U2 Small Nuclear RNA Is Remarkably Conserved between *Schizosaccharomyces pombe* and Mammals

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We report the molecular cloning and sequencing of the most abundant trimethylguanosine-capped small nuclear RNA from the fission yeast *Schizosaccharomyces pombe*, a highly conserved homolog of mammalian U2 small nuclear RNA. This RNA is 186 nucleotides in length, just 2 nucleotides shorter than its human counterpart; this is in contrast to *Saccharomyces cerevisiae* U2, which is 1,175 nucleotides long. Moreover, the secondary structure of *Schizosaccharomyces pombe* U2 is virtually identical to that of mammalian U2, including the 3' half of the RNA, which shows limited primary sequence identity. Northern (RNA) blot analysis revealed that the size of this RNA is conserved not only in fission yeasts but in many organisms, including other ascomycetes.

Recent studies of mRNA maturation have begun to describe in detail the functions of the RNA components of small nuclear ribonucleoproteins (snRNPs) in RNA processing reactions, particularly the removal of intervening sequences from mRNA precursors (for a review, see reference 17). U2, the subject of this report, is well conserved in size and sequence among higher eucaryotes. Ribonuclease protection assays have implicated this RNA in recognition of the site of lariat formation (3), and a proposed base-pairing scheme between U2 RNA and the branch point consensus (3) was confirmed by the construction of compensatory mutations in the *Saccharomyces cerevisiae* homolog (22). While it is clear that the basic mechanism of splicing is the same in budding yeast cells and mammals, there are differences which may become significant as the details of the process emerge. For example, splicing signals in *Saccharomyces cerevisiae* are rigidly conserved, whereas in mammals they are quite flexible, allowing the use of cryptic sites when the normal sequence is removed (e.g., see reference 25). Presumably because of the strict sequence requirements of the budding yeast splicing machinery, several attempts to express intron-containing genes from higher eucaryotes have failed (2, 14, 33). Paralleling the differences in pre-mRNA structure are differences in the budding yeast homolog of the U small nuclear RNAs (snRNAs), illustrated most dramatically in the case of U2, which at 1,175 nucleotides is over six times the length of its mammalian counterpart (1). In an effort to reconcile these differences, we examined the splicing apparatus of the fission yeast *Schizosaccharomyces pombe*. Like *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* is amenable to both classical and modern genetic analysis. However, while intervening sequences are rare in budding yeast genes, fission yeast protein-coding sequences often contain multiple interruptions (10, 11, 29). Moreover, the splicing signals in fission yeast cells more closely resemble those of metazoans and, in at least one case, *Schizosaccharomyces pombe* is capable of splicing a mammalian intron (13). On the basis of these observations, one might predict that U snRNAs, the core of the splicing machinery, would be well conserved between fission yeast cells and metazoans. We report here the structural charac-

terization of U2 from *Schizosaccharomyces pombe*, which supports this hypothesis.

Identification of a class of abundant snRNAs in *Schizosaccharomyces pombe*. We previously showed that one of the four abundant *Schizosaccharomyces pombe* RNAs in the size range of 150 to 260 nucleotides is cytoplasmic and a likely homolog of the 7SL RNA component of signal recog-

FIG. 1. Immunoprecipitation of fission yeast snRNAs with anti-TMG antibodies. Total fission yeast RNA was reacted with monoclonal antibodies directed against TMG (a kind gift of A. Krainer). (A) Lane TMG shows the pattern of TMG-capped RNAs after 3' end labeling with 5'-³²P-labeled pCp (9) and resolution on a 6% polyacrylamide-7 M urea sequencing gel. Lane M contains 5'-end-labeled 1-kilobase ladder fragments as size markers (numbers indicate nucleotides). Lane T is 3'-end-labeled fission yeast total RNA. (B) Ethidium bromide-stained 6% polyacrylamide-7 M urea gel of the same preparations of total (lane T) and anti-TMG-precipitated (lane TMG) RNA as in panel A. Braces indicate the positions of tRNAs in both panels.

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FIG. 2. Structure and organization of the fission yeast U2 gene. (A) Restriction map and sequencing strategy. The RNA coding sequence, indicated by the thick arrow, is located 120 nucleotides downstream of a *Bam*HI site in pMA2, which is a subclone containing a 2.7-kilobase *Ava*I fragment inserted into the *Sma*I site of pUC18. The numbers refer to the distance from the *Bam*HI site in the plasmid (which is a *Sau*3AI site in the genome). DNA sequence analysis was carried out on double-stranded DNA (6, 36) by using the strategy indicated by the arrows below the restriction map. The boundaries of the RNA coding sequence were determined as described in the text. (B) Genomic Southern blot. *Schizosaccharomyces pombe* genomic DNA was prepared by the method of Durkacz et al. (8) except that Novozyme (Novolabs) was used in place of Zymolyase 60000 (Kirin Brewery). After digestion with the indicated restriction enzymes (Bg, *Bgl*II; B/R, *Bam*HI + *Eco*RI; R, *Eco*RI; H, *Hind*III; A, *Ava*I), samples were electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane. The blot was probed with a nick-translated, 720-base-pair, *Bam*HI-*Xba*I subclone containing the U2 gene under the hybridization conditions previously described (34). The positions and sizes of the 1-kilobase ladder fragments are marked at right.

nitiation particle (5). To determine whether the three nuclear species carry the trimethylguanosine (TMG) cap which distinguishes U-class snRNAs, an immunoprecipitation was performed with a monoclonal antibody specific for TMG (kindly provided by A. Krainer) by a modification of the procedure of Riedel et al. (24). An ethidium bromide-stained gel (Fig. 1B) revealed a simple pattern of three abundant fission yeast capped snRNAs similar to that seen with in vivo ³²P-labeled RNA (5). Upon end labeling RNA from the same precipitation, a more complex pattern appeared (Fig. 1A), presumably due to preferential uptake of pCp by some of the less abundant snRNAs (cf. 30).

Characterization of the *Schizosaccharomyces pombe* U2 homolog. A gene encoding the most abundant snRNA, close in size to mammalian U2, was isolated from a *Sau*3AI partial library (16) by using 5'-end-labeled L15 oligonucleotide (3), which is complementary to a highly conserved portion of U2

FIG. 3. Primary sequence comparison of *Schizosaccharomyces pombe*, human, broad bean, and *Saccharomyces cerevisiae* U2 snRNAs. Numbering is with respect to the *Schizosaccharomyces pombe* sequence. Asterisks above the other sequences indicate identity with fission yeast U2 in that position. Sequences which occur in analogous positions in the secondary structures are aligned, and gaps have been introduced to maximize primary sequence conservation. The Sm-binding site, centered around nucleotide 100, is underlined in each sequence. The positions and orientations of the hairpin stems are indicated by arrows below the sequences.

S. POMBE U2

HUMAN U2

FIG. 4. Secondary structure comparison of fission yeast and human U2 snRNAs. The human U2 secondary structure is a composite of the 5' domain proposed by Ares (1) and the 3' domain proposed by Branlant et al. (4) (see text). The fission yeast structure is based on computer analysis (37) and phylogenetic conservation. Boxed regions correspond to areas of extended primary sequence identity. The sequences which have the potential to form a pseudoknot (see text) are indicated by braces.

RNA, as the probe. Soon after our initial characterization of *Schizosaccharomyces pombe* snRNAs, Ares (1) described the isolation of a gene encoding a homolog of U2 RNA from budding yeast cells and also demonstrated that fission yeast cells contain an RNA that hybridizes to L15. Sequence analysis of the relevant portions of our plasmid by using the strategy outlined in Fig. 2A revealed that it encoded the RNA that Ares had identified. We determined the 5' terminus of the RNA coding region by dideoxy RNA sequencing (20) on total fission yeast RNA by using end-labeled L15 as the primer. The 3' boundary of the gene was determined by comparison of the DNA sequence with the RNA sequence obtained by partial enzymatic digestion (7) of 3'-end-labeled RNA.

U2 RNA in mammalian cells is encoded by a tandemly repeated cluster of 10 to 20 genes (32), while in budding yeast cells it is transcribed from a single gene (1). To determine the copy number in *Schizosaccharomyces pombe*, a Southern blot of various restriction enzyme digests of genomic DNA was probed with a small subclone containing the U2 gene labeled by nick translation. The result, shown in Fig. 2B, was that a single band hybridized in each digest. By analogy to the case in *Saccharomyces cerevisiae*, we predict that disruption of this single-copy gene will be lethal to haploid cells.

A comparison of the primary structure of fission yeast U2

FIG. 5. Cap and modified nucleotide analysis of fission yeast U2 RNA. In vivo ^{32}P -labeled RNA was isolated by the guanidine thiocyanate-hot phenol method, eluted from a two-dimensional polyacrylamide gel, and digested to completion with P1 nuclease as described by Brennwald et al. (5). The products from the uniformly labeled RNA were resolved in two dimensions on a cellulose thin-layer chromatograph. Positions of the nonradioactive markers, visualized by UV, are indicated.

FIG. 6. Northern blot to identify U2 homologs from different organisms. Total RNA from each organism was separated on a 5% acrylamide-7 M urea gel and transferred to Hybond N. The blot was then probed under the conditions described by Patterson and Guthrie (23) with 5'-end-labeled L15 oligonucleotide (3), which hybridizes to a region of U2 conserved through evolution (1). The positions of 5.8S and 5S rRNA, visualized by ethidium bromide staining prior to transfer, are marked. Also indicated is the band corresponding to budding yeast U2, snR20 (22), also known as LSR1 (1). Lanes: Hu, human; XI, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Mz, maize; Sp, *Schizosaccharomyces pombe*; Tp, *Tetrahymena pigmentosa*; An, *Aspergillus nidulans*; Yl, *Yarrowia lipolytica*; Ec, *Escherichia coli*.

with the sequences of human, broad bean, and budding yeast homologs is shown in Fig. 3. The first 60 positions are largely invariant despite the wide phylogenetic distances spanned by this sample. The next ca. 40 nucleotides of the RNA constitute a region of intermediate conservation which includes the binding site for the Sm antigen, a core protein of snRNPs required for recognition by antisera from patients with autoimmune disease (15). The putative Sm site in *Schizosaccharomyces pombe* U2 diverges from the consensus AU₄₋₆G (4, 24) in that it begins with a G rather than an A residue. We have confirmed this assignment by direct RNA sequencing to discount the possibility that a mutation occurred during cloning (data not shown). The replacement of A with G does not appear to be a general property of Sm-binding sites in *Schizosaccharomyces pombe*, however, since we observed a more typical AU₅G in the appropriate region of the fission yeast homolog of U1 (G. Porter, P. Brennwald, and J. A. Wise, manuscript in preparation). Following the Sm site, there are only two patches of strong sequence identity among human, broad bean, and fission yeast U2 snRNAs.

Figure 4 depicts a comparison of the predicted folding patterns of human and fission yeast U2 snRNAs. The structure shown for human U2 is a composite of the 5' half proposed on the basis of phylogenetic conservation (1) and the 3' half deduced from enzymatic probing of end-labeled RNA (4); *Schizosaccharomyces pombe* U2 folds into a structure that is virtually identical. The conservation of sequence in the 5' domain of U2 extends far beyond the region which can base pair to the branch point, perhaps due to constraints imposed by intra- and intermolecular interactions; a specific tertiary structure may, for example, be required for recognition of the splicing substrate or catalysis of branch formation. Consistent with this notion, a portion of

the conserved region comprises the pseudoknot structure recently proposed by Ares and Igel (M. Ares and I. H. Igel, *Molecular Biology of RNA*, in press). Base pairing between the single-stranded region at the base of stem II (nucleotides 47 to 52) and an interior loop in this same stem (nucleotides 61 to 66) results in a new helix which could coaxially stack with stem II. Fission yeast U2 contains compensatory base changes at positions 48 and 65 which support the validity of the pseudoknot interaction. In *Schizosaccharomyces pombe*, and mammals, the right side of the interior bulge in stem II is 3 nucleotides long, while in *Saccharomyces cerevisiae* it is extended to 12 nucleotides; this is potentially significant, since the length of the crossing strand may affect the stability of the pseudoknot.

Our data provide strong phylogenetic support for the structural model proposed by Branlant et al. (4), since the 3' halves of *Schizosaccharomyces pombe* and human U2 snRNAs fold into a pair of stems of similar lengths; primary sequence identity in this domain is mostly limited to the hairpin loops. It is especially noteworthy that there is a clear conservation of both stem-loops III and IV in the fission yeast RNA. This is in contrast to the 148-nucleotide *Trypanosoma brucei* form of U2, in which stem-loop III appears to have been precisely deleted while the stem-loop IV structure is retained (31; Ares and Igel, in press). Similarly, *Saccharomyces* U2 has a potential stem-loop IV located near the 3' end but lacks the potential to form a convincing stem-loop III; deletion analysis reveals that the region which might correspond to the latter structure as well as the remaining extra sequences (relative to its alignment with metazoan U2) are dispensable for function (12, 27). The observed biphasic sequence conservation suggests that there may be two functionally distinct domains in U2. The 3' domain of the vertebrate RNA includes binding sites for two proteins unique to this snRNP, implying a role in assembling or stabilizing the particle (18). Alternatively, since deletions in the 3' portion apparently disrupt interactions with U1 snRNP (19), it is also possible that it functions in assembling other snRNPs and perhaps auxiliary factors (25) around the U2 snRNP during spliceosome assembly.

As noted above, it has been previously suggested that the mechanism of splicing in *Schizosaccharomyces pombe* may be more mammalianlike than splicing in budding yeast cells (13). Particularly relevant to the present study is the site of lariat formation, an invariant UACUAAC 10 to 57 nucleotides upstream of the 3' splice site in *Saccharomyces cerevisiae* (14, 22); *Schizosaccharomyces pombe* branch sites are more flexible, with as little as 4 nucleotides of identity with the *Saccharomyces* heptanucleotide (10, 11, 29). A more critical test of similarity is the ability to use cryptic splicing signals when the normal site is mutated. Although one report suggested that a double mutation in the UACUAAC box of a fission yeast intron did not result in the use of an alternate branch site (21), a more recent study has identified a cryptic site which is activated when the A which normally forms the branch is changed to a C (N. Kaufer, personal communication). Genetic analysis of the function of fission yeast U2 in branch site recognition should help to clarify discrepancies between *Saccharomyces cerevisiae* and mammals and perhaps identify features of mRNA splicing which are unique to this organism.

Modified nucleotide analysis. Though the function of modified nucleotides in snRNAs is not yet clear, they have been retained through evolution and have been documented in the snRNAs of metazoan (26), slime mold (35), and budding yeast (34) cells. In order to determine whether fission yeast

U2 is highly modified like its rat counterpart (26), we digested a uniformly ^{32}P -labeled sample with nuclease P1 and performed two-dimensional thin-layer chromatography (Fig. 5). Two important features were revealed: the nuclease-resistant bridge structure due to the presence of a TMG-capped terminus, and a high content of several modified nucleotides, including 2'-O-methylated derivatives of U, G, and C. It was not possible to determine whether pseudouridine, a common modification of rat U2, was present, because streaking from the uridine spot obscured this region of the chromatogram.

The length of U2 is conserved among many eucaryotes. The high degree of similarity in size and structure between fission yeast U2 and the corresponding RNAs from mammals, insects, and plants contrasted with the divergence of the U2 found in *Saccharomyces cerevisiae* led us to inquire further about the evolutionary conservation of this RNA. In order to examine size variations in U2 among an extended roster of eucaryotes, we performed the Northern (RNA) blot experiment shown in Fig. 6. The autoradiogram reveals a single band hybridizing with L15 in each of three metazoan samples (human, frog, and fruit fly) which corresponds to the known size of the U2 of that organism. Two hybridizing bands are observed in maize RNA, one of similar mobility and one of slightly lower mobility than metazoan U2. Skuzeski and Jendrisak previously showed that another plant, wheat, contains multiple U2-related RNAs in the size range of the mammalian cognate (28). A ciliated protozoan, *Tetrahymena pigmentosa*, contains a hybridizing band nearly identical in size to mammalian U2, as do three of the ascomycetes (*Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Yarrowia lipolytica*). Only after a longer exposure of the blot was a band corresponding to the 1,175-nucleotide LSR1 (snR20) RNA (1, 22) revealed in the *Saccharomyces cerevisiae* lane. No band was seen in the *Escherichia coli* lane with the same exposure as *Saccharomyces cerevisiae*, as was expected. We conclude that *Schizosaccharomyces pombe* is not unique among lower eucaryotes in its possession of a metazoan-sized U2 and that this conserved size is probably a consequence of the retention of its structure through evolution.

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