The RNA component of RNase P in *Schizosaccharomyces* species

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In the fission yeast *Schizosaccharomyces pombe*, the enzyme RNAse P copurifies with two RNAs, K1- and K2-RNA, which are identical except for their termini [1] and which are encoded by a single gene [2]. We have undertaken the cloning of the K-RNA genes in related organisms in order to gain comparative structural information. Because the K-RNA sequence is poorly conserved across species, we have cloned the K-RNA genes in the *Schizosaccharomyces* species *S. malidevorans, S. japonicus, S. versatilis,* and *S. octosporus*. Of the 4 species, only *S. octosporus* contains a K-RNA gene different from that in *S. pombe*; the gene diverges by 20%. Based on the two sequences, nuclease protection data and computer analysis, we have proposed a secondary structure model for the K-RNA. Northern analysis shows the K-RNA genes in all four *Schizosaccharomyces* species to be expressed as two RNAs, as in *S. pombe*.

RNAse P; tRNA; Structure; (*Schizosaccharomyces*)

1. INTRODUCTION

RNase P is the ribonucleoprotein enzyme responsible for maturation of the 5'-end of tRNAs during their biosynthesis. In eubacteria, where the enzyme has been extensively characterized, the RNA component alone is a true ribozyme, capable of catalytic activity under certain in vitro conditions [3-5]. Although much less is known about the eukaryotic enzyme, there seem to be fundamental differences from eubacterial RNase P. For example, in eubacteria the RNA component makes up the bulk of the enzyme (90% in *E. coli* and *B. subtilis*, refs. 6-9), while in eukaryotes the enzyme is at least 50% protein [10,11]. The size of the holoenzyme is also much larger in eukaryotes (roughly 450 kDa in *S. pombe*, ref [1]) than in eubacteria (140 kDa in *E. coli*, ref. [12]). Furthermore, none of the RNAs from the eukaryotic enzymes identified to date have been shown to be catalytic in vitro, the hallmark of eubacterial RNase P [1,11,13,14].

Nonetheless, there are similarities in RNase P which span species. Functionally the enzyme is well conserved; RNase P enzymes in general can correctly process tRNA precursors from a variety of organisms [15]. On the structural level, it has recently been shown that an immunological determinant of the protein component is common to *E. coli* and HeLa cells [16]. Thus, it is not yet clear to what extent RNase P diverges across species, and to what extent essential structural features have been conserved.

In the fission yeast *S. pombe*, nuclear RNase P activity copurifies with two RNAs, designated K1- and K2-RNAs. Differing only in chain length (285 nt. and 270 nt.), the two K-RNAs are encoded by the same single copy gene which is essential for viability of the organism [2]. More recently the RNA components of RNase P from two other eukaryotes have been identified. Both enzymes contain single RNA species; the *S. cerevisiae* RNA (*Sce* RNA) is 369 nt. long [14] while the HeLa cell RNA (H1 RNA) contains 340 nt. [11]. Between the *Sce* RNA and the K-RNA there is limited sequence conservation, while there is virtually no sequence similarity between H1-RNA and the other two RNAs.

Because of the remarkable diversity of RNase P enzymes, it is useful to study the enzyme in a variety of organisms. In regard to the RNA component, phylogenetic comparisons can help to elucidate secondary structure and define features which are conserved through evolution. With this goal we have attempted to clone genes homologous to the K-RNA from other eukaryotes. Here we report the cloning, sequencing, and expression of the K-RNA genes in 4 *Schizosaccharomyces* species and a secondary structure model based on the two different sequences.

2. MATERIALS AND METHODS

2.1. *Strains and organisms*

S. malidevorans, S. japonicus, S. versatilis and *S. octosporus* strains and genomic DNA were generous gifts from Dr Jürg Kohli (Universität Bern, Switzerland). Dr Herman Phaff (University of California, Davis) provided strains of *Endomyces geotrichum, Endomyces tetrasperma, Geotrichum* sp. and *Arthroascus javanensis*. Genomic *Neurospora crassa* and *Drosophila* DNA was obtained from Drs U. Rajbhandary (MIT) and W. McGinnis (Yale), respectively. DNA from *S. cerevisiae* and *Xenopus* was prepared by standard procedures [17].

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2.2. *Southern and Northern analyses*

Genomic DNA was harvested from stationary phase *Schizosaccharomyces* species, *E. geotrichum, E. tetrasperma, Geotrichum* sp. and *A. javanensis* as described [18]. Southern analysis was performed as described [17] with 10 μg DNA per lane in a 0.8% agarose gel. The DNA was blotted onto nitrocellulose (Millipore) and probed with a K-RNA probe. Routine washes were at 42°C with less stringent ones at room temperature. The probe was labeled by a T4 DNA polymerase chew-back/fill-in reaction [17] of the *Sph*I/*Pvu*II K-RNA fragment. The plasmid containing this fragment was created by primer-directed mutagenesis of the K-RNA fragment to provide *Sph*I and *Pvu*II restriction sites at the 5′ and 3′ ends, respectively. This fragment was then inserted into Bluescribe M13- (Vector Cloning Systems). The oligodeoxynucleotides 5′-CCAGCATGCTTCTTCGG (*Sph*I) and 5′-CCATACAGCTGAACCAC (*Pvu*II) were used for mutagenesis.

RNA was prepared by phenol extraction at 37°C of mid-logarithmic cultures. For Northern blots, RNA (20 μg per lane) was loaded on an 8% polyacrylamide urea gel and electroblotted onto Zetaprobe membrane (Biorad) according to manufacturers instructions with the following changes: incubations were at 42°C, 10× Denhardt's solution was substituted for BLOTTO, no carrier RNA was included and the prehybridization solution was also used for hybridization. The plasmid for probe synthesis was constructed by inserting the *Hin*fI/*Mlu*I K-RNA fragment which had been filled in and provided with *Eco*RI linkers, into the *Eco*RI site of pGEM3 (Promega Biotechnology). The antisense probe was then generated by cutting with *Sma*I, transcribing with T7 polymerase (Promega) according to the manufacturer's protocol, and purifying on a polyacrylamide urea gel.

2.3. *Cloning and Sequencing*

In order to clone the K-DNA gene, size selected libraries were constructed from *Eco*RI digested RNA of the *Schizosaccharomyces* species. Regions of a low melting agarose gel corresponding to hybridization on a Southern blot were excised. The DNA was extracted, ligated into Bluescribe M13- (Vector Cloning Systems) and screened by colony hybridization with Whatman filter paper [19] using the K-RNA probe described above for Southern blots. Positive colonies were verified by Southern analysis of minilysates [17]. The *Hin*dIII fragment of the *S. octosporus* K-RNA gene was subcloned and used for sequencing while for the other *Schizosaccharomyces* species, the *Eco*RI/*Cla*I fragment was subcloned.

Sequencing was done as described previously [20] and with Sequenase (US Biochemicals) according to manufacturers protocols. Internal primers used were 5′-GCGTGCTCGTGAGG, 5′-CCTCACGAGCACGC, 5′-CAGACATACAGACAA.

Since the original *S. pombe* K-RNA clone contained only 60 bp of 5′ flank, we wished to extend the upstream sequence. The *Cla*I fragment containing the K-RNA gene was cloned by colony hybridization and sequenced as described above. Sequences reported here have been submitted to GenBank (*S. pombe* K-RNA gene flanks, accession No. X52530; *S. octosporus* K-RNA gene, accession No. X52531).

Secondary structure was analyzed with the Zuker programs [21–23].

3. RESULTS AND DISCUSSION

3.1. *Poor conservation of K-RNA sequence across eukaryotic species*

In order to obtain structural information about the K-RNA, we attempted to clone equivalent genes from a variety of eukaryotes. Low stringency Southern hybridization experiments were performed first using a two thirds length antisense K-RNA probe (see Materials

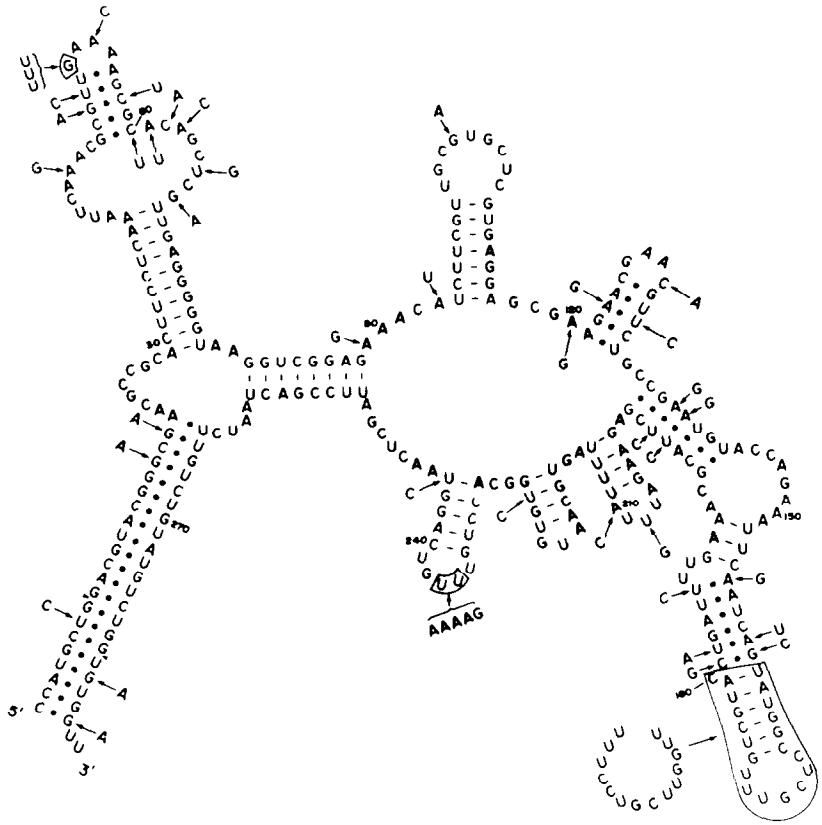

Fig. 1. Secondary structure model of the K-RNA. The *S. pombe* K-RNA sequence is shown, with the *S. octosporus* K-RNA sequence denoted by the changes. Bold dots mark the stems which are phylogenetically supported. The model was obtained with the use of a secondary structure prediction program [21–23] as described in the text.

and Methods). No cross-hybridization was observed with DNA from *Xenopus laevis, Drosophila melanogaster, Neurospora crassa*, or *Saccharomyces cerevisiae* (data not shown). Consequently we focused our search on other yeasts and yeast-like fission organisms which might be more closely related to *S. pombe*. Under the same conditions, Southern analysis showed no significant sequence similarities in the fission organisms *Endomyces geotrichum, Endomyces tetrasperma*, an uncharacterized *Geotrichum* species isolated from cactus rot and the yeast *Arthroascus javanensis*, whose life cycle is somewhat between fission and budding. In an attempt to increase specificity of hybridization, an oligonucleotide which is complementary to a possible conserved region between the K-RNA [1], H1 RNA [11] and *Sce* RNA [14] (5'-AITAGTCGGAATCGIGTT-3') was used to probe a Northern blot from the above-mentioned fission organisms. This conserved sequence is located between nt. 245 and 262 in the K-RNA (Fig. 1), and inosine was substituted in two of the degenerate positions. No cross-hybridization was seen (data not shown). Hence the K-RNAs in these organisms are too divergent to allow cloning with heterologous *S. pombe* probes.

3.2. *Cloning and sequencing of the K-RNA from four* Schizosaccharomyces *species*

Because of the poor sequence conservation, we cloned the K-RNAs from four other *Schizosaccharomyces* species: *S. maledivorans, S. versatilis, S. japonicus* and *S. octosporus*. Southern analysis showed cross-hybridization for all 4 species (Fig. 2). Interestingly, 3 of the yeasts (*S. maledivorans, S. versatilis, S. japonicus*) have identical blot patterns to *S. pombe* for all restriction digests, indicating extensive sequence identity for long distances in the flanks. In addition, the single bands in all digests suggest the gene is present in single copy, as has been shown for the *S. pombe* K-RNA [2].

Size-selected genomic libraries were constructed, and the K-RNA genes were cloned by colony hybridization and sequenced using internal primers as described in Materials and Methods. Not surprisingly, the K-RNA gene sequences of *S. maledivorans, S. versatilis* and *S. japonicus* are completely identical to those of *S. pombe*, including at least 50 nt. of the 5' flank and 40 nt. of the 3' flank. The *S. octosporus* sequence, however, differed significantly from *S. pombe*, with an identity of 80% within the sequence coding for the mature RNA (Fig. 1). The flanking sequence is conserved for approximately 10 base pairs on both sides of the gene and then diverges completely (Fig. 3).

3.3. *A secondary structure model of the K-RNA*

The original secondary structure model of the K-RNA was derived in part from enzymatic probing of isolated RNA [1]. We wanted to reexamine this structure in light of the *S. octosporus* sequence. Analysis was assisted by the secondary structure prediction computer programs [21–23]. On average, the best structure generated by this program contains 70% of the helices in the 'true' structure. In addition, it presents suboptimal RNA foldings. To find those pairing interactions which are phylogenetically supported, all the computer-generated structures of lowest 10% free energy were examined. Statistically, this subset should contain 90% of the stems in the true structure [22]. Four stems contain-

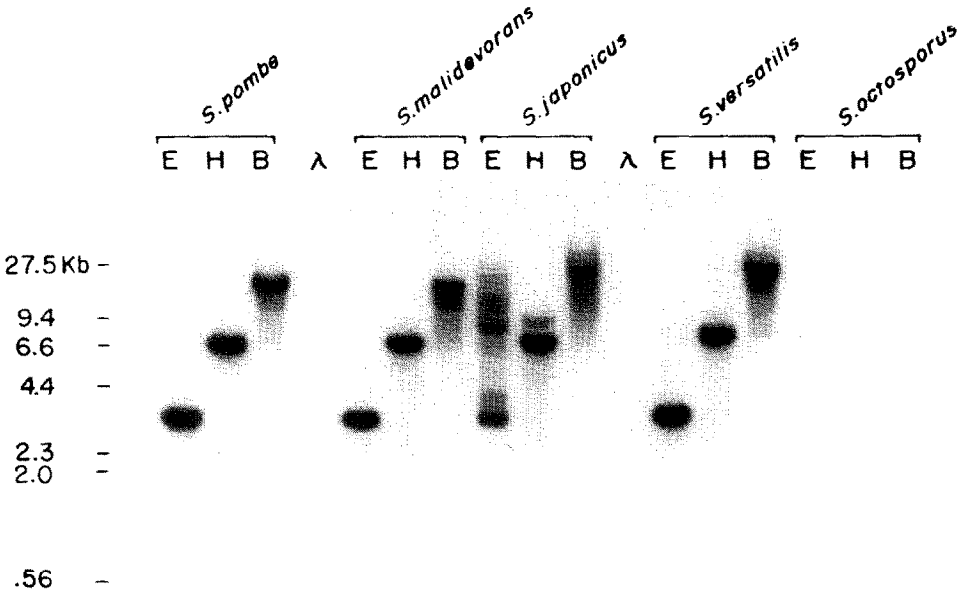

Fig. 2. Southern analysis of *Schizosaccharomyces* yeast genomic DNA with a K-RNA antisense RNA probe. The lanes of *S. octosporus* were derived from the same blot washed less stringently. E = *Eco*RI digest; H = *Hin*dIII digest; B = *Bam*HI digest; λ = phage lambda DNA digested with *Hin*dIII.

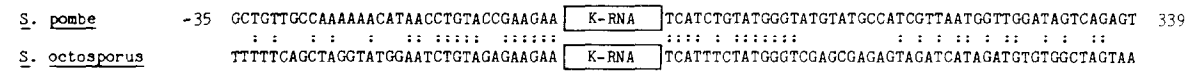

Fig. 3. Genomic flanking sequences of *S. pombe* and *S. octosporus* K-RNAs. Conserved nucleotides are denoted.

ed compensatory mutations, and in addition the 5′-3′ stem showed 4 independent nucleotide changes which were consistent with the pairing. These 5 stems were then required of the program. Of the resulting structures, we selected the second best one (Fig. 1) because it is more consistent with the previous nuclease protection data, which had shown heavy single stranded nuclease susceptibility in the region 100–112 [1]. This proposed model is one of many structural possibilities; the accumulation of further data will undoubtedly refine it.

Our model for the K-RNA differs from the ones for the H1 RNA and *Sce* RNA because it is not similar to the 'proven' structure in eubacteria [11,14,24]. We have opted not to confine the K-RNA to this general model. Since there is no sequence conservation of the RNAs between prokaryotes and eukaryotes, and there seem to be basic differences in RNase P in these two kingdoms, most notably size and protein content, we reason that the secondary structures of the RNAs may also be different. Therefore, we prefer the most energetically favorable configuration which fits our data. The portion of the structure represented by nucleotides 1–88 and 252–286 (the left hand side of Fig. 1) can be drawn similarly for the *Sce* RNA and H1 RNA.

3.4. *Expression of the K-RNA genes of the* Schizosaccharomyces *yeasts*

RNase P in *S. pombe* is unusual in that it is the only RNase P for which two RNA species have been reported. Therefore we wanted to see if the other *Schizosaccharomyces* yeasts also exhibit this unique feature. Northern analysis was performed with RNA of the other 4 *Schizosaccharomyces* species (Fig. 4). As in *S. pombe*, the K-RNAs in all 4 yeasts are present as two species; however, there are interesting differences. The proportions of K1- and K2-RNAs vary widely between species. Even between K-RNAs of identical sequence the ratio of K1 to K2 species reproducibly varies approximately thirtyfold, as shown by densitometric scanning of the autoradiogram. These proportions did not change when the experiment was repeated with RNA prepared from cells grown in minimal medium or to stationary phase in rich medium. It appears that the ratios of K1- to K2-RNA are fairly stable and species-specific.

Originally, it was assumed that the holoenzyme contained one molecule of each K-RNA [1]. However, the lack of stoichiometry of K-RNAs in several of the yeasts in this study argues against the assumption. Based on the enzyme's low buoyant density [10], we now predict that only one RNA is present per holoenzyme.

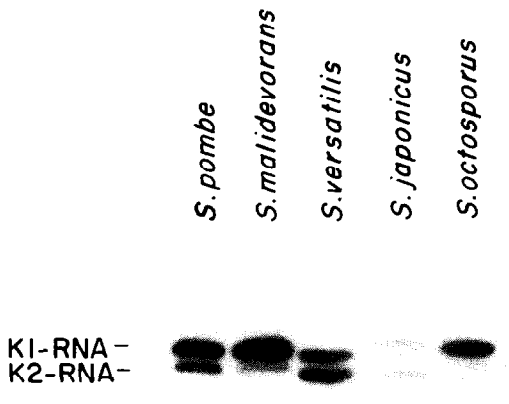

Fig. 4. Northern analysis of K-RNA genes of the *Schizosaccharomyces* yeasts with an antisense K-RNA probe.

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