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The rRNA-Processing Function of the Yeast U14 Small Nucleolar RNA Can Be Rescued by a Conserved RNA Helicase-Like Protein

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The phylogenetically conserved U14 small nucleolar RNA is required for processing of rRNA, and this function involves base pairing with conserved complementary sequences in 18S RNA. With a view to identifying other important U14 interactions, a stem-loop domain required for activity of *Saccharomyces cerevisiae* U14 RNAs (the Y domain) was first subjected to detailed mutational analysis. The mapping results showed that most nucleotides of the Y domain can be replaced without affecting function, except for loop nucleotides conserved among five different yeast species. Defective variants were then used to identify both intragenic and extragenic suppressor mutations. All of the intragenic mutations mapped within six nucleotides of the primary mutation, suggesting that suppression involves a change in conformation and that the loop element is involved in an essential intermolecular interaction rather than intramolecular base pairing. A high-copy extragenic suppressor gene, designated *DBP4* (DEAD box protein 4), encodes an essential, putative RNA helicase of the DEAD-DEXH box family. Suppression by *DBP4* (initially CA4 [T.-H. Chang, J. Arenas, and J. Abelson, Proc. Natl. Acad. Sci. USA 87:1571–1575, 1990]) restores the level of 18S rRNA and is specific for the Y domain but is not allele specific. DBP4 is predicted to function either in assembly of the U14 small nucleolar RNP or, more likely, in its interaction with other components of the rRNA processing apparatus. Mediating the interaction of U14 with precursor 18S RNA is an especially attractive possibility.

The eukaryotic large rRNA operon is transcribed as a large precursor which subsequently undergoes a series of cleavages and nucleotide modifications to produce the mature rRNAs. These processes take place in the nucleolus and require both protein factors and small nucleolar RNAs (snoRNAs) (9, 10, 27, 42, 46). The snoRNA populations are large, probably in excess of 100 different species, and are known to be involved in both processing and nucleotide modification. The number of snoRNAs currently linked to processing is quite small, only five in *Saccharomyces cerevisiae* and six in vertebrates (11, 27, 45, 46). A much larger number of species are known or predicted to provide sequence specificity for ribose methylation of rRNA (7, 21, 29, 30). Many other snoRNAs have yet to be tied to any process, but these species are also expected to have roles in ribosome synthesis, for example, in other modification reactions, folding of pre-RNA, or ribosome assembly (2, 27, 43). Like other cellular RNAs, the snoRNAs are presumed to function as snoRNP complexes rather than as free RNA.

All snoRNAs known can be classified into two large families, except for one species suspected of being a ribozyme (7-2/MRP) (4). These families are distinguished by the presence of conserved sequence elements, known as boxes C and D (the box C/D family), and the ACA box (the ACA box family). The box elements are required for snoRNA synthesis, in particular, processing of snoRNA precursors (4, 15, 47). Box D also influences formation of 5′ trimethylguanosine caps (44) where these occur, nucleolar localization (34, 44), and site selection for ribose methylation of rRNA (7, 21, 29, 30). snoRNAs required for processing exist in both families, whereas all snoRNAs linked to rRNA methylation are in the box C/D family.

U14 snoRNA, which is the subject of the present study, is required for processing of 18S rRNA in yeast (24) and for a cleavage in the 5′ external spacer in mouse (11). It is also predicted to have a role in ribose methylation (21). U14 has been found in a variety of organisms, including several yeasts, vertebrates, and plants (22, 27, 49). All U14 RNAs contain four common sequence elements and a 5′, 3′ terminal helix (see Fig. 1). The universal sequences include boxes C and D and domains A and B (19). Boxes C and D are joined by the terminal stem, and this box C/D-helix motif is required for processing and accumulation of U14 (3, 15, 47).

Domains A and B base pair with complementary elements in 18S rRNA, which are also conserved (26). Domain A is essential for processing activity (19). Domain B is dispensable but becomes conditionally essential when the interaction between domain A and rRNA has been weakened (26). The rRNA elements complementary to U14 are predicted to be in close proximity in the folded molecule, suggesting that U14 might bind to both elements simultaneously. In this context, U14 is viewed as a potential RNA chaperone involved in long-range folding and perhaps stabilization of pre-RNA. The postulated role in rRNA methylation is based on the presence of a motif which provides sequence specificity for this process (7, 21, 29, 30). The common motif consists of a long sequence complementary to rRNA followed closely by a box D/D′ sequence. The domain B-box D elements conform to this motif, and a 2′-O-methylated nucleoside occurs in the expected position in rRNA (35). The fact that domain B is dispensable in yeast U14 argues that the predicted methylation function is not essential.

The focus of the present report is another U14 structural domain that is present in yeasts and perhaps plants but absent in U14 homologs from vertebrates. This structure is called the

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Y domain (yeast specific) and is required for activity (23). The Y domain is a highly folded stem-loop structure and accounts for nearly all of the approximately 40-nucleotide (nt) difference in size between *Saccharomyces cerevisiae* and vertebrate U14 homologs. Large deletions and substitutions in the Y domain disrupt processing of 18S RNA (23). The importance of this domain was also demonstrated in a study which showed that mouse U14 was inactive in yeast but functional when the Y domain from *S. cerevisiae* was inserted at the appropriate site (23).

Y-domain structures have been identified in U14 RNAs from five yeasts. In addition to *S. cerevisiae*, these include *Kluyveromyces lactis*, *Pichia pastoris*, *Candida albicans*, and *Schizosaccharomyces pombe* (38). Comparison of the Y-domain sequences from the five homologs showed that the lengths and nucleotide sequences of the stems are not conserved; the loop, however, contains eight completely conserved nucleotides, including the pentanucleotide 5′-GAACC-3′ (see Fig. 1). Conservation of the loop sequence and the overall similarity of the stem-loop structures suggest that the vital Y-domain function involves some intra- or intermolecular interaction, with the loop sequence serving as a recognition signal (38).

The present study was undertaken to define the structural requirements for Y-domain activity and to attempt to identify components that interact with it. Our strategy was to perform an extensive mutational analysis of the Y domain and then search for both intragenic and extragenic suppressors that would rescue defects in this domain. Several essential nucleotides were identified within the loop. Near-lethal combinations of point mutations in this segment were found to be rescued by intragenic mutations near the primary mutation site and by overexpression of extragenic suppressor genes. Characterization of one extragenic suppressor gene revealed it to encode a putative, essential RNA helicase protein.

MATERIALS AND METHODS

Strains and media. Yeast strain YS153 is *MAT*α *ura3 trp1 his3 HIS3::GAL1::U14*. Wild-type U14 in this strain is expressed from a galactose-inducible allele encoded in the chromosome (19). Strain YS626, which was derived from YS153, is *MAT*α *leu2 ura3 trp1 his3 HIS3::GAL1::U14*. YS628 is an isogenic strain of YS626, differing only in mating type (*MAT***a**). Cells were cultured in medium containing 0.67% yeast nitrogen base (amino acid free), 2% vitamin-free Casamino Acids, and 2% glucose or galactose, supplemented with uracil (200 μM) or tryptophan (400 μM) as needed. Cells and cultures were manipulated as described by Sherman et al. (41).

DNA manipulations and Northern hybridization. Plasmid pJZ45 is a yeast low-copy plasmid containing a *TRP1* gene and a 1.3-kb yeast genomic DNA fragment which includes the U14 gene (19). This plasmid was used for in vitro mutagenesis as described previously (19). In short, a single-stranded uracil-containing template of pJZ45 was isolated by infection of helper phage R408 (Stratagene). Purified template was then annealed with a mutagenic oligonucleotide, and the second strand was synthesized by T4 DNA polymerase. The reaction mix was transformed into *Escherichia coli*. U14 genes from individual clones were sequenced or were introduced directly into a *GAL1-U14* test strain (YS153). In the latter case, transformants were initially grown on galactose medium, where the chromosomal U14 gene is expressed, and then replica plated to glucose medium and incubated at 15, 30, and 37°C to screen for growth defects. Plasmids unable to support growth of the test strain on glucose were isolated, and the growth defects were confirmed by restreaking transformants on glucose plates. The U14-containing plasmids from these cells were isolated, shuttled into *E. coli*, and subjected to DNA sequencing. DNA subcloning was performed according to standard procedures (1).

RNA was extracted from cells grown to an optical density at 660 nm of 1 to 2. Cells were resuspended in NaCl buffer and vortexed in hot water-saturated phenol, followed by several phenol-chloroform extractions (14). RNA blotting and hybridization were carried out as previously reported (3). DBP4 mRNA was detected with a probe derived by digestion of a 1.2-kb fragment of *DBP4* with *Bgl*II. A 0.6-kb *Xho*I-*Kpn*I fragment from the second exon of the *ACT1* gene was used to detect actin mRNA. DNA fragments were radiolabeled by using a random primer kit (NEBlot) from New England Biolabs.

Identification of intragenic and extragenic suppressors of mutant U14. The search for intragenic suppressors was based on a strategy described by others (50). Briefly, strain YS626 with a *URA3* plasmid containing the A64U, C65U mutant U14 allele was mutagenized with ethyl methanesulfonate (EMS), at a concentration which led to 90% mortality, and spread on glucose medium. To distinguish intragenic from extragenic mutations, surviving cells (a total of around 25) were mated with a YS626 isogenic strain (YS628) which harbored a single-copy *TRP1* plasmid containing the A64U, C65U mutant U14 allele. The resulting diploid cells were grown on galactose medium containing 5-fluoro-orotic acid to cure the *URA3* plasmid, and cells lacking this plasmid were then streaked on glucose. Failure to grow on glucose suggested that the *URA3* plasmid was responsible for the suppression, indicating the likelihood of an intragenic suppressor mutation. Plasmids from these candidate strains were then shuttled into *E. coli* and sequenced to map the sites of the mutations. To confirm that suppression was indeed intragenic, the plasmids recovered from *E. coli* were also reintroduced into the initial *GAL1-U14* test strain (YS626). Growth phenotypes of transformants were examined on glucose medium at 15, 30 and 37°C.

Extragenic suppressor genes were identified by transforming a genomic library in a high-copy vector (Yep24) into YS626 cells containing the C66U, C67G mutant U14 allele in a single-copy, *TRP1* plasmid. The library was constructed by Carlson and Botstein (6). The transformants were grown on glucose at 15°C for 20 days, and fast-growing colonies were isolated. Plasmids of interest were shuttled into *E. coli* and subjected to further analysis.

Sequence analysis of the *DBP4* gene. DNA fragments containing the *DBP4* gene were subcloned into the pBluescript KS+ plasmid and sequenced by a combination of primer walking and nested deletion methods (1). A database search was performed with the Blast program provided by the National Center for Biotechnology Information, and an analysis of the DNA sequence was carried out with programs from the University of Wisconsin Genetics Computer Group. *DBP4* was also independently sequenced in the laboratory of J. Abelson by C. O'Day (as CA4 DNA) (32). Sequence data from the two sources are in complete agreement.

Nucleotide sequence accession number. The GenBank accession number for *DBP4* is U72149.

RESULTS

Y-domain function requires a stem-loop structure and conserved loop sequence. The importance of the Y domain was initially demonstrated by deletion mutations (23); however, in no case was the domain excised perfectly. To rectify this, we constructed two new deletions. One removed the segment from position 49 (G) to 81 (U) (G49–U81), which defines the Y domain, and the second removed G50–C80, which leaves the first and last nucleotides of the Y-domain stem (Fig. 1). These alleles were transformed into a *GAL1-U14* test strain (YS153), in which the chromosomal U14 gene is repressed by growth on glucose-containing medium. Neither deletion variant supported growth on glucose, verifying that the Y-domain sequence is essential. Northern hybridization data showed that the levels of the mutant U14 RNAs were equivalent to the level of wild-type U14 in control cells, ruling out the possibility that the growth defect was due to instability of the mutant U14 RNA (data not shown).

Results from earlier structure analysis with chemical and enzymatic probes predicted that the Y domain of *S. cerevisiae* consists of two stems of 7 and 4 bp, separated by a bulge of 4 nt on one side and 2 nt on the other and topped with a loop of 5 nt (3) (Fig. 1). The importance of the short stem was tested by eliminating three of the four potential base pairs in one mutant and restoring base pairing with complementary mutations in a second mutant. Neither of these substitutions affected growth of the test strain on glucose (Fig. 2, mutants I and II), indicating that base pairing in this region is not necessary. The dispensability of this shorter helix is also indicated by phylogenetic data from the other yeast homologs. *S. pombe* U14 cannot form this stem and yet is able to substitute for *S. cerevisiae* U14 in vivo (38). Furthermore, only three of the five yeast U14 sequences available have the potential to form this shorter stem (38). We conclude that a Y domain containing a single stem of 7 bp and a loop of 19 nt is active.

The importance of the conserved stem was also examined with substitution mutations. Two substitutions which replaced

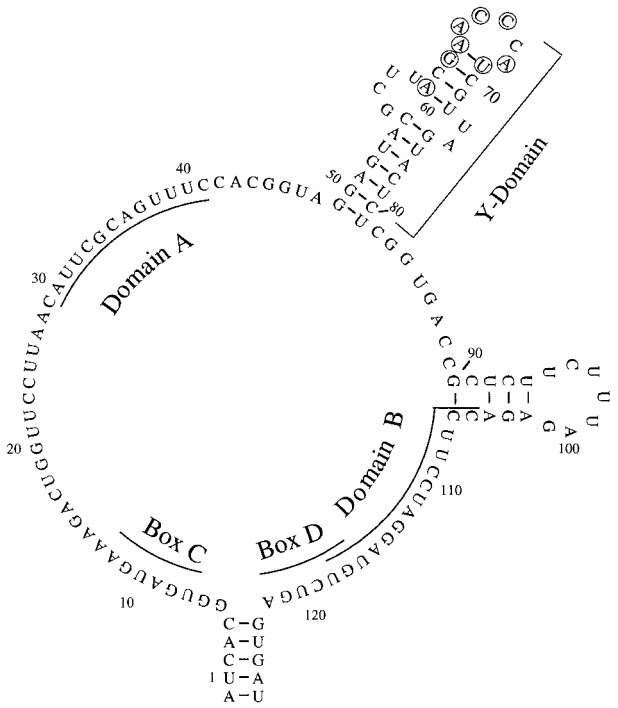


FIG. 1. Secondary structure of *S. cerevisiae* U14. The structure shown was deduced from direct probing analysis (3). The molecule contains four conserved sequence elements: box C, box D, domains A and B (underlined), and a highly folded yeast-specific structure called the Y domain. Boxes C and D and the terminal stem are required for U14 production. Domains A, B, and Y are required for U14-dependent rRNA processing activity. Nucleotides conserved among the Y domains of five yeast species are circled (38).

separate blocks of 3 nt did not affect function (Fig. 2, mutants III and IV). However, two longer substitutions, which eliminated five potential base pairs or changed six base pairs and part of the adjacent loop (U73–C80) (23), yielded cold-sensitive growth phenotypes (Fig. 2, mutants V and VI). These effects suggest that the stem is necessary at lower temperatures and that 4 bp are sufficient for function even at the reduced temperature. One earlier deletion (U53–U59), predicted to yield a 4-bp stem and 18-nt loop, was known to yield functional RNA (Fig. 2, mutant VII) (23). Since nearly all of the nucleotides in the stem can be substituted and many can be deleted, we postulate that these nucleotides have a structural role rather than serve as a recognition element. Thus, the function of the stem could be to present the loop segment for some vital inter- or intramolecular interaction.

The importance of the loop sequence was examined next. We determined that shortening the loop from 19 nt to 15 nt can be tolerated (Fig. 2, mutant VIII). However, a longer deletion (U53–A60 and U72–A77) predicted to leave a 7-bp stem and 5-nt loop was lethal, as was a deletion expected to leave a 4-bp stem and 5-nt loop (Fig. 2, mutants IX and X). Since substitution mutations that disrupt three of the seven potential base pairs of the stem are fully functional (Fig. 2, mutant III and IV), the lethality of the last mutation (mutant X) is likely to be due to the shortened loop rather than the shortened stem.

The loop sequences of the five different yeast U14 RNAs examined contain eight conserved nucleotides (5′-A-GAACC-AU-3′), including the pentanucleotide sequence GAACC at positions 62 to 66 in *S. cerevisiae* (Fig. 1). A previously constructed substitution that overlapped this sequence in *S. cerevisiae* U14 at positions 64 to 68 created a lethal phenotype (Fig. 2, mutant XI) (23). To further define the nucleotide requirements for this segment, we mutagenized this 5-bp stretch of the Y loop and analyzed a mix of variants containing mostly single and double mutations. Transformants unable to grow on glucose were isolated, and the growth defects were confirmed by restreaking on glucose medium. The plasmids from these cells were isolated, shuttled into *E. coli*, and subjected to DNA sequencing. Eleven types of unique mutations were identified among the 37 isolates characterized (Fig. 3A). The results showed that single point mutations giving rise to growth defects were limited to positions A64, C65, and C66 and that double mutations could exacerbate the deleterious effect. Interestingly, at positions 65 and 66, only mutations from C to A or C to G were among the single point mutations identified, suggesting that mutations to U at these positions do not affect U14 activity. This inference was confirmed by constructing a C-to-U mutation at each position and demonstrating that neither mutation affected growth (Fig. 3B). These results suggest that nucleotides C65 and C66 may be involved in base pairing and interact with G residues either in the same or in a different RNA molecule. Consistent with this possibility, mutations from C to U would still permit U-G base pairing, whereas mutations to A or G would disrupt such pairing and eliminate U14 function.

Intragenic mutations can suppress the lethal phenotype conferred by a double mutation in the Y loop. Isolation of intragenic suppressor mutations can reveal important intramolecular interactions in RNA molecules. To determine if the Y loop is involved in intramolecular base pairing, we searched for intragenic mutations that could suppress lethal point mutations within the Y loop. Two alleles with double point mutations, the A64U, C65U allele and the C65G, C66A allele, were analyzed. Intragenic suppressors were recovered with the former allele, which was introduced on a *URA3*-containing plasmid into a *GAL1-U14* strain; no isolates were recovered with the second allele, and the reason for this is not clear. Transformants were subjected to EMS mutagenesis, and colonies able to grow on glucose medium were selected. Intragenic suppressors were distinguished from extragenic mutations by mating the surviving strains with the isogenic *MAT***a** *GAL1-U14* strain containing the A64U, C65U U14 allele on a *TRP1* plasmid. After curing of the *URA3*-marked plasmid, the cells were analyzed for growth on glucose medium. Failure to grow on glucose suggested that the *URA3*-marked plasmid contained an intragenic suppressor mutation.

Interestingly, the results from this genetic analysis suggested that all of the suppressor mutants contained intragenic rather than extragenic mutations. To examine this, the *URA3*-containing plasmids from 12 of 25 randomly chosen suppressor strains were shuttled into *E. coli* and subjected to DNA sequencing. The results showed that the original U14 mutations had reverted to wild-type U14 in two plasmids; however, the other 10 contained intragenic mutations at three different positions near the primary mutation (Fig. 4, mutants XIII to XV). Three plasmids that represented each of the different types of intragenic mutations were retransformed into the wild-type *GAL1-U14* test strain and shown to support growth. This result confirmed that suppression was indeed intragenic. Northern analysis showed the amount of U14 RNA produced by strains containing the various intragenic suppressor genes remained the same as the amount in strains with the original A64U, C65U mutation (data not shown). This last result rules out the possibility that suppression was due to overexpression of the mutant U14.

Each of the three mutations able to suppress the lethal

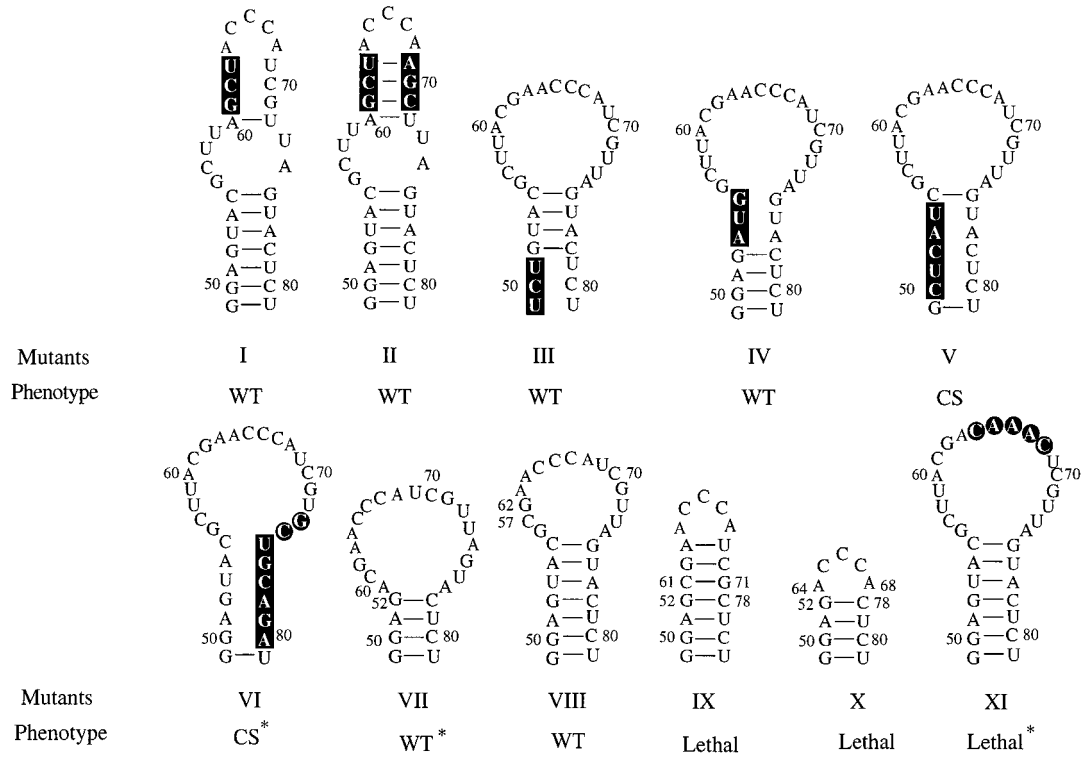


FIG. 2. Mutational analysis of the Y-domain stem. Eight U14 mutant alleles containing different substitution or deletion mutations were constructed and analyzed for activity in a *GAL1-U14* test strain. Nucleotide changes are indicated in boxes. The substitutions in mutants I, III, and IV to VI are predicted to disrupt base pairing. Mutant II contained substitutions predicted to restore the base pairing lost for mutant I but with different sequences. Deletion mutants VII (U53–U59) and VIII (U58–C61) contain hypothetical stems of 4 and 7 bp. Deletion variants IX (U53–A60, U72–A77) and X (U53–A63, U69–A77) maintained long and short stems in conjunction with a 5-bp loop. Mutant XI contained a substitution in the loop (A64CCCA68→C64AAAC68). Growth phenotypes were examined by incubating transformants containing plasmid-encoded mutant alleles on glucose plates at 15, 30, and 37°C. WT, wild type growth at all temperatures; CS, cold sensitive at 15°C. Asterisks indicate results from a previous report (23).

phenotype occurred in the Y loop, at positions where nucleotide substitutions are permitted. The fact that none occurred in more distant portions of the molecule suggests that the essential Y-loop nucleotides are not involved in vital intramolecular base pairing and that the Y loop interacts with some other molecule, possibly an RNA. Of course, this interpretation is based on a negative result and on use of a mutagen that produces transitions only. In support of this conclusion are the following results: (i) the high yield of suppressor mutations in the loop region (10 mutations) and (ii) the fact that the mutant sequence contains both a transition and a transversion and that single point mutants are able to grow (Fig. 3). The intragenic suppressor mutations did not create any recognizable new element, such as the conserved GAACC, making it unlikely that suppression resulted from acquisition of a new functional element. Because all of the intragenic suppressor mutations occurred within 6 nt of the original mutations in the loop sequence, we suggest that suppression involves a change in conformation that restores contact between the loop nucleotides and an interacting partner.

An extragenic suppressor encodes an essential RNA helicase-like protein. One approach to identify components that interact with the Y domain is to select for genes which, when overexpressed, can suppress a defective phenotype associated with mutations in this structure. The rationale behind this approach is that overexpression of an interacting molecule can drive an unfavorable equilibrium associated with a mutant RNA toward the formation of a productive complex. To this end, we asked if extragenic suppressors could be isolated that suppress a plasmid-encoded C66U, C67G allele with weak activity. Transformants of this allele in the *GAL1-U14* test strain grew extremely slowly at low temperatures but slightly better at higher temperatures. We transformed into this strain a yeast genomic library in a multicopy vector. The number of transformants was estimated to be approximately eight genome equivalents, based on plating an aliquot of transformed cells on galactose medium. Seven fast-growing colonies were selected after 20 days of growth at 15°C on glucose medium. The plasmids in these isolates were shuttled into *E. coli* and subjected to restriction analysis. The restriction pattern predicted two different groups of suppressor genes. Northern analysis showed that the level of U14 RNA in both types of suppressor mutants was normal, indicating that suppression was not caused by overexpression of U14 (data not shown). One of the suppressor genes was characterized further; the second gene was reserved for later study.

A genomic fragment encoding suppressor function was mapped by subcloning, complementation analysis, and DNA sequencing (Fig. 5). This fragment could suppress not only the C66U, C67G U14 allele but also the A64U, C65U U14 allele, which was used for subsequent complementation analysis. Suppression activity disappeared when the fragments were subcloned by either *Eag*I or *Nhe*I digestion, suggesting that a coding sequence spans these sites. A 3.4-kb genomic *Xba*I-*Bam*HI fragment was sequenced and an open reading frame of 2,331 nt was identified. This open reading frame is flanked by portions of two previously identified genes, known as *MAD2* and *KAY2* (25, 31) (Fig. 5A).

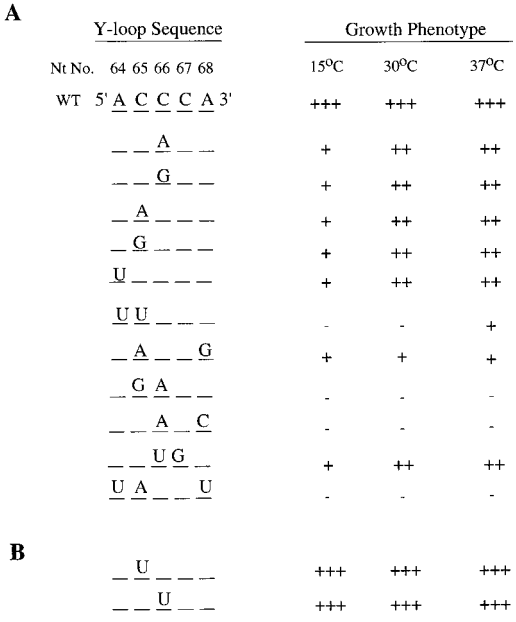


FIG. 3. Effects of Y-loop mutations on U14 activity. (A) A pool of plasmids containing mostly single and double point mutations in the Y loop was screened for U14 activity by incubating transformants at the indicated temperatures on glucose plates after replica plating from galactose medium. (B) Activity of C→U mutations at positions 65 and 66. The mutational effects observed for these positions suggest a possible role in RNA base pairing. The test strain (YS153) containing a plasmid-encoded wild-type U14 gene served as the positive control. Sequence changes found in the growth-defective mutants are indicated; a blank indicates no change from the wild-type sequence. Growth phenotypes were confirmed by restreaking. +++, wild-type growth; ++, good growth, but slower than wild type; +, extremely slow growth; −, lethal. Nt No., nucleotide position number.

The predicted protein contains all eight conserved motifs found in a family of DEAD box RNA helicases, suggesting that the suppressor gene encodes an RNA helicase (Fig. 5B). The carboxyl-terminal half of the putative protein is interspersed with both basic and acidic amino acids, implying that this region may be involved in protein-RNA or protein-protein interactions. A search of the GenBank database showed that

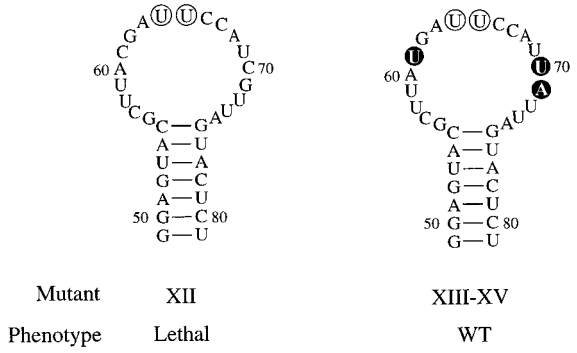


FIG. 4. Intragenic suppressor mutations of a lethal Y-loop allele. Strain YS626 bearing a lethal U14 A64U, C65U allele (circles) (mutant XII) was mutagenized with EMS and then grown on glucose medium. Surviving cells were mated with the isogenic strain YS628 for identification of intragenic suppressors (see Materials and Methods). Plasmids from suppressor strains were then shuttled from yeast into *E. coli* and subjected to DNA sequencing analysis. All second site mutations occurred in the Y-loop region and corresponded to single point mutations at positions 61 (XIII), 70 (XIV), and 71 (XV) (black circles). WT, wild type.

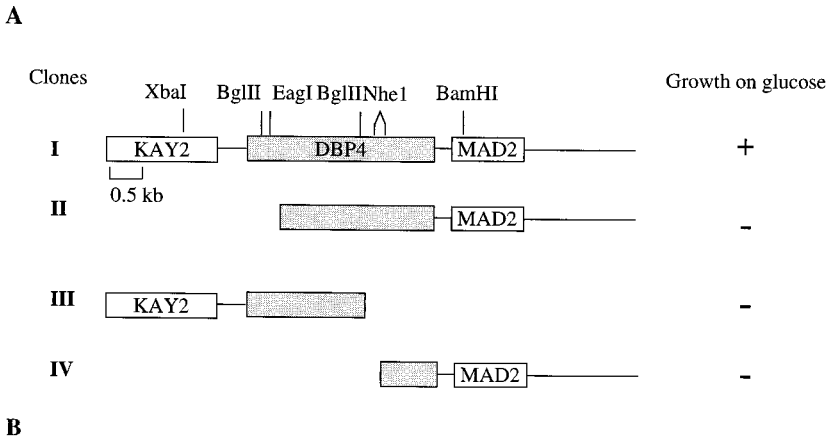
the suppressor gene is identical to a gene designated earlier as *CA4*. The latter gene was discovered by sequencing a PCR-amplified genomic DNA fragment made with primers complementary to conserved helicase domains that are widely spaced (8). Our search of the database also revealed a human gene homolog, called DDX10 (39). This gene encodes a DEAD box protein of 875 amino acids, and the two protein sequences are 78% similar (62% identical), with highest relatedness in the DEAD box segments. Because the yeast gene conforms so well to the consensus sequence for DEAD-DEXH box proteins, we prefer to designate it *DBP4* (DEAD box protein 4); three other yeast *DBP* genes have already been described (17, 18, 33).

To test the essentiality of the *DBP4* gene, we disrupted the coding sequence by inserting a *HIS3* marker at a *Bgl*II restriction site. This construct was used to replace one copy of the *DBP4* gene in diploid cells, as confirmed by Southern analysis (data not shown). Tetrads dissected from this diploid showed a 2:2 segregation pattern for the ability to grow on rich medium, with all surviving haploid cells auxotropic for histidine. The disruption results indicate that the *DBP4* gene is essential for growth, consistent with the importance implied by high homology with the human *DBP4*-like coding sequence.

Suppression by *DBP4* is specific for the Y domain but is not allele specific. One concern in characterizing genetic suppressors is the possibility that suppression may occur through an indirect mechanism, for example, bypass suppression. In the present case, overexpression of *DBP4* in the U14 mutant strain could bypass the functional requirement for U14. To examine the suppression specificity, a high-copy plasmid containing the *DBP4* gene (Yep24-*DBP4*) was introduced into the yeast test strain along with a number of plasmid-encoded U14 alleles harboring mutations in different vital regions, including domains A and B, boxes C and D, and the Y loop. After initial growth on galactose medium, transformants were streaked onto glucose-containing plates (Fig. 6A). The multicopy *DBP4* plasmid was only able to rescue growth of cells containing the tested Y-domain mutation (A64U, C65U), ruling out the possibility of bypass suppression and showing that suppression is specific to the Y domain.

Given that *DBP4* can rescue the A64U, C65U mutation but not mutations in other essential domains, it was then logical to ask whether suppression involves contact of the DBP4 protein with specific Y-domain nucleotides. To this end, we introduced the Yep24-*DBP4* plasmid into strains containing additional Y-domain mutations. The test included the following: (i) a number of double point mutations within the Y loop (Fig. 6B), (ii) the cold-sensitive substitution allele (mutant V, Fig. 2), (iii) two lethal deletion alleles (mutants IX and X, Fig. 2), and (iv) a U14 variant containing a complete deletion of the Y domain (Fig. 6C). Strikingly, all of these mutants could be rescued to various extents, including the allele lacking the entire Y domain. This last result indicates that direct interaction between DBP4 and the Y domain is not necessary for suppression. Taken together, the suppression results show that overexpression of the *DBP4* gene can alleviate the defect of Y-domain function and that dosage-dependent suppression by *DBP4* is domain specific but not allele specific.

***DBP4* rescues 18S rRNA synthesis.** Loss or inactivation of U14 is known to disrupt specific cleavages of pre-rRNA, resulting in reduced levels of mature 18S rRNA. Since the *DBP4* gene was isolated based on its ability to alleviate the U14 mutant phenotype, it was relevant to determine if the steady-state level of 18S RNA is restored when *DBP4* is overexpressed in cells containing the U14 A64U, C65U mutation. RNA was extracted from such cells harboring the *DBP4* allele in a high-copy vector. Northern analysis showed the level of *DBP4*



B

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                                                                                                      M   A   K   K
      20                  40                  60                  80                 100                 120
aatagattgaacacaactcaaagaaagactttacgtcaaaaggaagatgagtacatcgaaaacttgaaaaccaaaattgatgagtatgaccctaagataaccaaggccaagttttcaaa
N   R   L   N   T   T   Q   R   K   T   L   R   Q   K   E   D   E   Y   I   E   N   L   K   T   K   I   D   E   Y   D   P   K   I   T   K   A   K   F   F   K
    140                 160                 180                 200                 220                 240
gatctccccattagcgatcccacattaaaaggtttaagagaatcatccttttataaaacttactgagatccaagccgattctattcccgtgtccctgcaaggtcatgatgtattggcggcc
D   L   P   I   S   D   P   T   L   K   G   L   R   E   S   S   F   I   K   L   T   E   I   Q   A   D   S   I   P   V   S   L   Q   G   H   D   V   L   A   A
    260                 280                 300                 320                 340                 360
gcaaagacaggttccggtaagacattagcatttttagttccagtaattgaaaagctataccgcgaaaaatggaccgaattcgatgggttaggtgccctttatcatatctccaacaagagag
A   K   T   G   S   G   K   T   L   A   F   L   V   P   V   I   E   K   L   Y   R   E   K   W   T   E   F   D   G   L   G   A   L   I   I   S   P   T   R   E
    380                 400                 420                 440                 460                 480
ttggctatgcaaatatatgaggttttgacgaaaataggtagtcacacttcattttcagctgggttagttatcggtggtaaagatgttaaattcgaactagaaaggatatcaaggattaac
L   A   M   Q   I   Y   E   V   L   T   K   I   G   S   H   T   S   F   S   A   G   L   V   I   G   G   K   D   V   K   F   E   L   E   R   I   S   R   I   N
    500                 520                 540                 560                 580                 600
attcttatcggtacacctggtcgtatttttacaacatttagatcaagccgtaggtctaaatacttccaatttgcagatgcttgttcttgacgaagctgatagatgtttagacatggggttt
I   L   I   G   T   P   G   R   I   L   Q   H   L   D   Q   A   V   G   L   N   T   S   N   L   Q   M   L   V   L   D   E   A   D   R   C   L   D   M   G   F
    620                 640                 660                 680                 700                 720
aaaaaaactctggatgccatcgtaagcacattgtcaccatctagacaaacactttttatttttcagcgactcaatcacaatctgttgcagacttggctaggctttccttgacagattataaa
K   K   T   L   D   A   I   V   S   T   L   S   P   S   R   Q   T   L   L   F   S   A   T   Q   S   Q   S   V   A   D   L   A   R   L   S   L   T   D   Y   K
    740                 760                 780                 800                 820                 840
accgttggtacacatgatgtgatggatggtcggttaacaaagaagcatctactccagaaacttgcaacaattctcacattgaagttccttggcagataaattggatatccttttcagt
T   V   G   T   H   D   V   M   D   G   S   V   N   K   E   A   S   T   P   E   T   L   Q   Q   F   Y   I   E   V   P   L   A   D   K   L   D   I   L   F   S
    860                 880                 900                 920                 940                 960
tttataaaatcacatttaaaatgcaaaatgatcgttttcttatcaagttccaaacaagtgcattttgtctatgagacatttaggaagatgcagcctggtatttctttaatgcatttacac
F   I   K   S   H   L   K   C   K   M   I   V   F   L   S   S   S   K   Q   V   H   F   V   Y   E   T   F   R   K   M   Q   P   G   I   S   L   M   H   L   H
    980                1000                1020                1040                1060                1080
ggtagacagaaacaacgtgctaggacagaaacttggacaaattcaatcgtgctcaacaggtgtgtctttttgctaccgatgtcgttgctagaggtattgactttcctgcagtcgattgg
G   R   Q   K   Q   R   A   R   T   E   T   L   D   K   F   N   R   A   Q   Q   V   C   L   F   A   T   D   V   V   A   R   G   I   D   F   P   A   V   D   W
    1100                1120                1140                1160                1180                1200
gttgttcaagtggattgtccagaggacgttgatacatatatccacagagtcggtagatgtgctcgttaccggtaaaaagggtaaatctttaatcatgttgactccacaagagcaggaagcg
V   V   Q   V   D   C   P   E   D   V   D   T   Y   I   H   R   V   G   R   C   A   R   Y   G   K   K   G   K   S   L   I   M   L   T   P   Q   E   Q   E   A
    1220                1240                1260                1280                1300                1320
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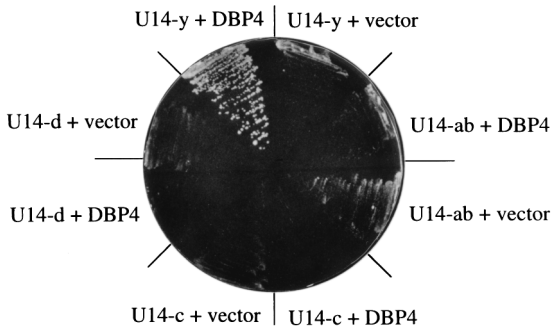
F   L   K   R   L   N   A   R   K   I   E   P   G   K   L   N   I   K   Q   S   K   K   K   S   I   K   P   Q   L   Q   S   L   L   F   K   D   P   E   L   K
    1340                1360                1380                1400                1420                1440
tatctaggacaaaaggcttttatatcgtacgtgagatctatatatgtgcaaaaggacaaagaagttttcaaattcgatgagttaccaacagaagaattcgcttattctcttggtttacca
Y   L   G   Q   K   A   F   I   S   Y   V   R   S   I   Y   V   Q   K   D   K   E   V   F   K   F   D   E   L   P   T   E   E   F   A   Y   S   L   G   L   P
    1460                1480                1500                1520                1540                1560
ggggccccaaaaatcaaaatgaagggtatgaagaccattgagcaggccaaagaaagaaagaatgctccaagacagctagctttgttatcaaaagcaaatgaagatggtgaagtaattgaa
G   A   P   K   I   K   M   K   G   M   K   T   I   E   Q   A   K   E   R   K   N   A   P   R   Q   L   A   L   L   S   K   A   N   E   D   G   E   V   I   E
    1580                1600                1620                1640                1660                1680
gataagtcgaaacaaccaagaacaaaatatgataaaatgtttgaacgtaaaaatcaaaccatcttaagtgaacactatttgaatataacgaaggctcaagcacaagaagacgaggatgac
D   K   S   K   Q   P   R   T   K   Y   D   K   M   F   E   R   K   N   Q   T   I   L   S   E   H   Y   L   N   I   T   K   A   Q   A   Q   E   D   E   D   D
    1700                1720                1740                1760                1780                1800
gatttcatttctgtcaagcgtaaagatcatgaaataaatgaagctgagttaccagctctaactcttccaacttcaaggcgagcacagaagaaagcgctttcaaagaaggcatctctggcc
D   F   I   S   V   K   R   K   D   H   E   I   N   E   A   E   L   P   A   L   T   L   P   T   S   R   R   A   Q   K   K   A   L   S   K   K   A   S   L   A
    1820                1840                1860                1880                1900                1920
tccaagggtaacgctagcaaattaatatttgatgatgaaggcgaagcccatccagtttatgaacttgaagatgaagaagaatttcataagagaggtgatgcggaggtacaaaagacagaa
S   K   G   N   A   S   K   L   I   F   D   D   E   G   E   A   H   P   V   Y   E   L   E   D   E   E   E   F   H   K   R   G   D   A   E   V   Q   K   T   E
    1940                1960                1980                2000                2020                2040
tttttaactaaagaatctgcagtaatggcagacattgacaatattgataagcaggtggctaaagaaaaaaacaagagaagaagaggaaaagacctagaagccatgagaagggaaatggag
F   L   T   K   E   S   A   V   M   A   D   I   D   N   I   D   K   Q   V   A   K   E   K   N   K   R   R   R   G   K   D   L   E   A   M   R   R   E   M   E
    2060                2080                2100                2120                2140                2160
gctgctatggaagaagaaatttctggagatgaagaagaagggaagacagtagcatatttaggaacaggaaacttgagcgatgatatgagcgacggtgatatgcctgactcggaaggccac
A   A   M   E   E   E   I   S   G   D   E   E   E   G   K   T   V   A   Y   L   G   T   G   N   L   S   D   D   M   S   D   G   D   M   P   D   S   E   G   H
    2180                2200                2220                2240                2260                2280
ttgaaaaagaaagctagaactgttgactattcccacggccacaatccatccaattcagttgatgatgacattattgaagtggaagaaccacaaacttttggaggatttggaatctttgaca
L   K   K   K   A   R   T   V   D   Y   S   H   G   H   N   P   S   N   S   V   D   D   D   I   I   E   V   E   E   P   Q   T   L   E   D   L   E   S   L   T
    2300                2320                2340                2360                2380                2400
gctaaattaatccagggttaaaccctcctgcatagtctaacctatagtatttagaacttgtatacatctattctctacactaatatacttatttctttgttcaaatcatcggtaatatta
A   K   L   I   Q   G   *
    2420                2440                2460                2480                2500
caataaaagaaaaatatgcatatactttcgtgcattgaaggtgtcaaatgaaatcccgcttatagtgaacaggcttccatcatatatacatatatat
```

FIG. 5. Identification of an extragenic suppressor gene of the U14 C66U, C67G mutation. (A) Complementation analysis of the *DBP4* gene. The initial genomic fragment was subcloned into Yep24 by using the indicated restriction sites. The resulting plasmids were introduced into the test strain containing the U14 A64U, C65U allele, and the transformants were streaked on glucose for phenotypic observation at 30°C. DNA sequencing identified the *DBP4* open reading frame and revealed the *DBP4* gene to be flanked by the *KAY2* and *MAD2* genes (25, 31). *KAY2* lies 0.6 kb upstream and *MAD2* lies about 0.15 kb downstream of the 2.3-kb *DBP4* coding sequence. (B) Nucleotide sequence of the *DBP4* gene and deduced amino acid sequence of the DBP4 protein. Eight sequence motifs conserved in the family of DEAD box RNA helicases are underlined. The carboxyl-terminal half of the DBP4 protein is enriched with charged amino acids, in particular, arginine, aspartic acid, glutamic acid, and lysine.

A



B

Y-loop Sequence Nt No. 64 65 66 67 68	Growth Phenotype	Suppression Phenotype
WT 5' A C C C A 3'	+++	+++
U U _ _ _	-	++
_ _ A _ C	-	++
_ _ U G _	+	++
U A _ _ U	-	+

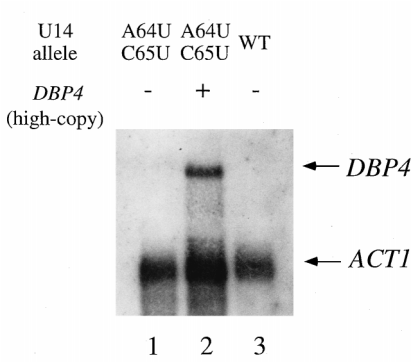
C

Mutation	Substitution (S); Deletion (D)	Growth Phenotype	Suppression Phenotype
GAGUA54-> CUCAU	S (V)	CS	++
U53-A60, U72-A77	D (IX)	-	++
U53-U63, A69-A77	D (X)	-	++
G49-U81	D	-	+

FIG. 6. Suppression of U14 mutations by *DBP4* is domain specific. (A) A Yep24 plasmid bearing the *DBP4* gene was transformed into the *GAL1-U14* test strain (YS153) harboring different mutant U14 alleles in a low-copy, *TRP1* plasmid. Transformants were grown on glucose at 15, 37, and 30°C. Test strains are designated as follows: U14-y + DBP4 and U14-y + vector, the lethal U14 Y-domain A64U, C65U allele with a high-copy *DBP4* gene and the Yep24 vector, respectively; U14-ab + DBP4 and U14-ab + vector, a lethal U14 domain A mutation (G34C) in conjunction with a replacement of domain B (26) with a high-copy *DBP4* gene and the Yep24 vector, respectively; U14-c + DBP4 and U14-c + vector, a lethal U14 box C mutation (G8C) with a high-copy *DBP4* gene and the Yep24 vector, respectively; U14-d + DBP4 and U14-d + vector, a lethal U14 box D mutation (G121U) with a high-copy *DBP4* gene and the Yep24 vector, respectively. (B) Suppression of Y-loop mutations by *DBP4*. A Yep24 vector harboring the *DBP4* gene was transformed into strain YS153 harboring different Y-domain mutants, and transformants were streaked and grown on glucose plates at 15, 30, and 37°C. Suppression phenotypes were compared with those of mutant cells containing vector alone. The growth phenotypes for all but C66U, C67G U14 were similar at the three temperatures. The latter strain exhibited a severe growth defect at the low temperature and a mild defect at the higher temperature (Fig. 3A). The defect at 15°C could be rescued by overexpression of *DBP4*, whereas suppression was not evident at the higher temperature. (C) Suppression of Y-domain substitution and deletion mutations by *DBP4*. Suppression was assayed as described for panel B. Note that overexpression of DBP4 can partially rescue a complete deletion of the Y domain (deletion G49-U81). +++, wild-type; ++, good growth but slower than wild type; +, extremely slow growth; −, lethal; CS, cold sensitive at 15°C.

mRNA in these cells was indeed manyfold higher than that in the mutant cells harboring an empty plasmid (Fig. 7A). The level of 18S RNA was analyzed by fractionating total RNA in a 1% agarose gel and staining with ethidium bromide. The results showed that the level of 18S RNA was restored to near normal in the mutant cells when the *DBP4* gene was overexpressed compared to its expression in mutant cells containing a single chromosomal allele and an empty vector (Fig. 7B, lanes 1 and 2); no effect on U14 level was observed for either genetic condition (20). These results lead us to speculate that the DBP4 protein may function in pre-rRNA processing by acting together with the U14 snoRNP.

A



B

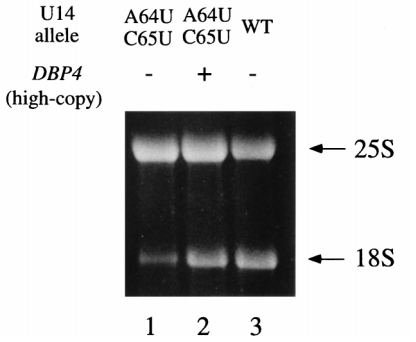


FIG. 7. Overexpression of *DBP4* restores production of 18S rRNA. RNA was extracted from A64U, C65U U14 mutant cells harboring a *DBP4* gene in a high-copy plasmid. Cells were grown in glucose at 30°C for 40 h. (A) Northern analysis of *DBP4* messenger RNA. A blot prepared with 5 μg of RNA separated in an agarose-formaldehyde gel was probed with a ^{32}P-labeled DNA fragment from the *DBP4* gene. Actin mRNA served as an internal control. The RNA samples shown were derived from A64U, C65U U14 mutant cells containing either the Yep24 plasmid or Yep24 with the *DBP4* gene (lanes 1 and 2) or from the *GAL1-U14* test strain (YS153) containing a plasmid-encoded wild-type U14 gene and the Yep24 plasmid (lane 3). (B) Steady-state level of 18S rRNA. An agarose-formaldehyde gel prepared as described for panel A was stained with ethidium bromide. WT, wild type.

DISCUSSION

U14 RNA from *S. cerevisiae* has very little secondary structure except for the Y domain, which forms a highly folded stem-loop structure (3). Functional studies with deletion and substitution variants and hybrid yeast-mouse RNAs have demonstrated that this region of *S. cerevisiae* U14 plays an essential role, and precise deletion of the Y domain led to lethality (reference 23 and the present study). In protein-free RNA, this Y domain forms a structure consisting of an 11-bp stem disrupted by bulges and topped by a terminal loop of 5 nt (3) (Fig. 1). Our mutagenesis results indicated that nucleotides in the upper portion of the stem can be substituted such that base pairing is almost completely disrupted without affecting U14 function. This suggests that the functional requirement can be satisfied with a contiguous 7-bp stem and loop of 19 nt. The dispensability of base pairing in the upper portion of the stem is also supported by phylogenetic evidence. While three of five yeast U14 species examined have the ability to form a longer,

imperfect stem and smaller loop, others can only form a shorter, contiguous stem and loop structure. Moreover, *S. pombe* U14 RNA, which can only form a contiguous 6-bp stem without any of the bulges predicted for the *S. cerevisiae* homolog, is able to functionally substitute for the *S. cerevisiae* U14 (38).

The length requirement for the stem is quite flexible; a stem of 4 bp is sufficient to provide normal function. The nucleotide sequence of the stem also does not appear to be critical for function. Two blocks of 3 nt could be substituted on one side of the stem without effect, and substitutions of 5- or 6-nt blocks on the opposite side led only to cold sensitivity. Also consistent with the absence of a sequence requirement for the stem is the fact that no sequence conservation is apparent among the five yeast U14 species examined (38). Since the requirements for the stem are so flexible, we favor the notion that it functions as a spacer which displays the important loop element.

In contrast to the stem, both the size and nucleotide content of the loop are important for function. Deletions predicted to produce an abbreviated Y-domain structure consisting of a stem of 4 or 7 bp and a 5-bp loop were lethal. Additionally, many single and double base changes in a mutagenized 5-nt stretch of the loop led to a lethal phenotype, implying that the loop nucleotides are required for internal base pairing or interaction with another molecule. At least two of the nucleotides in this segment, C65 and C66, are speculated to interact with a G residue in another RNA, based on the fact that base changes from C to A or G were lethal, whereas mutations to U at these positions could be tolerated (Fig. 3). Logical candidates include rRNA or another snoRNA(s). Another snoRNA which joins the processome complex (12) early, such as U3 or snR30 (5, 16, 28), is an attractive candidate. The fact that all intragenic suppressors of a lethal mutation in the loop were confined to the Y loop, within six nt of the primary mutation, also argues against intramolecular base pairing and supports the view that the loop nucleotides interact with other molecules. Suppression here is most likely related to a conformational change that leads to better presentation of the mutant loop sequence. Intermolecular interactions are expected to be important for many U14 activities, including U14 snoRNP assembly, association of the U14 snoRNP with the multi-snoRNP pre-rRNA processome complex, base pairing of U14 with complementary elements in 18S RNA, and release of the U14 snoRNP from processing and nucleoside modification complexes.

A major finding of this study is that overexpression of a gene encoding an RNA helicase-like protein can suppress a variety of Y-domain mutations but not mutations in other essential U14 domains. This suppression restores the steady-state level of 18S RNA, suggesting that the U14 activity required for processing of 18S RNA is rescued. The suppression phenotype is consistent with the known requirement for U14 snoRNA in 18S RNA synthesis. The fact that a mutant allele lacking the Y domain can be suppressed argues that the Y domain does not have an essential catalytic role in ribosome synthesis. Its function is probably less direct, related to the formation of a vital complex.

The *DBP4* gene encodes eight conserved sequence motifs found in a family of DEAD box RNA helicases and encodes amino acids highly charged at the carboxyl-terminal half. RNA helicases are known to play important roles in processes such as translation initiation, pre-mRNA splicing, mRNA decay, and ribosome synthesis (13, 40). It is reasonable to expect that RNA helicases might function in every major process of ribosome biogenesis. In the present case, the DBP4 protein can be imagined to function in synthesis or activity of the U14 snoRNP. A possibility we disfavor is that DBP4 unwinds the Y-domain stem. Two circumstances argue against this hypothesis. One is that *DBP4* will suppress an *S. cerevisiae* U14 allele which contains a complete deletion of the Y domain. Second, *DBP4* appears to be conserved in humans, yet the Y domain is absent in all known vertebrate U14 RNAs. This last consideration would be negated, of course, if one or both helicases are multifunctional.

The most attractive hypothesis is that DBP4 plays a role in melting in or melting out the duplexes formed between 18S RNA and the two conserved U14 domains A and B. The interaction through domain A is essential for successful processing, and that involving domain B is important when pairing at domain A has been weakened (26). As noted previously, the sequences in 18S RNA known to interact with U14 are located in close proximity in the secondary structure, suggesting that binding and release of the individual U14 elements from 18S RNA may be highly coordinated (26). Mutations in the Y domain could impair movement of U14 into or away from these contact sites. Defective complexes might then be rapidly degraded, leading to decreased production of fully processed 18S RNA. Overexpression of DBP4 protein might restore 18S RNA synthesis by facilitating the binding or release of U14 and 18S RNA. This hypothesis is consistent with both the conserved nature of the *DBP4* gene and the domain specificity of *DBP4* gene suppression. Other possible roles for DBP4 include remodeling 18S RNA and mediating the assembly of functional U14 or 18S RNP complexes. Including DBP4, five putative RNA helicases have thus far been linked to processing of rRNA; the four others are Rrp3, Drs1p, Spb4p, and Dbp3p (33, 36, 37, 48). Spb4p and Drs1p are involved in processing of 25S rRNA from 27S pre-rRNA, and Rrp3, like DBP4, is involved in processing of 18S rRNA. However, specific details about how these proteins affect processing are still lacking. An early aim in the future characterization of DBP4 will be to determine if it is associated with U14, either stably or transiently, and if it is associated with rRNA precursors and perhaps other snoRNAs involved in rRNA synthesis.

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