More than half of yeast U1 snRNA is dispensable for growth

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ABSTRACT

Yeast U1 snRNA (568 nucleotides) is 3.5-fold larger than its mammalian counterpart (164 nucleotides) and contains apparent sequence homology only at the 5' and 3' ends. We have used deletion analysis to determine whether the yeast-specific U1 sequences play essential roles *in vivo*. Yeast cells carrying a deletion of more than 60% (355 nucleotides) of the single-copy U1 gene are viable, though slow-growing, while a deletion of 316 nucleotides allows essentially wild-type growth. The boundaries of the viable deletions define a dispensable internal domain which comprises sequences unique to yeast. In contrast, the essential 5' and 3' terminal domains correspond to phylogenetically conserved sequences and/or structures previously implicated in RNA:RNA and RNA:protein interactions. The minimal essential sequences of yeast U1 can be drawn in a secondary structure which resembles metazoan U1 in four of seven structural domains.

INTRODUCTION

Splicing of pre-messenger RNAs (pre-mRNAs) takes place in a large multi-component complex called the spliceosome (1, 2, 3). Major constituents of the spliceosome are ribonucleoprotein particles (snRNPs) comprised of U1, U2, U4/U6 and U5 small nuclear RNAs (snRNAs), each of which are complexed with a set of common (so-called Sm) and snRNP-specific proteins (4). Although the RNA components have been conserved across evolution, there are marked differences between yeast and mammals in the sequence, and in some cases, the size of the spliceosomal snRNAs (reviewed in reference 5). The most striking differences are found for U1 and U2 snRNAs; the yeast RNAs are 3.5- and 6-fold larger than their metazoan counterparts, respectively. In contrast, yeast and mammalian U6 snRNAs are highly similar in size and sequence. These comparisons raised

the possibility that some of the yeast snRNAs perform different or additional functions compared to their roles in mammalian splicing. Alternatively, U1 and U2 may be relatively tolerant to alterations of size and sequence, while U6 is structurally and functionally constrained.

Evidence that the basic functions of the snRNAs have been conserved comes from genetic and biochemical analysis. In both yeast and mammals, base-pairing interactions have been established between U1 and the 5' splice site (6, 7, 8) and U2 and the branchpoint region (9, 10, 11). In yeast U2, the base-pairing region falls within a 120 nucleotide 5' domain with high sequence and structural homology to mammalian U2. In addition, yeast U2 contains a large, phylogenetically conserved 3' terminal stem/loop (12). Deletion analyses have shown that virtually all of the yeast-specific sequences (~ 1kb) can be deleted from yeast U2 RNA without affecting growth rate (13, 14). Moreover, it was subsequently demonstrated that the human U2 gene can efficiently complement the lethal phenotype of a yeast U2 deletion (15).

In contrast to the situation with U2 snRNAs, the sequence identity between yeast and metazoan U1 is confined to three short clusters: 1) the first 10 nucleotides, which include the complementarity to the 5' splice site consensus sequence (16, 17); 2) a 9 out of 10 match to a conserved loop sequence, which comprises part of the binding site of the U1 snRNP-specific 70K protein (18, 19, 20); and 3) a 6 out of 8 match to the consensus binding site for the common (Sm) proteins (16, 17). Because these homologies are located within the first 50 and last ten nucleotides of the 568 nucleotide yeast RNA, it has been particularly difficult to identify additional regions of significant structural (and presumably functional) homology.

Recently, a secondary structure of yeast U1 has been derived from phylogenetic arguments and structure probing (21). According to this model, yeast U1 is built from a conserved cruciform structure (the 'universal core'), elaborating from which are a series of yeast-specific helices (see Figure 5). Based on this structure, localized mutagenesis of a conserved and a yeast-

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specific region was performed. However, these regions, when mutated individually (22) or removed as a group (23), had no significant impact on growth.

We have now undertaken a systematic deletion analysis of the yeast U1 gene. Our results show that more than half of the sequence of U1 snRNA can be removed without producing a growth phenotype. As with U2, the deletions define a large, internal, dispensable domain comprised solely of yeast-specific sequences. By defining the required regions of yeast U1 RNA, such experiments greatly facilitate the search for essential interactions in the spliceosome.

MATERIALS AND METHODS

Deletion construction and viability determination

Standard molecular cloning techniques were employed (24). Deletions were generated in yeast U1 by removing the Apa I to Asu II restriction fragment ($\Delta 249-396$, see Figure 1), and by deleting from these sites with nuclease Bal-31 (BRL). Sal I linkers (New England Biolabs) were inserted at the site of deletion and deletion endpoints were determined by DNA sequencing. U1 deletion mutations were assayed for U1 activity by using the 'plasmid shuffle' technique (25) to generate yeast strains in which the deleted U1 is the sole source of U1 RNA. Because U1 RNA is essential for yeast viability (8, 7), U1 deletion mutations disrupting essential functions give rise to strains which are unable to live in the absence of wild type U1. However, deleted U1 RNAs which provide U1 function generate viable U1 deletion mutation strains. Doubling times were determined by growth in rich media (YEPD, 26) at permissive (30°C) or restrictive (37°C) temperatures.

RNA analysis

To examine U1 RNA, RNA extracted from U1 deletion mutants was separated on a 6% polyacrylamide gel, transferred to nylon membrane (Hybond, Amersham), and probed with an oligonucleotide complementary to yeast U1 RNA (27, 16). For *RPL32* Northern, RNA has been isolated from U1 deletion mutation strains at 30°C and 37°C and electrophoresed on a formaldehyde gel (28). After transfer to nylon membrane (Hybond, Amersham), the samples were probed with an oligonucleotide complementary to the intron-containing *RPL32* mRNA. Temperature sensitive strains were grown to log phase at a permissive temperature, spun down, resuspended in pre-warmed media, and grown at 37°C for two hours before RNA extraction.

RESULTS

Deletions in U1 RNA define a dispensable internal domain

To identify functionally important sequences in the nonconserved regions of yeast U1 RNA, we have generated a series of internal deletion mutations in U1. Because yeast U1 RNA is essential for viability, we presume that any internal deletions which are viable have not disrupted a region of yeast U1 RNA which is critical for U1 function. A series of internal deletions in yeast U1 RNA was constructed and introduced into yeast cells. The internal deletions were tested for viability by using the plasmid shuffle assay (25) to create strains in which the deleted U1 is the only source of U1 RNA. We find that while some regions of U1 RNA can be deleted without loss of viability, these viable deletions are not fully wild type. Strains carrying viable deletions

all exhibit growth defects including longer doubling times and temperature sensitivity. Representative U1 RNA deletion mutations are shown in Figure 1. For viable deletions, the doubling time of cells carrying U1 deletion mutations grown at their permissive temperatures, relative to wild type cells, is shown.

The deletions identify one essential region of yeast U1 RNA extending from the 5' end to nucleotide 170. Deletions removing sequences 5' of nucleotide 170, such as $\Delta 158-324$, are lethal. In contrast, those deletion mutants which retain the 5' essential domain, such as $\Delta 171-310$, are viable. Deletion mutations which do not impinge on the 5' essential domain, e.g. $\Delta 184-312$, exhibit near-wild type viability. Interestingly, $\Delta 168-301$, which retains most of this 5' essential region, is viable but has a severe growth defect. Strains containing this U1 deletion mutation form micro-colonies after long incubation on agar media, and grow so slowly in liquid media that a doubling time could not be determined.

Figure 1. Deletions in Yeast U1 RNA. A schematic map of yeast U1 RNA is shown. Shaded boxes represent primary sequence homologies to the 5' splice site interaction (5'SS) and loop I (loop I) regions of metazoan U1 and black boxes represent potential binding sites for the Sm antigen. The Apa I and Asu II restriction sites used as starting points for the deletions are indicated. Sequences deleted are represented by black bars. Deletions are listed in ascending order of their left-hand endpoints. Deletion names indicate the first and last nucleotides which are deleted. Also shown for each deletion are the number of nucleotides removed and the viability in strains with the deletion as the sole source of U1 RNA. Lethal deletions are indicated by a '—' while the doubling time (relative to wild type at 30°C) is given for viable deletions. Although $\Delta 168-301$ is viable, this strain has an extreme growth defect which prevents determination of the doubling time (ND, not determined). A scale in nucleotides is shown.

The lethal phenotype of deletions at the 3' end indicates that these sequences are also required (data not shown). Although not tested here, we believe that the Sm binding site (nucleotides 552–558) is essential, as lethal mutations have been constructed in the Sm sites of the yeast U5 and U2 snRNAs (29, and M. H. Jones and C.G., personal communication).

While the sequences at the 5' and 3' ends of yeast U1 RNA are essential, much of the central portion of yeast U1 can be removed without loss of viability (Figure 1). Deletion $\Delta 187-509$ removes 323 nucleotides from the center of yeast U1 RNA and has only a minimal effect on growth rate (doubling time $1.4 \times$ wild type). The largest viable deletion, $\Delta 185-539$, removes 355 nucleotides from U1 RNA while retaining the essential 5' and 3' domains. We believe the resultant 215 nucleotide U1 RNA approximates the smallest functional yeast U1 which can be generated.

In some cases, the severity of the growth phenotype does not correlate with the number or nature of sequences deleted. For example, the lethality of $\Delta 249-396$ suggests that an essential RNA sequence lies within this region. However, larger deletions which fully include this region (e.g. $\Delta 249-443$ and $\Delta 213-417$) are viable. The lethal phenotype of $\Delta 249-396$ most likely results from a structural defect caused by the novel fusion junction or the juxtaposition of inappropriate RNA sequences. We therefore obtained a set of deletions in each area of interest. Because multiple deletion alleles in a given area will have different novel fusion junctions, such potential artifacts can be identified. Thus, the consistently lethal phenotypes of deletions in the region of nucleotide 170 argues that an essential sequence has been disrupted, and that these deletions are not lethal due to fortuitous reasons.

Figure 2. Deletion U1 RNAs are of the predicted size and accumulate to normal levels. A Northern blot probed with a ^{32}P -labelled oligonucleotide complementary to nucleotides 43 to 26 of yeast U1 RNA is shown. RNA isolated from deletion strains as indicated above each lane or from wild type (WT). Total RNA (25 micrograms) was loaded in each lane. By staining with ethidium bromide, each lane contained approximately the same amount of RNA. M, molecular weight markers; Bluescript II KS (Stratagene) digested with Hpa II and end-labelled with ^{32}P .

Deletion U1 RNAs are stable and expressed at normal levels

Both viable and non-viable U1 deletion mutations were examined by Northern blot analysis to document the presence of U1 RNAs. All U1 deletion strains were examined and all were found to synthesize U1 RNAs of the predicted length and in steady state amounts approximately equivalent to the wild type (Figure 2 and data not shown). Nonviable deletions were also examined (as obligate heterozygotes with wild type U1) and found to synthesize deleted RNAs of the predicted sizes (data not shown). Thus, regardless of their effects on viability, the U1 internal deletions do not grossly affect the synthesis or stability of U1 RNA. Lethal

Figure 3. Deletion strains are temperature sensitive for growth. Growth curves are shown for wild type and deletion strains grown in rich media at 30°C (open symbols) and at 37°C (filled symbols). Deletion strains $\Delta 192-507$ (circles) and $\Delta 187-509$ (diamonds) have minimal growth defects compared to an isogenic wild type strain (squares) when grown at 30°C. Strains were grown in rich media (YEPD).

Figure 4. U1 deletions cause splicing defects. A Northern blot probed for the spliced mRNA *RPL32* is shown. The positions of unspliced pre-mRNA and mRNA are indicated. RNA was isolated from wild type (WT) and deletion strains grown at 30°C and 37°C. For 37°C growth, strains were grown to log phase at 30°C, spun down, resuspended in pre-warmed media, and grown for 2 hours at 37°C before RNA isolation. Deletion names and growth temperature are indicated above each lane. Each lane was loaded with 25 micrograms of total RNA. The *RPL32* probe was stripped and the blot rehybridized with an oligonucleotide complementary to the actin mRNA to demonstrate that all lanes contained approximately equal amounts of RNA (data not shown). The efficiency of splicing in each strain can be judged by the ratio of pre-mRNA to mRNA.

deletions in yeast U5 RNA have also been shown to accumulate normal amounts of U5 RNA (D. Frank and C.G., personal communication). In addition, the Northern blots demonstrate that there is no wild type U1 RNA in the deletion strains. We therefore conclude that the phenotype of the U1 deletion mutation strains reflects the level of function of the deletion U1 allele rather than the level of transcription or stability of the deleted RNA.

Internal deletions of U1 RNA affect growth rate and splicing

All viable U1 deletion mutation strains exhibit increased doubling times at 30°C (Figure 1); moreover, these strains are temperature sensitive (Figure 3). The severity of the growth defects at 30°C ranges from minimal (e.g. $\Delta 192-507$, 1.1 $\times$ slower than wild type) to severe (e.g. $\Delta 213-417$, 5.2 $\times$ slower than wild type). As seen in Figure 3, even U1 deletion mutations which cause

slight increases in doubling time at 30°C (e.g. $\Delta 192-507$) grow much more slowly at elevated temperatures (37°C).

Splicing defects can be demonstrated for deletion strains using Northern blot analysis with probes for intron-containing mRNAs such as the *RPL32* gene product (Figure 4). In wild type cells, the majority of the steady state *RPL32* transcript is in the form of mature mRNA, with a detectable amount of unspliced pre-mRNA present. Judged by the decrease in the ratio of spliced to unspliced *RPL32* mRNA, splicing is impaired in deletions $\Delta 249-418$ and $\Delta 213-417$. This splicing defect is more extreme in $\Delta 213-417$ when grown at 37°C, indicating that splicing is strongly inhibited in this strain at high temperature (this strain does not grow at 37°C). Correlation of the U1 deletion with the growth phenotype and the splicing defect demonstrates that the deletions disrupt U1 function.

Figure 5. Minimal yeast U1 RNA structurally resembles metazoan U1 RNA. The secondary structure of human U1 RNA of Mount and Steitz (33) is shown along with the secondary structure of yeast U1 RNA proposed by Kretzner et. al. (21, 22) and a proposed structure for the smallest viable U1 deletion allele ($\Delta 185-539$). The stem/loop structures are indicated according to (5). A. The secondary structure of human U1 RNA. Regions of high sequence conservation to yeast U1 RNA are boxed. The Sm binding site is enclosed in hatched boxes. Indicated on the structure are the following regions: 5' SS, 5' splice site interaction domain; LRI I, 5' side of the long range interaction domain; stem/loop I; stem/loop II; stem/loop III; LRI II, the 3' side of the long range interaction domain; Sm, the binding site for the Sm proteins; and stem/loop IV. TMG; m2,2,7 G cap. B. The universal core of yeast U1 including the 5' and 3' essential domains. The dashed vertical lines at nucleotides 167 and 545 indicate the position of stem/loop III structures in parts C. and D. Indicated on the structure are the regions of homology to metazoan U1, Sm binding site, and structural features defined in the legend to part A. Yeast U1 has no structure equivalent to the stem/loop IV of metazoan U1. The structure of stem/loop II and the long range interaction are modified from (21, 22) by omission of the proposed atypical $U_{63}G_{152}$, $A_{64}C_{151}$, $G_{65}G_{150}$, $U_{66}G_{149}$, and $U_{90}U_{108}$ base pairs. C. The stem/loop III structure of intact yeast U1 RNA from Kretzner et. al. (21). The dashed vertical lines indicate where this structure is positioned to the core structure in part A. Roman numerals indicate helices in the yeast specific sequences (21, 22). D. The stem/loop III structure of $\Delta 185-539$, the smallest viable U1 RNA. The deletion endpoints are numbered and the Sal I linker inserted at the site of deletion is shown in bold type. The dashed vertical lines indicate where this structure is positioned to the core structure in part A.

DISCUSSION

The largest viable deletion of U1 ($\Delta 185-539$) removes 355 nucleotides from the center of the 568 nucleotide RNA (see Figure 1). While these cells grow very poorly, a slightly smaller deletion of 316 nucleotides ($\Delta 192-507$) produces only a 10% increase in doubling time (at 30°C). Taken together, these experiments define a ~350 nucleotide dispensable region. This region includes a yeast-specific 'core domain' (helices VII and VIII in Figure 5C) identified on the basis of its presence in three related yeast species (21). Previous mutagenesis of this region revealed it to be dispensable (22). Recently, Goguel et. al. (23) reported the viability of a 345 nucleotide deletion ($\Delta 188-534$) removing stems VI through X. Our results now extend the dispensable region to include all sequences which comprise stems V through X of the proposed secondary structure. Furthermore, we show growth and splicing defects caused by deletions in U1 RNA.

The dispensable domain contains most of the sequences unique to yeast (see below). Thus, as has been found for U2 RNA, the majority of yeast-specific nucleotides do not mediate functions that are essential for efficient growth under standard laboratory conditions. Our experiments do not address the possibility that these sequences contribute to the performance of the snRNA under sub-optimal conditions. For example, Rosbash and colleagues have presented evidence that alterations within the yeast-specific domain of U1 can have a significant impact on growth when splicing is made rate-limiting by the addition of mutations in the conserved portion of the snRNA (22).

Figure 5 shows existing secondary structure models of U1 from humans (Fig. 5A) and from yeast (Fig. 5B). Mammalian U1 can be subdivided into seven structural domains (see reference 5): 1) the 5' splice site base-pairing region (5'SS); 2) a long-range interaction that forms a helix juxtaposing the 5' and 3' domains (LRI); 3) stem/loop I, which is the binding site of the 70K protein; 4) stem/loop II, the binding site of the U1 snRNP-specific protein A; 5) stem/loop III, 6) the single-stranded binding site for the Sm proteins (Sm site); and 7) the 3'-terminal stem/loop IV. Based solely on sequence alignments (16, 17, 5), the full-length yeast RNA contains readily identifiable homologues of four of these domains (5'SS, LRI, stem/loop I, and Sm site), while it lacks the 3' terminal stem/loop. It has remained unclear whether the 'extra' nucleotides in yeast U1 contain structurally altered versions of the other two domains (stem/loops II and III), or whether these domains have been substituted by unrelated structures in the yeast RNA.

Our deletion data are consistent with the conclusion that the four domains with clear-cut homology to the metazoan structure are essential for viability. This case is strongest for the 3'-boundary of the internal dispensable domain, in that the endpoint of $\Delta 185-539$ and $\Delta 207-539$ corresponds almost precisely to the start of the 3' segment of the long-range interaction (LRI) helix. Moreover, we presume that the adjacent Sm site is itself essential, since point mutations in the Sm site of U5 (29) and U2 (M. H. Jones and C.G., unpublished observations) are inviable. This conclusion is also consistent with our observation that deletions coming in from the 3' non-coding region are all inviable; in these constructs, however, we cannot rule out additional effects on transcription and/or processing of the RNA.

The 5' boundary is defined by viable deletions $\Delta 171-310$ and $\Delta 168-301$, which are extremely slow-growing, and $\Delta 184-312$,

which grows with a doubling time close to that of wild-type. We know that the 5' SS domain is essential, since point mutations in this region have been shown previously to be inviable or temperature-sensitive (8). By the argument invoked above, we believe that the 5' section of the LRI is also essential. The requirement for stem/loop I is still to be directly demonstrated. Deletion of the highly conserved loop has been shown to result in wild-type growth rates (22) despite the fact that point mutations in this sequence prevent binding of metazoan U1 70K protein to U1 RNA *in vitro* (18, 20). On the other hand, a putative homologue of the yeast snRNP protein has recently been identified and cells deleted for this single copy gene are inviable. These results could be reconciled by arguing that the efficiency of 70K binding to a truncated stem/loop I *in vivo* is sufficient for normal doubling times, given that U1 snRNA levels can be decreased ten-fold without impact on growth rate (22).

The position of the 5' boundary argues that ~120 nucleotides 3' to stem/loop I are also essential for viability. According to the structure in Figure 5B, these sequences can form a long, irregular duplex. The position of this domain is analogous to that of stem/loop II in metazoan U1. In addition to considerable length differences (40 nucleotides in human vs. 120 nucleotides in yeast), however, these structures lack obvious sequence identity in unpaired nucleotides. Further experiments will be required to determine whether these domains are functionally homologous. In mammalian U1, this is the binding site for the U1 A snRNP protein (30); as yet, no candidate for the U1 A protein has been identified in yeast. Alternatively, this structure may comprise the binding site for an unrelated protein. In that a 'swap' of the yeast and human U2 snRNAs resulted in efficient growth (15), it is conceivable that construction of chimeric yeast/human U1 RNAs (together with co-expression of the appropriate human proteins) will allow us to dissect the role of this domain (and others) in snRNP function *in vivo*.

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