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Saccharomyces cerevisiae U1 small nuclear RNA secondary structure contains both universal and yeast-specific domains

(phylogenetic studies/splicing)

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ABSTRACT **The five small nuclear RNAs (snRNAs) involved in mammalian pre-mRNA splicing (U1, U2, U4, U5, and U6) are well conserved in length, sequence, and especially secondary structure. These five snRNAs from *Saccharomyces cerevisiae* show notable size and sequence differences from their metazoan counterparts. This is most striking for the large *S. cerevisiae* U1 and U2 snRNAs, for which no secondary structure models currently exist. Because of the importance of U1 snRNA in the early steps of "spliceosome" assembly, we wanted to compare the highly conserved secondary structure of metazoan U1 snRNA (≈165 nucleotides) with that of *S. cerevisiae* U1 snRNA (568 nucleotides). To this end, we have cloned and sequenced the U1 gene from two other yeast species possessing large U1 RNAs. Using computer-derived structure predictions, phylogenetic comparisons, and structure probing, we have arrived at a secondary structure model for *S. cerevisiae* U1 snRNA. The results show that most elements of higher eukaryotic U1 snRNA secondary structure are conserved in *S. cerevisiae*. The hundreds of "extra" nucleotides of yeast U1 RNA, also highly structured, suggest that large insertions and/or deletions have occurred during the evolution of the U1 gene.**

The nuclei of eukaryotic cells contain a group of RNA molecules known as small nuclear RNAs (snRNAs). Among higher eukaryotes, the five splicing snRNAs are well conserved in length, sequence, and especially secondary structure. They interact with pre-mRNA, are required for splicing, and are found associated with the large macromolecular structure called the "spliceosome," which contains pre-mRNA and within which the two cleavage and ligation reactions characteristic of splicing take place (1–4). Arguably, the best evidence for an snRNA–pre-mRNA interaction during mammalian splicing was a genetic experiment demonstrating base pairing between the 5′ end of human U1 snRNA and the pre-mRNA 5′ splice site (5). This experiment confirmed the original predictions of such a base-pairing interaction (6, 7) and the *in vitro* experiments that had demonstrated an association between the pre-mRNA 5′ splice site and U1 snRNA (8).

In the yeast, *Saccharomyces cerevisiae*, pre-mRNA splicing occurs by a very similar pathway to that described for metazoan cells (9, 10). Even yeast spliceosome assembly (11, 12) is remarkably similar to that described for mammalian cells (e.g., refs. 13 and 14). Yet several of the five yeast splicing-associated snRNAs show only limited sequence conservation with their homologs in higher eukaryotes (for review, see ref. 15). Indeed, two of these snRNAs (U4 and U5) were initially identified only by their association with yeast spliceosomes (16), and one (U1) by its association with pre-mRNA during *in vitro* splicing (17, 18). Most striking in their divergence from vertebrate snRNAs are yeast U2 (19), 1175 nucleotides long compared to 187 nucleotides for human U2, and yeast U1 (18, 20), 568 nucleotides long compared to 164 nucleotides for human U1. For both of these yeast RNAs, most of their sequence show no obvious similarity to their metazoan counterparts, and no secondary structure models for either of them have yet been reported.

For yeast U1 RNA, a base-pairing interaction occurs between the 5′ end of U1 and the 5′ splice junction (21, 22), as in higher eukaryotes. Furthermore, this interaction precedes other detectable steps in *in vitro* spliceosome assembly (21, 23), indicating that U1 RNA plays a prominent early role in splicing (see also ref. 24). For these reasons, we were interested in determining which elements of the well-conserved secondary structure of metazoan U1 snRNA might be present in the large yeast U1 snRNA, as well as how the "extra" yeast sequences are organized relative to any such conserved metazoan structural elements. Our results show that most of the core elements of higher eukaryotic U1 RNA structure are indeed present in yeast U1 RNA. Those sequences unique to yeast can be described as two highly structured insertions into (or, for metazoa, deletions from) an ancestral U1 RNA molecule.

MATERIALS AND METHODS

Cloning Other Large U1 Genes. Southern blotting with the *S. cerevisiae* U1 gene (*SNR19*, refs. 18 and 20) as a probe revealed that the *Saccharomyces uvarum* U1 gene is single copy and contained on a 3-kilobase *Hin*dIII–*Pst* I fragment. A *Hin*dIII–*Pst* I *S. uvarum* minilibrary in the vector pTZ19R (United States Biochemical) was probed with the *SNR19* gene, and several candidate clones were isolated and sequenced (25) completely in both directions.

Probing for the *Kluyveromyces lactis* U1 with *SNR19* yielded weak signals and high background. We, therefore, tested oligodeoxynucleotides complementary to sequences at the 3′ end of the *S. cerevisiae* U1 RNA, to see if any would prime reverse transcription from *K. lactis* RNA. One primer, complementary to nucleotides 545–529 of *S. cerevisiae* U1, generated a long extension product, and sequence information was obtained. From these data we designed two primers that were used for polymerase chain reaction (PCR) amplification of the U1-specific *K. lactis* genomic DNA (a thoughtful suggestion of Ana Regina Campos). PCR-generated material was gel-purified, labeled with random primers (26), and used to probe *K. lactis* genomic Southern blots. This showed that the *K. lactis* U1 gene is a single-copy gene and is contained within an ≈1.5-kilobase *Bgl* II–*Hin*dIII genomic

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Abbreviations: snRNA, small nuclear RNA; PCR, polymerase chain reaction.

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fragment. A minilibrary of this specificity was constructed and probed with PCR-generated DNA. A clone for the *K. lactis* U1 gene was isolated and sequenced completely in both directions (25).

Computer Analysis. Sequence data from the three yeast U1 RNAs were aligned by computer to determine primary sequence conservation. They were then analyzed independently with the FOLD program of the Sequence Analysis Software Package (version 5) from the Genetics Computer Group, University of Wisconsin Biotechnology Center (Madison, WI) (27). The predicted structure of *S. cerevisiae* and *K. lactis* U1 RNAs contained helices I, II, the proximal region of III, and IV (i.e., the boxed region in Fig. 1, referred to in the text as the universal core). The predicted structures of *K. lactis* U1 also contained helices V–VIII essentially as shown in Fig. 1*C*, whereas the predicted structures of *S. cerevisiae* and *S. uvarum* U1 RNAs were different, and different from each other, in this region of the molecule. Yet we were able to fold nucleotides 180–325 of *S. cerevisiae* and *S. uvarum* into helices V–VIII as predicted for *K. lactis* whereas the converse was not possible. Folding *S. uvarum* U1 into helices V–VIII then also allowed for the folding of helices I–VI into a structure essentially identical to that of the other two species. The variable regions, including helices IX and X and the distal regions of helices III and VI, are as computer-predicted for *S. cerevisiae*, except for the distal region of helix III that has been modified to account for the S1 nuclease probing data (Fig. 2) in the vicinity of loop IIIa. The distal regions of helices III and VI as well as helices IV and X of *K. lactis* were left unstructured (Fig. 1*C*). This is because of the absence of sequence data from a close relative of *K. lactis* and the absence of structure-probing data for this molecule. However, we note that the computer prediction of *K. lactis* helix III contains three loops, one of which has sequence similarity to *S. cerevisiae* loop IIIa. The distal regions of *K. lactis* helices VI and IX also resemble reasonably well the corresponding *Saccharomyces* structures (Fig. 1 and data not shown).

Enzymatic and Chemical Analysis of RNA. All RNA used for direct structure probing was phenol-extracted from *S. cerevisiae* or *K. lactis* splicing extracts (28). For enzymatic probing or chemical modification (29), RNA (5 μg) was used per time point. Nuclease V1 (Pharmacia) was used at concentrations ranging (in different experiments) from 0.007 to 0.02 unit/μg of RNA; S1 nuclease (Pharmacia) was used at concentrations ranging from 0.015 to 1 unit/μg of RNA. Soluble carbodiimide was from Merck and dimethyl sulfate was from Aldrich. In some experiments (data not shown), RNA was denatured and renatured prior to probing; no change in enzyme susceptibility was observed.

RESULTS

Cloning Other Large U1 RNA Genes. Preliminary low-stringency Northern blot analyses of the RNA from a variety of budding yeasts were performed with the *S. cerevisiae* U1 gene (*SNR19*) as a probe (data not shown). These experiments suggested that at least two yeast species, *S. uvarum* and *K. lactis*, contained U1-like RNAs in the size range of 500–600 nucleotides—i.e., similar to *S. cerevisiae* U1 RNA. Cloning and sequencing of the *S. uvarum* U1 gene revealed a U1 RNA of 573 nucleotides, assuming a 3′ end position identical to that of *S. cerevisiae*, which had been determined directly (18). The transcribed region of the *S. uvarum* U1 gene has 87% sequence conservation with *S. cerevisiae* U1. The highest degree of conservation occurs in the 5′ and 3′ ends of the RNAs and in one internal region. Specifically, there are no nucleotide changes in the first 63 nucleotides, the last 50 nucleotides, or in any nucleotides between positions 250 and 309 (Fig. 1 and data not shown). Cloning and sequencing of the *K. lactis* gene indicated that it is 529 nucleotides long (again, by analogy to *S. cerevisiae* U1) and has an overall sequence conservation of only 47% with *S. cerevisiae* U1 RNA. Like *S. uvarum*, the similarity with *S. cerevisiae* is greatest at the 5′ and 3′ ends of the molecule and also in the region of nucleotides 250–310 (Fig. 1 and data not shown).

Secondary Structure Model of *S. cerevisiae* U1 RNA. Each of the three yeast U1 sequences was analyzed with a computer folding program based on the algorithm of Zuker and Stiegler (27). These initial foldings were then inspected manually and redrawn into similar structures (Fig. 2). This analysis revealed a highly structured *S. cerevisiae* U1 RNA molecule with ≈70% of the nucleotides paired. There are 10 major helices (often interrupted by bulged nucleotides, internal loops, or both) with six or more consecutive base pairs (Fig. 1*B*).

The *S. cerevisiae* U1 structure can be divided into three parts. Helices I–IV, including only the proximal region of helix III, constitute the "universal core." This domain (boxed regions of Fig. 1) is present in all U1 RNAs and contains its four helices in the cruciform structure characteristic of the smaller U1 RNAs (Fig. 1*A*). Helices V–VIII, including only the more proximal region of helix VI, constitute the "yeast core" that is highly conserved between the three yeast molecules. Primary sequence conservation is most notable in these two domains. The third part consists of regions less-well conserved (at both the primary and secondary structure levels) among the three yeast molecules (shaded area in Fig. 1*B* that also indicates that the secondary structure of this domain is not well-established, see below). These include the distal region of helix III (outside the boxed region of Fig. 1), the distal region of helix VI, and helices IX and X. The predicted structures in these regions of the *K. lactis* U1 molecule are sufficiently different from those of *Saccharomyces* that they are not shown in Fig. 1*C*.

Phylogenetic Comparisons. Structural models of many RNAs have been built, refined, or confirmed based on sequence comparisons between different organisms (e.g., refs. 30–34). A common criterion of phylogenetic proof of a putative helix is the occurrence of two or more compensatory base-pair changes within it (35). On this basis, all of the universal core except helix I can be considered proven (Table 1), and helix I is well supported by comparison to higher eukaryotes (Fig. 1 and ref. 10). All of the yeast core except helix VII is also supported by these comparisons (Table 1). Helix VII, although conserved in primary sequence, contains one compensatory base-pair change at the base of the stem (G-257·U-277 of *Saccharomyces*, to U-248·A-268 of *K. lactis*) as well as an additional single base change (U-278, *Saccharomyces*, to A-269, *K. lactis*) that creates a new potential base pair in *K. lactis* (Table 1). We also note that the sequence of loop VII is identical and that of loop VIII is similar between *Saccharomyces* and *K. lactis*. Comparisons in the more variable regions (restricted to a comparison of the two *Saccharomyces* species) have less phylogenetic support (Table 1).

Structure Probing of *S. cerevisiae* U1 RNA. To examine the regions of the molecule with less convincing phylogenetic support, we undertook structure probing using single- and double-strand-specific nucleases (S1 and V1 nucleases, respectively), as well as modifying chemicals specific for unpaired nucleotides (dimethyl sulfate, for adenosines and cytidines, and soluble carbodiimide, for guanosines and uridines; ref. 29). Nuclease or chemical treatment of RNA was followed by reverse transcriptase extension of labeled primers complementary to specific regions of U1 RNA (36).

The data obtained are in good agreement with the proposed structure. Nucleotides on one side of helix VII, for which phylogenetic "proof" is lacking, are nuclease V1 sensitive

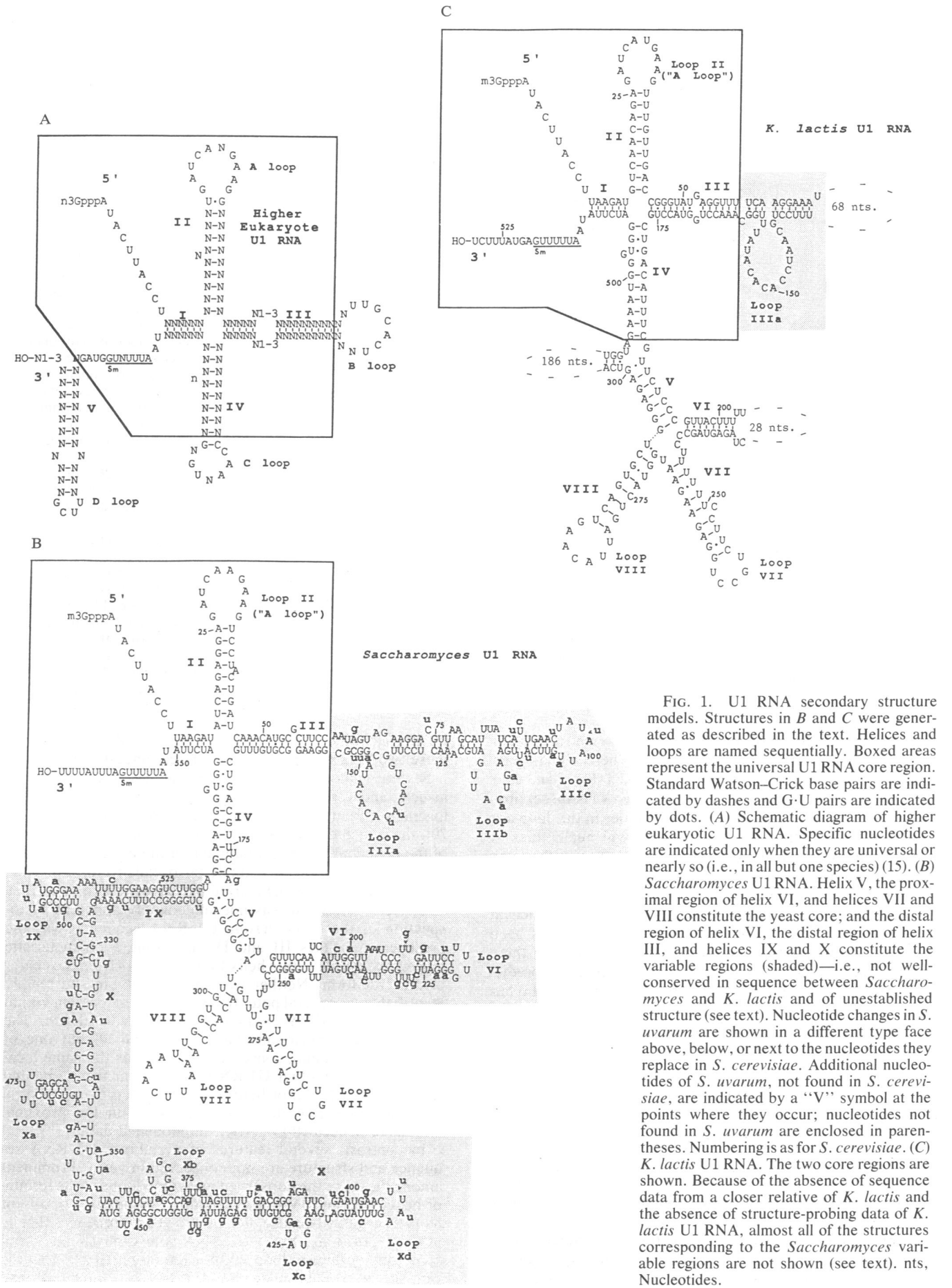

FIG. 1. U1 RNA secondary structure models. Structures in *B* and *C* were generated as described in the text. Helices and loops are named sequentially. Boxed areas represent the universal U1 RNA core region. Standard Watson–Crick base pairs are indicated by dashes and G·U pairs are indicated by dots. (*A*) Schematic diagram of higher eukaryotic U1 RNA. Specific nucleotides are indicated only when they are universal or nearly so (i.e., in all but one species) (15). (*B*) *Saccharomyces* U1 RNA. Helix V, the proximal region of helix VI, and helices VII and VIII constitute the yeast core; and the distal region of helix VI, the distal region of helix III, and helices IX and X constitute the variable regions (shaded)—i.e., not well-conserved in sequence between *Saccharomyces* and *K. lactis* and of unestablished structure (see text). Nucleotide changes in *S. uvarum* are shown in a different type face above, below, or next to the nucleotides they replace in *S. cerevisiae*. Additional nucleotides of *S. uvarum*, not found in *S. cerevisiae*, are indicated by a "V" symbol at the points where they occur; nucleotides not found in *S. uvarum* are enclosed in parentheses. Numbering is as for *S. cerevisiae*. (*C*) *K. lactis* U1 RNA. The two core regions are shown. Because of the absence of sequence data from a closer relative of *K. lactis* and the absence of structure-probing data of *K. lactis* U1 RNA, almost all of the structures corresponding to the *Saccharomyces* variable regions are not shown (see text). nts, Nucleotides.

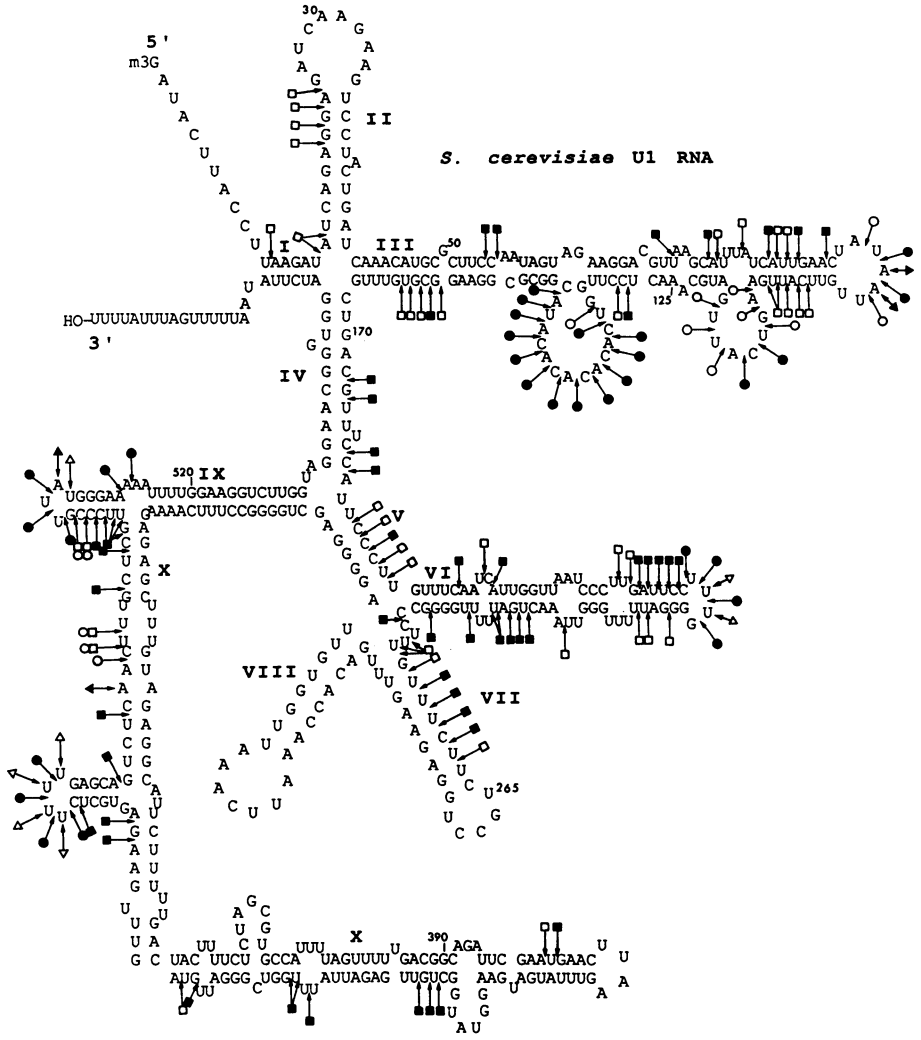

FIG. 2. Summary of chemical and enzymatic structure probing. All sites of modification or cutting are indicated by arrows. Solid box, moderate to strong V1 nuclease cut; open box, weak V1 cut; solid circle, strong S1 nuclease cut; open circle, weak S1 cut; solid triangle, dimethyl sulfate modification; open triangle, soluble carbodiimide modification. Weak and strong cuts are determined relative to the background (control lane) for each experiment. Primer summary: With a primer complementary to nucleotides 44–27, positions 1–26 were analyzed with nuclease V1; with a primer complementary to nucleotides 147–131, positions 50–120 were analyzed with nucleases V1 and S1, and positions 75–120 were analyzed with chemicals; with a primer complementary to nucleotides 280–266, positions 90–265 were analyzed with nuclease S1, positions 125–265 were analyzed with nuclease V1, and positions 180–265 were analyzed with chemicals; with a primer complementary to nucleotides 545–529, positions 390–525 were analyzed with nuclease V1, and positions 460–525 were analyzed with chemicals.

(Fig. 2). A substantial portion of the variable region (the distal regions of helices III, VI, and IX as well as helix X) has been analyzed, and the data fit well with the model (Fig. 2 and data not shown). For example, *S. cerevisiae* loop VI is susceptible to S1 nuclease cleavage, and two nucleotides in this loop are modified by dimethyl sulfate. Also, several nucleotides in helix VI are V1 nuclease-sensitive, consistent with the view that they are involved in base-pairing interactions. [We note that there are nuclease V1 cut sites in some "unpaired" nucleotides, suggesting that this region may be in a helical configuration (37)]. The distal region of helix III has been carefully probed with nuclease S1; the data indicate three loops (IIIa, -b, and -c), consistent with the computer prediction. However, the precise size of the first of these (IIIa) has been modified slightly to account for the details of the nuclease S1 data and for the fact that a similar loop IIIa is visible in the same region of the *K. lactis* molecule (Fig. 1*C*).

DISCUSSION

In overview, the *S. cerevisiae* U1 RNA molecule can be viewed as a metazoan U1 RNA that has experienced two insertion events: a major insertion of helices V–X into the terminus of metazoan helix IV (the C loop) and a relatively minor insertion event into the terminus of helix III (the B loop) that extends substantially the length of this stem. Given the precedent established in evolutionary comparisons of rRNAs (e.g., refs. 38–40), the yeast-specific sequences can be viewed as two "expansion segments." Alternatively, the U1 RNA of higher eukaryotes can be viewed as having lost these two "extra" regions from a larger ancestral U1 RNA.

Several features of the much smaller U1 RNA of plants, insects, and vertebrates are conserved across the vast phylogenetic distance to the yeast U1 RNA. As indicated (18, 20), these include the conserved 10 nucleotides at the 5′ end of the molecule, helix II, and much of the primary sequence of the terminal loop of helix II (also called loop A). Also present is the long-range pairing that gives rise to helix I, as suggested (18). What was not immediately apparent by inspection of the *S. cerevisiae* U1 RNA sequence was the presence of helices III and IV, including a characteristic bulged nucleotide within helix IV 3 base pairs from the center of the cruciform. Neither was the correct Sm site readily identifiable, as several potential Sm sites were present within the 568-nucleotide *S. cerevisiae* U1 RNA sequence. The phylogenetic comparisons have eliminated all but one of these. The remaining conserved Sm site has the same location as that in all other U1 RNAs—i.e., near the 3′ end and directly downstream of helix I. Also conserved are several key nucleotides, purines, or pyrimidines in the proximal regions of helices II–IV (A.K., unpublished data).

In contrast, several features of vertebrate U1 RNA sequence and structure are not conserved in yeast. Prominent among these are the conserved metazoan loops at the termini of helices III and IV (loops B and C, respectively), absent almost certainly because of the extra yeast sequences that lie at these positions. Yeast U1 RNA is also missing a 3′ end stem-loop structure (loop D), present downstream from the Sm site in higher eukaryote U1 RNAs. Similar 3′ end

Table 1. Helix-maintaining changes in yeast U1 RNAs

Region	Helix	Sc/Kl changes		Sc/Su changes	
		Double	Single	Double	Single
Universal core	I	—	—	—	—
	II	4/4	1/1	—	—
	III p	8/8	1/1	—	—
	IV	2/2	—	—	—
Yeast core	V	2/2	1/1	—	—
	VI p	3/3	—	—	1/1
	VII	1/1	1/1*	—	—
	VIII	3/3	1/1	—	—
Variable regions	III d	†	†	1/1	0/3
	VI d	†	†	2/3	4/6
	IX	†	†	2/2	3/4
	X	†	†	6/11	12/18

Sc, *S. cerevisiae*; Su, *S. uvarum*; Kl, *K. lactis*. Values given are number of changes that maintain pairing divided by the total number of changes within the indicated *S. cerevisiae* helices (p, proximal; d, distal; as defined in Fig. 1*B*). Nucleotides considered "bulged" in both species were not included in the comparisons. Base pairs considered were G·C, A·U, and G·U, based on the structures in Fig. 1. Dashes indicate that no changes occur within a given helix between the two species being compared. Note that the comparisons of single and double changes between *S. cerevisiae* and *S. uvarum* in helices VI and X were done conservatively—i.e., they took into account nucleotides that may be paired in *S. uvarum* although they are drawn as bulges for *S. cerevisiae* in Fig. 1*B* (e.g., nucleotides 209–210 and 227–229 of *S. cerevisiae* are shown unpaired in that species but may form consecutive base pairs in *S. uvarum*). These changes contributed to the denominator but not the numerator.

*The change referred to is described in the text and creates an additional base pair in *K. lactis*.

†Variable regions (shaded in Fig. 1*B*) were compared for the two *Saccharomyces* species only, as alignment with *K. lactis* in these portions of the molecule is ambiguous.

stem-loops are characteristic of other metazoan snRNAs and are also absent from their yeast homologs (15). Also notably missing from the *K. lactis* sequence (based on both DNA and RNA analysis) is a conserved bulged nucleotide within helix II, 5 base pairs from the base of the cruciform. This is the first such U1 RNA of almost 20 that have been sequenced.

Although the universal core and the yeast core of *S. cerevisiae* U1 are well-supported by the phylogenetic comparisons, the proposed structures of much of the variable regions are not (Table 1 and data not shown) and must, therefore, be considered tentative (the major reason for the shading in Fig. 1*B*). Some of these structures may differ between the three yeast U1 RNAs. Alternatively, they may correspond poorly to a more conserved set of structures only present within the U1 snRNP particle.

Although there are no certain mechanistic differences in U1 snRNP function between yeast and vertebrates, it is tempting to postulate that the well-conserved yeast domain may play a specific role in splicing. Alternatively, some more subtle aspect of snRNP assembly and/or metabolism may be different in yeast and requires at least some of these domains, perhaps in association with yeast-specific U1 snRNP proteins. A careful assessment of splicing and snRNP metabolism in strains that carry mutations in the yeast domain should clarify this issue.

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