyond helix Ib formation, these allelic constraints suggest that the gua- nine of the AGC triad serves an additional, purine-dependent role in splicing that is distinct from its role in helix Ib formation.
tant U2-G21C, which reforms a Watson–Crick base pair, and by U2-G21U, which forms a wobble in helix Ib (Fig. 2D). In addition, the mild temperature sensitivities of U6-C61A and -C61U are suppressed by compensatory mutations at U2-G21 (data not shown). We conclude that the primary function of the C of the AGC triad is to base pair with U2. Still, given the strict conservation of the C of the AGC triad (Gu et al. 1998), we anticipate that this C, as for the A and the G of the AGC triad, likely plays an additional role beyond helix Ib formation under certain conditions.

The role of the AGC triad in base-pairing with U2 appears to function redundantly with the additional, essential role of the AGC triad. Although some AGC triad mutations are sick or lethal, consistent with the importance of helix Ib, all U2 mutations that disrupt helix Ib are viable (Fig. 2C,D; data not shown; Madhani and Guthrie 1992), indicating that helix Ib is nonessential. Field and Friesen (1996) found that U2 mutations in helix Ib and helix II are synthetically lethal, suggesting that helix Ib merely serves a function redundant to that of helix II. Restoration of helix Ib, however, is sufficient to suppress most AGC mutants, indicating that helix Ib becomes essential when a critical role of the AGC triad is compromised. We conclude that helix Ib reinforces this essential function of the AGC triad (discussed below).

We have begun an investigation to determine which stage in splicing requires helix Ib, and we found that the helix Ib base pair between U6-A59 and U2-U23 is required specifically for exon ligation. The U6-A59C allele (Fig. 2I; cf. Fabrizio and Abelson 1990) and the U2AFD-U23C and -U23G alleles (Fig. 2I; Madhani and Guthrie 1992) show reduced mRNA levels and increased lariat intermediate levels, indicating a defect in exon ligation. Importantly, combining U6-A59C and U2-U23G to restore helix Ib results in mutual suppression of their exon ligation defects. We conclude that the adenosine of the AGC triad functions in exon ligation, in part, by base-pairing within U2/U6 helix Ib. Because the first-step defect observed for mutations in the last two positions of the AGC triad (Fabrizio and Abelson 1990) may reflect a defect in formation of the U6 3′ stem or U4/U6 stem I (Fig. 1), rather than helix Ib, it remains to be determined whether the other base pairs in helix Ib are required for 5′ splice site cleavage or exon ligation. We are currently distinguishing between these possibilities in vitro.

Restoring the 3′ stem of U6 fails to suppress AGC triad mutations

As restoration of U2/U6 helix Ib is not sufficient to suppress three AGC mutants and the phenotypes of helix Ib mutants are asymmetric (Fig. 2B–D), the AGC triad likely plays a critical role beyond helix Ib formation. For example, the AGC triad may also extend the 3′ stem of U6 (Fig. 1B). Indeed, in mammals, where evidence for helix Ib has been lacking, suppression of several mutations in the AGC triad by downstream U6 mutations (Sun and Manley 1995) has suggested a critical function for the AGC triad in extending the 3′ stem of U6. In yeast, however, we do not observe significant suppression by any downstream U6 mutations (Fig. 2F–H). Furthermore, compensatory mutations at U6-G86 enhance, rather than suppress, U6-C61 mutations; for example, at 25°C, where U6-G86C grows well and U6-C61G grows slowly, the double mutant does not grow at all (Fig. 2H). These in vivo results are consistent with in vitro studies that similarly failed to find evidence for the 3′ stem extension (Ryan and Abelson 2002). Finally, U6 compensatory mutations fail to synergize with U2 compensatory mutations in suppressing AGC mutants (data not shown). We conclude that formation of the U6 3′ stem extension is not an important function of the AGC triad in yeast.

Although the AGC triad may function through alternative structures in the yeast and mammalian systems, formation of U2/U6 helix Ib may also be important in the mammalian system. First, the apparent discrepancies between the yeast (this work; Madhani and Guthrie 1992; Ryan and Abelson 2002) and mammalian (Sun and Manley 1995) systems may simply reflect differing rate-limiting steps during splicing. Second, although double mutations in the last two positions of the AGC triad in the mammalian system are not suppressed by compensatory U2 mutations (Sun and Manley 1995), tests for suppression of single mutations in these positions have not yet been reported. Conservation and cross-linking in the U12-dependent spliceosome (Tarn and Steitz 1996; Frilander and Steitz 2001) further support the significance of helix Ib. Significantly, the formation of helix Ib requires unwinding of the upper portion of U2 stem I, whereas formation of the U6 3′ stem extension does not. Thus, helix Ib formation necessitates a significant rearrangement in the activation of the spliceosome, suggesting that U2 stem I is a potential target for one of the splicesomal DExD/H-box ATPases (for review, see Silverman et al. 2003).

Restoring all AGC-containing structures fails to suppress three lethal AGC mutants

As restoration of both U2/U6 helix Ib and the U6 3′ stem fails to suppress three lethal mutations in the AGC triad (data not shown), the triad likely interacts with another component. U4 is a candidate, as the AGC triad is complementary to U4 within U4/U6 stem I (Fig. 1G; Brow and Guthrie 1988). Reformation of U4/U6 stem I alone via U4 compensatory mutations is not sufficient to suppress any AGC triad mutant (data not shown). Thus, we tested whether restoration of all three AGC-containing structures can augment or confer suppression of triad mutants (Fig. 3A,B). We found that a compensatory mutation in U4 does augment suppression by U2. Specifically, although the combination of U2 and U6 compensatory mutations alone fails to suppress U6-G60A at 37°C, the addition of U4-C59U...
Roles for the invariant AGC triad of U6

The partial redundancy between the essential, unknown function of the AGC triad and U2/U6 helix Ib (Fig. 2B–D; Madhani and Guthrie 1992) suggests that the essential function acts within the context of helix Ib. For example, both the helical configuration of helix Ib and the specific major groove functionalities of the AGC triad could contribute simultaneously and redundantly to the binding of a base or an important metal. Significantly, mutation of the helix that includes the AGC triad in domain V of group II introns exhibits a similar redundancy between the helical context and the identity of the AGC triad (Boulanger et al. 1995; Peebles et al. 1995). As the functional groups on the major groove face of the AGC triad in group II introns perform a catalytic role in splicing (Konforti et al. 1998), this parallel suggests that the major groove face of the U6 AGC triad, within the context of helix Ib, also plays a catalytic role in splicing.

The permitted mutations in the AGC triad (Fig. 3D) are consistent with a role for the major groove face of the AGC triad. The purine requirement at U6-G60 (Fig. 2C) suggests that functional groups common to purines, such as the N7, in the major groove, and/or the N3, in the minor groove, play an important role at the central G. As the major groove face of adenosine can be mimicked by cytosine in the syn conformation, the tolerance of the U6-A59C mutation (Fig. 3A, 3B) is consistent with a role for the major groove face of the AGC triad, within the context of helix Ib, could coordinate one of the catalytically important metals required for leaving-group stabilization during pre-mRNA splicing (Sontheimer et al. 1997; Gordon et al. 2000). We are currently performing in vitro experiments to elucidate the additional, essential role of the AGC triad.

Noncompensatory, weak suppressors of AGC mutants

We found that mutations that disrupt U4 stem I (Fig. 3C; Myslinski and Branlant 1991) can contribute to suppression of an AGC triad mutant, which disrupts the mutually exclusive U4/U6 stem I. Specifically, any mutation at U4-C59 suppresses U6-G60A at 37°C (Fig. 3B) in the context of the

confers wild-type growth (Fig. 3B). These data support the importance of U4/U6 stem I formation.

The U4/U6 interaction, however, does not account for the lethality of AGC mutants refractory to suppression. Even in the presence of U2, U4, and U6 compensatory mutations, the triad mutants U6-A59U, -G60C, and -G60U are lethal (Fig. 3A, 3B). Furthermore, the severity of mutations in vivo parallels their splicing defects in vitro (Fabrizio and Abelson 1990). Consistent with the strict conservation of the specific sequence of the AGC triad, these results suggest that U6-A59 and -G60 of the AGC triad perform a function beyond simply base-pairing with these snRNAs. We favor the hypothesis that the AGC triad interacts with yet another component, such as a critical nucleotide or an essential metal ion.

Interestingly, a purine preference at the central position of the AGC triad is also observed in a similar structure in the catalytic domain of group II introns. In the group II intron a15y, all mutations of the central G of the AGC triad inhibit splicing, but an A-C wobble can substitute for the wild-type G-U wobble (Boulanger et al. 1995; Peebles et al. 1995). In the mammalian spliceosome, mutation of the central G of the AGC triad to an A has no effect on splicing in vivo, whereas mutation to a C or U completely inhibits splicing (Datta and Weiner 1993), consistent with a purine-dependent role for the guanosine. Curiously, however, in the mammalian spliceosome the C mutation can be suppressed by a downstream mutation in U6 (Sun and Manley 1995), possibly reflecting different rate-limiting steps in yeast and mammalian splicing. Still, a purine-dependent function for the central residue of the AGC triad is implicated for both the yeast spliceosome and group II introns, consistent with a common, RNA-based mechanism for the catalysis of splicing.

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FIGURE 4. Mutations that destabilize U2 stem I suppress AGC mutants. (A) Suppression of U6-A59C, -G60A, and -C61A by mutations that destabilize U2 stem I. (B) Suppression of U6-C61A by additional mutations that destabilize U2 stem I. The matrices are labeled as in Figure 2 and show the growth phenotypes at 34°C in yHM118. Arrows mark the position of nucleotide changes in U2 stem I. 1 Compensatory mutation U6-C92U maintains U2/U6 helix II in the presence of the U2-G13A mutation.

U2 compensatory mutation that alone suppresses U6-G60A at lower temperatures (Fig. 2C). Although only the compensatory U4 allele C59U, which restores U4/U6 stem I, suppresses U6-G60A strongly, U4-C59A and -C59G suppress U6-G60A weakly (Fig. 3B). As wild-type U4-C59 stabilizes U4 stem I, the U4-C59 mutations likely weaken U4 stem I, thereby promoting the interaction of the AGC triad of U6 with U4. These data suggest that U4 stem I antagonizes U4/U6 formation and that the interaction between the AGC triad and U4 helps drive U4/U6 formation.

Mutations that destabilize U2 stem I, which is mutually exclusive with helix Ib, also weakly suppress AGC triad mutants. Surprisingly, we found that U6-G60A is suppressed more robustly by U2-C22U, which restores helix Ib but disrupts U2 stem I, than by U2-C22U/G13A, which restores helix Ib and maintains U2 stem I (Fig. 4A). Suppression of G60A, although enhanced by disruption of U2 stem I, requires maintenance of helix Ib. Other AGC mutants, however, are suppressed by disruption of U2 stem I alone in the absence of helix Ib repair. Specifically, each of the stem I-disrupting mutations U2-C14G, -C14U, -G21A, -G21C, -G21U, and -A25U suppresses the mutant U6-C61A (Fig. 4A,B); some of these U2 alleles, such as U2-G21U and -A25U, also suppress U6-C61G and -C61U growth defects (data not shown). At 15°C, U2-G21U, which maintains helix Ib, suppresses U6-C61A more robustly than G21A or G21C (data not shown), underscoring the importance of helix Ib; additionally, U2 mutations that disrupt a second base pair within helix Ib, such as U2-C22U, are synthetically lethal with C61A (Fig. 4A). The mutation U2-A25U also suppresses the mutant U6-A59C (Fig. 4A). These results indicate that U2 stem I competes specifically with U2/U6 helix Ib (cf. Wu and Manley 1992), suggesting that U2 stem I unwinding may be regulated to control activation of the spliceosome.

In summary, we have shown that the AGC triad, in forming U2/U6 helix Ib, forms an important interaction with U2. We also found that formation of helix Ib is coupled to U2 stem I unwinding, suggesting a mechanism for regulating the function of the AGC triad. Additionally, our data indicate that the sequence tolerance of the AGC triad in U6 and in group II introns is similar. Finally, we found that the AGC triad functions beyond helix Ib formation in an essential role that may be involved in the catalysis of splicing.

MATERIALS AND METHODS

Strains and plasmids

yPS628 (MATa ade2-101; his3Δ200; leu2Δ1; lys2-801; trp1Δ63; ura3-52; snr6::LEU2; snr14::KanMX4; snr20::LYS2; [pJPS467]) was derived from yHM118 (Madhani and Guthrie 1994). pU2U6U in yHM118 was exchanged for pJPS467, and the chromosomal copy of SNR14 was disrupted with KanMX4 (Wach et al. 1994). To make pJPS467, we amplified wild-type U4 from a pSE362-derived vector (Madhani et al. 1990) by PCR to add flanking XbaI sites (primers 5’-CCCCCTCTAGAGAATTCGGTGAAAAAGAAAAAGAAAAATATGG-3’ and 5’-CCCCGGCTCGAGGAATTCGGTGAAAAAGAAAAAGAAAAATATGG-3’), and cloned the digested product into the XbaI site of pU2U6U.

pSX6 (Madhani and Guthrie 1992) and a pSE362-based plasmid (pPS216; Shuster and Guthrie 1988) were modified by QuikChange mutagenesis (Stratagene) to create U6 and full-length U2 mutant alleles, respectively. pES143 (Shuster and Guthrie 1988) was modified by QuikChange to create the wild-type and mutant U2ΔFD alleles by deleting the nonnative residue U2-T6 and introducing the appropriate mutations. To create pJPS464, we amplified wild-type U4 from a pSE362-derived vector (Madhani et al. 1990) by PCR to add flanking SacI and Aval sites (primers 5’-CCCCAAAGGCTCATCATAAGTGCGTTCGAGGAAATAC-3’ and 5’-CCCCCTCTAGAGAATTCGGTGAAAAAGAAAAAGAAAAATATGG-3’), and cloned the digested product into the XbaI site of pU2U6U.

Growth assays

To combine U2 and U6 alleles, we cotransformed yHM118 with pPS216 and pSX6 variants. To combine U2, U4, and U6 alleles, we cotransformed yPS628 with pPS216, pSX6, and pJPS464 variants. Transformations were plated on media that selected for both the transformed and resident wild-type plasmids. Cells were grown overnight in liquid media selecting for all plasmids. Overnight cultures were diluted in rich media and grown for 3.5 doublings at 30°C. Cultures were diluted to equivalent optical densities and
spotted onto media containing 5-fluoroorotic acid (5-FOA; Sikorski and Boeke 1991). Growth phenotypes were assayed at 15°, 20°, 25°, 30°, 34°, and 37°C for 2–15 d.

**Primer extension analysis**

Cotransforming constructs were streaked onto 5-FOA media and colony-purified on rich media. Cells were grown in rich media at 30°C and harvested during log phase after a 4-h shift to 37°C. Total RNA was isolated, and specific RNA species were assayed by primer extension (Stevens et al. 2002) using 32P-end-labeled primers complementary to the 3’ exon of RPS1A (5’-CTTAGAAGCAACGCTTGACGG-3’) and to U14 (5’-AGCAGAGTGGTCTGAACGGTAC TCCTACCGTGG-3’). Products were separated on a 12% polyacrylamide gel, developed by PhosphorImager, and quantitated by ImageQuant 1.2 (Molecular Dynamics).

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