The yeast homolog of the U1 snRNP protein 70K is encoded by the *SNP1* gene

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ABSTRACT

The product of the yeast *SNP1* gene has high homology to two domains of the metazoan U1 snRNP protein 70K, which binds to stem/loop I of the U1 RNA. However, the absence of other domains conserved in metazoan 70K and the minimal effect of yeast U1 RNA stem/loop I deletion make the assignment of *SNP1* as yeast 70K less clear. To address this question, we have expressed the *SNP1* gene as a fusion protein in *E. coli* and developed a gel shift assay for U1 RNA binding. We show here that the product of the yeast *SNP1* gene binds directly and specifically to the first 47 nucleotides of yeast U1 RNA, which include the stem/loop 1 structure. We therefore conclude that the *SNP1* gene product is the yeast 70K homolog. This is the first yeast protein to be identified as a homolog of a metazoan snRNP protein.

INTRODUCTION

Pre-mRNA splicing requires the small nuclear ribonucleoprotein particles (snRNPs), which consist of small nuclear RNA molecules (snRNAs) and their specific sets of stably complexed proteins. During splicing, snRNPs form specific associations with one another and with conserved sequences in the intron (1, 2). The U1 snRNP, for example, binds to the 5' splice site of the pre-mRNA (3, 4); this binding requires both the 5' end of the U1 RNA (5) and the protein components of the snRNP (6). The U1 RNA base pairs with the 5' splice site (7), but the ways in which the U1 snRNP proteins contribute to 5' splice site recognition are unknown.

Metazoan U1 snRNPs contain the 164 nucleotide U1 snRNA, which adopts a cruciform-like secondary structure with 3 major internal stem/loop structures. Bound to the U1 RNA are the seven Sm proteins which are found in all snRNPs involved in splicing and three U1-specific proteins termed 70K, A, and C. The 70K protein binds directly to stem/loop I of U1 snRNA (8, 9, 10).

The gene encoding the 70K protein has been cloned from humans (11, 12, 13), mice (14), *Xenopus* (15), and *Drosophila* (16) and is highly conserved among these species (88% amino acid identity between human and *Xenopus*, for example; 15, 16). 70K contains an 80 amino acid RNP consensus domain

(Figure 1), a sequence feature common to many RNA-binding proteins (9, 17, 18). The RNP consensus domain is necessary and sufficient to bind U1 RNA specifically (9, 10).

In addition to the RNP domain, two regions rich in arginine are found in 70K from all metazoans. Similar arginine-rich regions are found in many proteins involved in RNA processing including the splicing factors ASF/SF2 and U2AF⁶⁵ (19) and *Drosophila* proteins involved in alternative splicing such as *su(w^o)* (20, 21) and *tra* (22). 70K also contains a glycine-rich domain and a region of 50% homology to the p30^{gag} protein of C type retroviruses (12). While the significance of this relationship is unknown, the retroviral protein may have RNA binding capability.

Yeast U1 RNA is fundamentally similar to human U1 in that both RNAs adopt an equivalent cruciform structure (23, 24, 25). Sequence and structural conservation of stem/loop I, the 70K binding site, is very high, including nine out of ten precisely conserved residues in loop I (26, 27). Mutagenesis of this highly conserved loop I sequence in yeast gave the surprising result that severe mutations in loop I were viable, even though they did reduce splicing efficiency (24). This finding is unexpected because mutagenesis of stem/loop I in metazoan U1 RNA abolishes 70K binding (9, 10), and therefore (presumably) U1 snRNP function. This result suggests that yeast U1 snRNPs do not contain 70K, or do not require its presence for U1 snRNP function. Alternatively, yeast 70K could remain snRNP-associated without binding to stem/loop I, possibly by interacting with other U1 snRNP proteins or with additional parts of the U1 RNA.

Recently, a yeast gene (*SNP1*) with strong homology to the RNP domain of human 70K was discovered during the sequencing of yeast chromosome IX (28). *SNP1* exhibits only 30% overall amino acid identity to human 70K, but contains regions of 50 and 30 amino acids having homologies of 80% and 86%, respectively (Figure 1). In addition to its homologous RNP domain, *SNP1* has a glycine-rich region which is very similar to that of human 70K. However, *SNP1* is shorter and lacks the arginine-rich regions and the p30^{gag} homology found in metazoan 70K.

The gene disruption experiments reported by Smith and Barrell show that *SNP1* encodes a protein essential for yeast viability (28). To test the relationship between *SNP1* and metazoan 70K,

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the *SNP1* RNP domain was replaced with that of human 70K. This hybrid gene complemented an *SNP1* gene disruption, indicating that the human and yeast RNP domains are not only homologous but also functionally interchangeable. Smith and Barrell were unable to demonstrate RNA binding by the *SNP1* gene product (28).

The presence of a strongly 70K-like RNP domain and the functional substitution of the human 70K RNP domain for the *SNP1* RNP domain argue that *SNP1* is the yeast 70K homolog. However, the lack of other conserved regions and the low overall homology complicate the assignment of *SNP1* as yeast 70K. Furthermore, the viability of U1 stem/loop 1 mutants raises the possibility that yeast U1 snRNPs may not possess or require a 70K homolog. Definitive assignment must be based on demonstration of direct binding to yeast U1 RNA by the *SNP1* gene product. Therefore, we set out to test the ability of the *SNP1* gene product to bind specifically to stem/loop 1 of yeast U1 RNA, as this binding is the *sine qua non* of 70K. We demonstrate here that *SNP1* binds specifically to a fragment of yeast U1 RNA which includes stem/loop 1, and conclude that *SNP1* is the yeast 70K homolog. This is the first identification of the yeast homolog of a mammalian snRNP protein, and sets the stage for the functional analysis of snRNP protein using genetic techniques available in yeast.

MATERIALS AND METHODS

In vitro transcription

Plasmids pT719 and 20pUC18 were kind gifts from D. McPheeters. The plasmid pT719 contains the yeast U1 gene under the control of phage T7 RNA polymerase promoter. When linearized with restriction enzyme Bcl I, this construct generates the U1/Bcl I RNA which encompasses the first 47 nucleotides of yeast U1 RNA. Nucleotides 17 to 45 contain the stem-loop I structure. Linearized template was transcribed *in vitro* as described (29). The specific activity of U1/Bcl I transcript was 1.4×10^7 cpm/ μ g. Plasmid 20pUC18 contains the yeast U2 gene under the control of phage T7 RNA polymerase promoter. This construct was linearized with Taq I and transcribed to generate a 259 nucleotide RNA fragment.

Construction of recombinant plasmids and preparation of the trpE-*SNP1* fusion protein

The cloned *SNP1* gene was provided by V. Smith and B. Barrell. A BamH I restriction site immediately upstream of the *SNP1* AUG codon was created by PCR. A BamH I-Cla I fragment containing the *SNP1* sequence was inserted into the pATH3 vector BamH I-Cla I interval so that the *SNP1* protein is in frame with trpE protein. The resultant construct was used to transform *E. coli* strain CAG456. Overexpression of trpE-*SNP1* fusion was performed essentially as described (30, 31) except after the final spin, the pellet fraction was resuspended in 6M urea, 10 mM Tris-HCl pH 7.4, 0.2 mM EDTA and then dialyzed against 10 mM Tris-HCl pH 7.4, 0.2 mM EDTA, 0.05% NP-40, 0.05 mM PMSF for 3.5 h at 4°C. After dialysis, samples were spun at 14,000 rpm for 5 minutes to remove insoluble material and the supernatant was used for the mobility shift assay.

Mobility shift assay

In vitro synthesized U1/Bcl I RNA fragment was denatured at 70°C and renatured slowly in the presence of 1.5 mM MgCl₂ before use. A typical reaction containing 0.25 mg/ml of pellet

fraction, 1.0–2.4 nM U1/Bcl I RNA fragment, 10 mM Tris pH 8.0, 60 mM NaCl, 0.05% NP-40 in 20 μ l reaction mix was incubated at room temperature for 30 minutes. For competition experiments, competitors were added together with ³²P-labelled U1/Bcl I RNA fragment. Poly(rA) and *E. coli* tRNA were purchased from Sigma. The concentrations of competitors used are indicated in the legend to Figure 4. After incubation, the trpE-*SNP1* fusion-RNA complex was resolved by electrophoresis through 5% non-denaturing polyacrylamide (acrylamide: bisacrylamide ratio of 29.2:0.8) gels run at 400 V for 5 minutes followed by 175 V for 2.5 h at 4°C using 1×TBE running buffer (32). The gels were dried and visualized by autoradiography.

UV Crosslinking

Samples from RNA binding assays were crosslinked at 254 nm with a Stratalinker UV crosslinker (Stratagene) at a distance of 10 cm at 0°C for 10 minutes. RNA was digested for 20 minutes at 37°C in the presence of pancreatic RNase A (1 mg/ml). Addition of 100 units of RNase T1 (350,000 units/mg) to the RNase A digestion showed no additional digestion. SDS sample buffer was added and the samples were heated to 100°C for 5 minutes. The samples were then subjected to 10% SDS-PAGE along with ¹⁴C molecular weight markers (Amersham). The gels were dried and autoradiographed.

Northwestern analysis

Proteins (10 μ g of each fraction) were fractionated by 10% SDS-PAGE and transferred to a nitrocellulose membrane using Towbin transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol; 33). The membrane was blocked by incubation in binding buffer (5× Denhardt's solution, 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) overnight at 4°C on a shaking platform (34). The filter was probed in 10 ml of binding buffer containing 5×10^5 cpm labeled U1/Bcl I RNA at room temperature for 1 hour. The membrane was washed once in binding buffer, then four times (10 minutes each) with 10 mM Tris-HCl pH 7.5, 50 mM NaCl and 1 mM EDTA. After air drying, the membrane was autoradiographed.

RESULTS

Expression of a trpE-*SNP1* fusion protein in *E. coli*

The *SNP1* gene was fused to the trpE gene of *E. coli* in the pATH3 vector. The fusion gene, along with the trpE control, was expressed in the protease-deficient *E. coli* strain CAG456. This gene fusion is predicted to produce a polypeptide with a molecular weight of 66 kD (34 kD from trpE, 32 kD from *SNP1*). A prominent protein of this molecular weight was produced in cells carrying a plasmid encoding the trpE-*SNP1* fusion (Figure 2), but not in cells with a trpE plasmid or no plasmid (data not shown). Upon separation of pellet and supernatant fractions, the fusion protein was found exclusively in the pellet fractions (Figure 2), indicating that it is insoluble. Treatment of the pellet fraction with 6M urea efficiently resolubilized the fusion protein. After dialysis to remove the urea and allow renaturation of the protein, the fusion protein was used to test the trpE-*SNP1* gene product for RNA binding.

The trpE-*SNP1* fusion protein binds U1 RNA specifically

We developed a gel mobility shift assay to test the fusion protein for the ability to bind U1 RNA. In this assay, a radio-labelled fragment encompassing the first 47 nucleotides (U1/Bcl I RNA)

Figure 1. Homology between human and the yeast *SNP1* gene product. A diagram of the human 70K protein is shown with the domains conserved among metazoans indicated. Domains shown are gag, p30gag homology; RNP, RNP domain; G, glycine-rich region; and Arg, arginine-rich regions. Below is shown a diagram of the yeast *SNP1* gene product shaded to indicate the degree of similarity to human 70K. Identities were calculated for ten amino acid blocks, and are shaded according to the scale shown below. The alignment of conserved domains between the yeast and human proteins is shown by lines between the two diagrams.

Figure 2. Fractionation of overexpressed *trpE-SNP1* protein. Total lysate was prepared from CAG456 transformed with *trpE-SNP1* plasmid and fractionated as described. Samples representing equal amount of cells were loaded in each lane. T, total lysate (lane 2); S, supernatant (lane 3); P, pellet (lane 4). Arrow indicates the overexpressed *trpE-SNP1* protein. Molecular weight standards are given in kD (lane 1).

was synthesized using T7 RNA polymerase. This U1/Bcl I RNA was incubated with the pellet fractions from untransformed *E. coli* cells, *E. coli* cells transformed with the *trpE* control, and *E. coli* cells transformed with *trpE-SNP1*. The resultant complexes were resolved on native gels (Figure 3). Incubation with the pellet fraction of cells carrying the *trpE-SNP1* fusion caused the formation of a retarded complex (Figure 3, lane 4). This complex was not detected in pellet fractions from the *E. coli* strain, with or without a plasmid expressing *trpE*. Because the only difference between these pellet fractions is the inclusion of the *trpE-SNP1* fusion protein, we conclude that the complex results from RNA binding activity of the *trpE-SNP1* fusion. This finding demonstrates that the *SNP1* gene product binds to U1 RNA.

To determine if *SNP1* binds U1 RNA specifically, complex was formed in the presence of competitor RNAs (Figure 4). Unlabelled U1/Bcl I RNA added to the binding reactions

Figure 3. Binding of *trpE-SNP1* protein to U1 stem/loop I *in vitro*. Radioactively labeled U1/Bcl I RNA fragment, transcribed by T7 RNA polymerase was incubated without protein (lane 1) or with pellet fractions from the CAG456 strain (lane 2), or from CAG456 containing the *trpE* plasmid (lane 3), or from CAG456 containing the *trpE-SNP1* plasmid (lane 4) in the presence of 200 fold excess concentration of *E. coli* tRNA. The U1/Bcl I-*trpE-SNP1* complex was visualized by 5% nondenaturing polyacrylamide gel electrophoresis followed by autoradiography. Free probe and complex are indicated.

Figure 4. Specificity of *SNP1* RNA binding. U1/Bcl I RNA (2.4 nM) was incubated with the pellet protein fraction isolated from *trpE-SNP1* fusion-containing cells. Competition was carried out by co-incubating the U1 RNA fragment with increasing concentrations of competitors. Complex was resolved on a 5% nondenaturing polyacrylamide gel. The gel was dried and subjected to autoradiography. Lane 1: U1/Bcl I RNA fragment alone. Lane 2: no competitor. Lanes 3-5: competition with 5x-, 20x-, and 100x-molar excess of cold U1/Bcl I RNA. Lanes 6-8: competition with 20x-, 100x-, and 200x-molar excess of cold tRNA. Lanes 9-11: competition with 20x-, 100x-, and 200x-molar excess of cold U2 RNA. Lanes 12-14: competition with 20x-, 100x-, and 200x-molar excess of cold poly(rA) RNA. We believe that the two complexes seen result from the binding of full length *trpE-SNP1* fusion and from the partial break down product of the fusion protein.

Figure 5. Crosslinking of trpE-SNP1 protein to U1 stem/loop I. RNA-protein binding reactions containing 0.25 mg/ml of pellet fraction and 1.0 nM U1/Bcl I RNA fragment were mixed at room temperature for 30 minutes followed by UV-irradiation for 10 minutes. The mixtures were treated without (lane 2) or with (lane 3) 1 mg/ml RNase A. Samples were resolved on 10% SDS-PAGE and autoradiographed. Sizes of protein molecular weight standards are given in kD (lane 1). Arrow indicates the crosslinked native trpE-SNP1 protein. The lower band is a crosslinked partial breakdown product of the trpE-SNP1 protein. The bands at 200 kD are due to material stuck in the wells. Control experiments in which the U1/Bcl I RNA alone is UV-crosslinked and RNase digested show no bands, indicating that the labelled bands seen when extract is included do not result from crosslinked RNA (data not shown).

Figure 6. U1/Bcl I RNA fragment binds to trpE-SNP1 protein immobilized on nitrocellulose membrane. Protein (10 μ g) from different fractions was resolved by SDS/PAGE, transferred to nitrocellulose membrane, and probed with labelled U1/Bcl I RNA. The arrow indicates the trpE-SNP1 fusion protein, as visualized by imidazole black staining of the filter. The low molecular weight bands seen in each lane are non-specific RNA binding proteins. When nonspecific competitor RNA was included in binding buffer, only the trpE-SNP1 protein bound U1/Bcl I RNA (data not shown). Lane 1: CAG456 total extract. Lane 2: CAG456 supernatant. Lane 3: CAG456 pellet. Lane 4: CAG456/trpE total extract. Lane 5: CAG456/trpE supernatant. Lane 6: CAG456/trpE pellet. Lane 7: CAG456/trpE-SNP1 total extract. Lane 8: CAG456/trpE-SNP1 supernatant. Lane 9: CAG456/trpE-SNP1 pellet.

competed complex formation strongly. Addition of U1/Bcl I RNA at 20 $\times$ the labelled U1/Bcl I RNA concentration greatly reduced complex formation (Figure 4, lane 4), while inclusion of 100 fold excess unlabelled RNA virtually eliminated complex formation (Figure 4, lane 5).

In contrast, inclusion of nonspecific competitor RNAs in the binding reaction did not reduce complex formation. Addition of a 200-fold molar excess tRNA (Figure 4, lanes 6–8), U2 RNA (Figure 4, lanes 9–11), or poly(rA) (Figure 4, lanes 12–14) did not diminish complex formation. Although addition of a 100-fold excess of tRNA appeared to slightly reduce the amount of complex formed (Figure 4, lane 7), no further reduction was seen when the amount of tRNA was increased to 200-fold (Figure 4, lane 8) or 800-fold (data not shown). These three nonspecific competitors represent three different types of RNA sequence complexity and secondary structure. The use of different competitor RNAs is important to rule out non-specific binding activities such as that exhibited by single-stranded RNA binding proteins. Because complex formation was strongly competed by the U1/Bcl I RNA, but not by any of three nonspecific competitor RNAs, we conclude that the trpE-SNP1 fusion protein binds U1 RNA specifically.

The trpE-SNP1 fusion protein binds U1 RNA directly

To prove that the fusion protein binds RNA directly, a UV-crosslinking experiment was performed (Figure 5). Complex between the U1/Bcl I RNA and trpE-SNP1 pellet fraction was allowed to form as above, and then irradiated with UV light to generate covalent crosslinks between RNA and protein residues. Resolution of these crosslinked complexes on SDS/PAGE revealed a broad high molecular weight band, representing crosslinked RNA and protein (Figure 5, lane 2). Digestion of the crosslinked complexes with RNase removed any RNA residues not closely associated with the fusion protein, leaving several labelled ribonucleotides covalently attached to the protein. Resolution of the crosslinked, RNase-digested proteins on SDS/PAGE allowed identification of the proteins which bind directly to the RNA. As seen in Figure 5, lane 3, a 70 kD band is labelled. This band is the trpE-SNP1 fusion protein crosslinked to the RNA, and is larger than the 66 kD fusion due to the crosslinked ribonucleotides. Also seen is a labelled band at 65 kD, corresponding to the fusion protein breakdown product seen in some preparations. We conclude that the trpE-SNP1 fusion protein binds directly to a fragment containing stem/loop I of U1 RNA.

We also demonstrated direct binding of the trpE-SNP1 fusion protein to the U1/Bcl I RNA using Northwestern blotting (Figure 6). In these experiments, extracts from untransformed *E. coli* cells, *E. coli* cells transformed with the trpE control, and *E. coli* cells transformed with the trpE-SNP1 plasmid fusion were separated by SDS/PAGE and blotted to a nitrocellulose membrane. The membrane was probed with the labelled U1/Bcl I RNA, and bands detected only in the lanes containing the total and pellet extracts from the trpE-SNP1 strain (Figure 6, lanes 7 and 9, respectively). The low molecular weight bands seen in all lanes are *E. coli* proteins which bind RNA nonspecifically. Inclusion of competitor tRNA in the binding reaction eliminated these bands but did not diminish the intensity of the trpE-SNP1 bands (data not shown). Thus, the Northwestern blot provides an independent assay demonstrating the direct and specific binding of SNP1 to U1 RNA.

DISCUSSION

The *SNP1* gene product binds specifically and directly to a fragment of yeast U1 RNA containing stem/loop I and is therefore the yeast 70K homolog. This is the first identification of the yeast homolog of a known metazoan snRNP protein.

Assignment of *SNP1* as yeast 70K argues that the viability of the U1 stem/loop 1 mutant can not be due to the absence of a yeast 70K homolog. Because *SNP1* is reported to be an essential gene (28), it also seems unlikely to be dispensable for U1 snRNP function. Therefore, the most likely explanation of the viability of stem/loop I mutations is that 70K remains associated with the stem/loop1 mutant U1 RNAs through accessory contacts with other parts of the U1 RNA or with other U1 snRNP proteins.

The structure of *SNP1* is unexpected in that not all domains of metazoan 70K are represented. Most strongly conserved is the RNP domain which, in mammalian 70K, is necessary and sufficient to bind 70K to U1 RNA (9, 10). RNP domains are found on a wide variety of RNA binding proteins which are involved in diverse processes. Thus RNP domains are unlikely to have any activities in addition to RNA binding. Presumably, with the *SNP1* RNP domain involved only in binding 70K to U1 RNA, whatever functions the 70K protein effects must be carried out by other domains of 70K. An intriguing candidate for such a functional domain is the glycine-rich domain, which is also well conserved from human to yeast.

Other conserved domains of metazoan 70K are not found in *SNP1*. Most obvious is the lack of the arginine-rich domains which are highly conserved among metazoan 70K. Although their functions are unknown, arginine-rich domains are found on a number of other proteins involved in splicing (19–22). It therefore seemed likely that they would be involved in 70K function, and it was expected that yeast 70K would have them. It is possible that the important features of these domains are present in *SNP1*, but are conserved in a more subtle manner. Alternatively, the arginine-rich domains in yeast may be part of another (neighboring) snRNP protein. Lastly, it is possible that yeast 70K function is not entirely identical to that of human 70K.

The biochemical analysis of metazoan snRNPs has been facilitated by their high abundance and the availability of specific antisera from patients with autoimmune diseases. In contrast, low abundance and poor conservation have made the identification of yeast snRNP proteins arduous. While the functional analysis of snRNP proteins in mammalian systems is quite difficult, genetic analysis in yeast offers the potential to dissect function. Possible functions for 70K include those aspects of U1 snRNP function for which U1 RNA is known to be insufficient. For example, 70K might be used to correctly identify 5' splice sites within pre-mRNAs, to determine the precise site of 5' cleavage, or to allow the second step of splicing to proceed (35, 36). Using the yeast *in vivo* system, the role of 70K in these processes can now be tested.

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