ARTICLE

The Schizosaccharomyces pombe protein Yab8p and a novel factor, Yip1p, share structural and functional similarity with the spinal muscular atrophy-associated proteins SMN and SIP1

Stefan Hannus, Dirk Bühler, Marta Romano, Bertrand Seraphin† and Utz Fischer+

Max-Planck-Institute for Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany and †European Molecular Biology Laboratories, Meyerhofstraße 1, D-69117 Heidelberg, Germany

Received 23 November 1999; Revised and Accepted 21 January 2000

The motor neuron disease spinal muscular atrophy (SMA) is caused by reduced levels of functional survival of motor neurons (SMN) protein. Previous studies have shown that SMN binds to the SMN-interacting protein SIP1 and mediates the assembly of spliceosomal U snRNPs in the cytoplasm. In addition, a nuclear function for SMN in pre-mRNA splicing has recently been proposed. Here, we describe the analysis of the Schizosaccharomyces pombe protein Yab8p and provide evidence that it is structurally and functionally related to SMN found in higher eukaryotes. We show that Yab8p interacts via its N-terminus with a novel protein termed Yip1p. Importantly, Yip1p exhibits homology to SIP1, and the mode of binding to Yab8p is remarkably similar to the SMN–SIP1 interaction. Hence, Yip1p is likely to be the homologue of SIP1 in S.pombe. Yab8p and Yip1p localize predominantly in the nucleus. Genetic studies demonstrate that Yab8p is essential for viability. Strikingly, suppression of YAB8 expression in a conditional knock-out strain causes nuclear accumulation of poly(A) mRNA and inhibition of splicing. These data identify Yab8p as a novel factor involved in splicing and suggest that Yab8p exerts a function similar or identical to the nuclear pool of SMN. Our studies provide a model system to study the cellular function of SMN in yeast, and should help in understanding the molecular events leading to SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is a common autosomal recessive disease characterized by degeneration of motor neurons in the spinal cord (1–5). A human gene has been identified that is directly linked to SMA, termed survival of motor neuron (SMN) (6). Two copies of the SMN gene, SMN1 and SMN2, are located in a 500 kb inverted repeat on chromosome 5p13. These genes differ in that full-length protein is produced almost exclusively from the SMN1 gene. In contrast, the primary transcript of SMN2 undergoes alternative splicing of exon 7, which leads to the predominant expression of C-terminally truncated SMN. More than 95% of SMA patients carry deletions or mutation in SMN1, whereas the expression of SMN2 is unaffected (6,7). The resulting reduced levels of full-length SMN protein cause apoptotic cell death in motor neurons of the spinal cord and consequently lead to SMA (6,8). In contrast to humans, mice harbour only one copy of the SMN gene, which gives rise to the full-length protein only. Gene targeting studies in this organism revealed that SMN is essential for viability, i.e. mice that are null for the SMN gene die prior to implantation at the blastocyst stage. In contrast, SMN+/− mice are viable, and do not exhibit symptoms similar to SMA (9).

The SMN gene encodes a 294 amino acid protein that is expressed in most tissues. In somatic cells, the majority of SMN is found in the cytoplasm. However, SMN also localizes to the nucleus, where it is concentrated in novel structures of unknown function, termed gems (gemini of coiled bodies) (10). By two-hybrid screening, SMN-interacting protein 1 (SIP1) has been identified as a component that interacts with SMN. Additional biochemical studies showed that SMN and SIP1 are found in the cytoplasm in a large complex that also contains spliceosomal U snRNP proteins of the Sm class (hereafter termed the SMN complex) (11). A series of experiments in Xenopus laevis oocytes further revealed a critical role for SMN and SIP1 during the assembly of spliceosomal snRNPs U1, U2, U4 and U5. During this process, the nuclear-encoded m7G-capped U snRNA is transiently transferred to the cytoplasm, where the common Sm proteins B, B′, D1, D2, D3, E, F and G are stored (12–15). Hereafter, the Sm proteins assemble onto a U snRNA, thus forming the Sm core domain of the snRNPs U1, U2, U4 and U5 (16,17). This

*To whom correspondence should be addressed. Tel: +49 89 8578 2475; Fax: +49 89 8578 3810; Email: ufischer@biochem.mpg.de
domain is common to spliceosomal U snRNPs, and may serve as a structural framework for the fully assembled particles. Subsequent to Sm core assembly, the cap structure of U snRNAs is hypermethylated to the m_7G-cap. The assembled U snRNP is then transported to the nucleus, where it functions in pre-mRNA splicing. Antibody microinjection experiments in X.laevis oocytes revealed an essential role for SMN and SIP1 in the assembly of U snRNPs, specifically in the formation of the Sm core domain (20; for a review see ref. 21).

Although biochemical and genetic studies have provided considerable insight into the role of SMN in the cytoplasm, much less is known about its functional interactions in the nucleus. However, SMN has recently been attributed a function in nuclear pre-mRNA splicing. This could be inferred from the observation that gels are in close proximity to coiled bodies, i.e. nuclear domains that have been implicated in splicing. Moreover, a monoclonal antibody directed against SMN interfered with splicing in vitro when pre-incubated with nuclear extract. It was therefore suggested that SMN recycles splicing factors after pre-mRNA processing (22). However, the precise mode of action of SMN and possible other components in this process remain elusive.

Domains in SMN that may serve specific functions in the aforementioned pathways have been described. A sequence located at the N-terminus of the protein and encompassing amino acids 2–49 (amino acid numbers according to the human sequence) is necessary to interact with SIP1 (11). The C-terminus (amino acids 242–278), on the other hand, has been implicated in SMN oligomerization. Interestingly, many intragenic mutations linked to SMA are located in the C-terminus and interfere with oligomerization (23,24). The central part of SMN extending from amino acid 90 to 160 constitutes a tudor domain based on its sequence homology with SIP1 and the way in which it binds to Sm proteins occurs via the tudor domain (27). Moreover, the critical part of YAB8 extending from amino acid 95 to 100 is conserved in higher eukaryotes such as man, mice, X.laevis, zebrafish and the nematode Caenorhabditis elegans (9,28).

RESULTS

Identification of a novel Yab8p-interacting protein with homology to SIP1

The question of whether Yab8p is related to SMN was investigated by a combination of biochemical and genetic approaches. Initially, we tested whether Yab8p interacts with components that are similar or identical to those components that have been reported to bind to human SMN, namely SIP1 and spliceosomal Sm proteins. Using Yab8p as bait in a yeast two-hybrid screen of an S.pombe cDNA library, we identified 95 clones coding for putative Yab8p-interacting proteins. Seven positive clones contained the same open reading frame (ORF) coding for 227 amino acids followed by a stop codon. The S' end of the coding region was subsequently determined by 5' RACE. The full-length ORF codes for a novel 235 amino acid protein, which we termed Yab8p-interacting protein 1 (Yip1p). Interestingly, sequence comparison showed that Yip1p is 20% identical and 45% similar to human or X.laevis SIP1 (Fig. 1). The homology extends over the entire length of the protein and is most pronounced within the N- and C-terminal regions of Yip1p. Despite the evolutionary distance, only one longer stretch of 25 residues (position 152–179) is lacking in Yip1p. Yip1p contains a putative leucine zipper, but, to our knowledge, does not contain any additional known sequence motifs. Yip1p is not homologous to Brr1p, the putative SIP1 homologue in Saccharomyces cerevisiae (see Discussion). The GenBank accession number for the YIP1 sequence is AJ252269.

Yip1p interacts with Yab8p in vitro

The Yip1p–Yab8p interaction identified in the yeast two-hybrid screen was tested in vitro. For this, both proteins were expressed in Escherichia coli as fusion proteins with two IgG-binding domains of protein A (ZZ-tag). The ZZ-tagged proteins were then immobilized on IgG–Sepharose beads and incubated with in vitro translated and 35S-labelled Yip1p and Yab8p, respectively. As a control, we incubated both proteins with ZZ-protein only, coupled to IgG–Sepharose. After extensive washing of the beads, the bound proteins were dissociated by
boiling in SDS-containing protein sample buffer, separated by denaturing SDS–PAGE and visualized by fluorography. Consistent with the two-hybrid interaction data, labelled Yip1p bound efficiently to ZZ-Yab8p and ZZ-Yip1p bound labelled Yab8p (Fig. 2A, lanes 8 and 13). In addition, ZZ-Yab8p could readily bind to labelled Yab8p, indicating that it forms dimers or oligomers (Fig. 2A, lane 7). In contrast, Yip1p did not oligomerize, as judged from the lack of binding of labelled Yip1p to immobilized ZZ-Yip1p (Fig. 2A, lane 14). None of the in vitro translated proteins bound to ZZ-protein alone, indicating that the observed associations were specific (Fig. 2A, lane 26).

Moreover, recombinant His-tagged Yab8p readily bound to immobilized ZZ-Yip1p (Fig. 2B, lane 2) and ZZ-Yab8p (lane 3), but not to ZZ-protein alone (lane 1). This shows that the Yab8p oligomerization and interaction with Yip1p is direct, and not mediated by proteins of the reticulocyte lysate. In positive control binding assays, we tested the interactions between SMN and SIP1 (Fig. 2A). As reported previously, SMN could oligomerize efficiently (Fig. 2A, lane 21) and also bound to SIP1 (lane 22). SMN and Yab8p can both oligomerize and bind to homologous proteins SIP1 and Yip1p, respectively. Given their similarity in binding studies, we next investigated whether Yab8p and Yip1p could form interspecies complexes with human SMN and SIP1 (Fig. 2A). As reported previously, SMN could oligomerize efficiently (Fig. 2A, lane 21) and also bind to SIP1 (lane 22). Thus, SMN and Yab8p can both oligomerize and bind to homologous proteins SIP1 and Yip1p, respectively. Given their similarity in binding studies, we next investigated whether Yab8p and Yip1p could form interspecies complexes with human SMN and SIP1 (Fig. 2A). ZZ-tagged proteins Yip1p, Yab8p and Yip1p were immobilized on IgG–Sepharose and incubated with the in vitro translated 35S-labelled SMN, SIP1, Yab8p and Yip1p proteins, respectively. In our hands, bacterially expressed ZZ-SIP1 is non-functional and hence could not be used for in vitro binding assays. As shown in Figure 2A, labelled SMN and SIP1 did not interact with ZZ-Yab8p (lanes 9 and 10), and similar results were obtained when binding to ZZ-Yip1p was tested (lanes 15 and 16). However, ZZ-SMN could bind a low but significant fraction of labelled Yab8p and Yip1p (Fig. 2A, lanes 19 and 20). The most likely explanation for these results is that the evolutionary distance between Schizosaccharomyces pombe and human proteins is too large to permit their efficient interaction.

SMN has previously been shown to interact via its N-terminus with SIP1 (11). Since this sequence is highly conserved in Yab8p, we investigated whether it can mediate the interaction with Yip1p. As shown in Figure 3A, binding of a Yab8p deletion mutant lacking the N-terminal 35 amino acids to ZZ-Yip1p was severely reduced when compared with the full-length Yab8p protein (lanes 4 and 5). This effect is specific for the Yab8p–Yip1p interaction, since deletion of the N-terminus did not impede the ability of Yab8p to oligomerize (Fig. 3A, lanes 6 and 7). Thus, the Yip1p binding site locates within the first 35 amino acids of Yab8p, and is remarkably similar in function to the SIP1 binding domain in SMN.

Yab8p and Yip1p are nuclear proteins and do not interact with Sm proteins in vitro

SMN and SIP1 form a complex that localizes to both the cytoplasm and the nucleus (10,11). Within the cytoplasm, SMN interacts directly with spliceosomal Sm proteins and mediates the assembly of the Sm core domain (11,20,27). However, apart from SIP1, no additional factors are currently known that functionally interact with nuclear SMN (22). Since the binding to Sm proteins is a hallmark for the role of SMN in U snRNP assembly, we tested whether Yab8p also interacts with this class of proteins. To address this question, 35S-labelled S. pombe and human SmB proteins were incubated with recombinant ZZ-Yab8p, which was immobilized on IgG–Sepharose (Fig. 2A). Surprisingly, Yab8p failed to bind efficiently to either Sm protein (Fig. 2A, lanes 11 and 12). Likewise, no interaction
Next, we used genetic approaches to assess the function of Yab8p. Initially, the YAB8 gene was inactivated by homologous recombination to investigate whether it is essential for viability, as had previously been reported for the murine SMN gene (9). Disruption of Yab8p was accomplished by replacement of the complete coding sequence of YAB8 with the kanamycin resistance cassette in the diploid S.pombe wild-type strain ZP94, thereby generating the strain ZP94-D1. Correct integration of the disruption cassette into the genome was confirmed by PCR, using primers complementary to sequences within the kanamycin cassette and the 5′-untranslated region (5′-UTR) upstream of the disrupted region, respectively. Tetrad analysis of the heterozygous yab8::Kanres/YAB8 diploids revealed that only two of four spores formed colonies, and none of the growing spores carried the kanamycin marker. Spores carrying the YAB8 deletion germinated, but growth was arrested after a few divisions (data not shown). Thus, we conclude that YAB8 is an essential gene required for cell viability.

To address the question of whether Yab8p fulfils a role in splicing, we generated a conditional knock-out of YAB8. For this purpose, YAB8 was cloned in S.pombe expression vector pNWL, which allows YAB8 expression under the control of the thiamine-repressible promoter nmt41 (33–35). Plasmid pNWL-YAB8 was transformed into strain ZP94-D1, thereby generating a strain which was subsequently submitted to random spore analysis to yield haploid progenies termed SP94-D1.1 (Table 1). Yab8p expressed from the plasmid is functional (data not shown). Production of plasmid-encoded Yab8p could readily rescue the lethal phenotype of YAB8 disruption, indicating that Yab8p expressed from the plasmid is functional (data not shown). Production of plasmid-encoded Yab8p was then suppressed by transferring strain SP94-D1.1 to minimal medium containing thiamine. As expected, cell growth was inhibited under these conditions, confirming the initial observation that Yab8p is essential for viability. However, cell growth slowed down only after 2 days, and complete inhibition of growth was observed after 3 days (Fig. 5). In contrast, both wild-type and the non-repressed mutant strain exhibited normal growth properties (Fig. 5).
reason for the unusually long lag phase between Yab8p repression and inhibition of growth is at present not clear.

Depletion of Yab8p causes accumulation of poly(A) RNA in the nucleus

Using the conditional knock-out strain for Yab8p, we next investigated the effect of Yab8p repression on pre-mRNA splicing. As previous studies have shown that inhibition of splicing leads to a nuclear accumulation of unspliced and partially spliced mRNA in the nucleus (36), we analysed the localization of poly(A) mRNA in S.pombe cells after suppression of Yab8p expression. Schizosaccharomyces pombe cells from Yab8p-repressed and control cultures were harvested and poly(A) mRNA localization was analysed. Cells were fixed and hybridized with an oligo-dT oligonucleotide coupled to the Cy3-fluorescence marker and analysed by fluorescence microscopy (Fig. 6). As expected, in control cells poly(A) mRNA was distributed over the entire cell, the nuclei being less stained than the cytoplasm (Fig. 6A, C and E). Strikingly, however, a strong nuclear accumulation of poly(A) mRNA was visible in >80% of cells in which the Yab8p expression was repressed (Fig. 6F, H and K). Thus, loss of Yab8p expression results in a growth defect concomitant with a dramatic nuclear accumulation of poly(A) mRNA.

Conditional knock-out of Yab8p displays splicing defect

The experiments described above do not permit a distinction to be made between a nuclear poly(A) mRNA accumulation caused by a defect in splicing or by other means, e.g. by inhibition of mRNA nuclear export. We therefore analysed by primer extension analysis whether nuclear splicing of intron-containing RNA was affected by suppression of YAB8 expression (37). Schizosaccharomyces pombe U6 snRNA was chosen as a substrate in these studies, since this abundant, stable RNA contains an intron 50 nucleotides in length that is constitutively spliced by the pre-mRNA splicing machinery (38,39).

Wild-type and YAB8-disrupted mutant strains were grown under conditions that either allow or suppress expression of extragenic YAB8, i.e. in the presence or absence of thiamine, respectively. After 60 h, total RNA was extracted from repressed and control cultures (see Fig. 5 for growth curves) and used as a template for primer extension analysis (Fig. 7). Three different primers were used to assess U6 snRNA splicing. The first primer, SpU6-E1, is complementary to nucleotides 30–44 of S.pombe U6 snRNA. SpU6-E1 serves as a control primer, since it gives rise to an extension product of 44 nucleotides, irrespective of whether U6 snRNA is spliced or not. The second primer, termed SpU6-I1, was designed to match nucleotides 62–79 in the intron and to recognize solely unspliced precursor and lariat species. An extension product 79

Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP94a</td>
<td>h’h’ ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18</td>
</tr>
<tr>
<td>SP94-1</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/</td>
</tr>
<tr>
<td>SP94-2</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/</td>
</tr>
<tr>
<td>SP94-1.1</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/(pNWL-LEU-ARS1 – YAB8)</td>
</tr>
<tr>
<td>SP94-1.2</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/(pRSP-LEU-ARS1 – YAB8)</td>
</tr>
<tr>
<td>SP94-1.3</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/(pNWL-LEU-ARS1 – YAB8-EGFP)</td>
</tr>
<tr>
<td>SP94-1.4</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/(pNWL-LEU-ARS1-EGFP-YIP1)</td>
</tr>
<tr>
<td>SP94-D1</td>
<td>h’/h’ ade6-M210/ade6-M216 leu1-32 ura4-D18/ura4-D18 yab8::kan”/YAB8</td>
</tr>
<tr>
<td>SP94-D1.1</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/yab8::kan”/(pNWL-LEU-ARS1-YAB8)</td>
</tr>
<tr>
<td>SP94-D1.2</td>
<td>h’ ade6-M210/leu1-32 ura4-D18/yab8::kan”/(pRSP-LEU-ARS1-YAB8)</td>
</tr>
</tbody>
</table>

*Kindly provided by Dr Zoi Lygerou (ICRF, UK).
nucleotides in length can be generated by this primer only from the unspliced pre-U6 snRNA. The third primer, SpU6-E2, hybridizes within exon 2 of U6 snRNA (nucleotides 140–120). Using this primer, two extension products of 140 and 90 nucleotides in length can be generated from unspliced precursor and mature U6 snRNA, respectively. The result of the primer extension analysis with the three primers is shown in Figure 7A. Strikingly, SpU6-I1 produced an extension product 79 nucleotides in length in the YAB8-repressed culture, indicative for unspliced U6-RNA (Fig. 7A, lane 6). In contrast, this extension product was essentially absent in both wild-type (Fig. 7A, lanes 7 and 9) and non-repressed null mutant strains (lane 8). Similarly, unspliced U6 snRNA could also be detected in the Yab8p-repressed strain using primer SpU6-E2. In this case, a 140 nucleotide transcript was generated, which was not detected in the control strains (Fig. 7A, compare lane 10 with lanes 11–13). Using the same primer, an extension product of 90 nucleotides was produced in similar amounts in all strains, resulting from spliced U6 snRNA (Fig. 7A, lanes 10–13). The large amount of spliced U6 snRNA in repressed and unpressed cells most likely reflects the fact that U6 is an extremely stable and abundant RNA, which is already present in a large amount before Yab8p depletion impairs the splicing reaction. As expected, control primer SPu6-E1 yielded one major extension product of similar intensity in all four strains, indicating that equal amounts of RNA were used in all experiments (Fig. 7A, lanes 2–5). Quantification of the primer extension analysis revealed in the case of SpU6-I1 a 9-fold, and in the case of SpU6-E2 a 15-fold, accumulation of unspliced U6 snRNA in the Yab8p-repressed strain compared with the control strains (Fig. 7B). Thus, repression of Yab8p causes an inhibition of nuclear splicing. This observation, together with its biochemical properties, suggests that Yab8p fulfills a function in S. pombe analogous to the role of SMN in the nucleus of higher eukaryotes.

DISCUSSION

Yab8p has initially been identified based on its sequence similarity to human SMN. However, since the homology between both proteins was restricted to the N- and C-terminal regions, it remained unclear whether Yab8p is indeed functionally related to SMN. In this study, we have investigated Yab8p by genetic and biochemical means. We reasoned that if Yab8p was the functional homologue of human SMN in S. pombe, it should be engaged in similar biochemical interactions and functional aspects as had been described for SMN.

By two-hybrid screening of an S. pombe cDNA library, we identified a novel gene coding for Yip1p. Strikingly, over the entire coding sequence, Yip1p exhibits similarity to the known SMN-interacting protein SIP1 with 20% identity and 45% similarity in a pairwise sequence comparison with human and X. laevis SIP1. Moreover, the way in which Yab8p binds to Yip1p is remarkably similar to the SMN–SIP1 interaction. In particular, the conserved N-termini of SMN and Yab8p are shown to be essential for the interaction with SIP1 and Yab8p, respectively. These data strongly suggest that Yab8p and Yip1p are the homologues of SMN and SIP1, respectively, in lower eukaryotes. The similarity between the human and S. pombe proteins is, however, not high enough to allow the efficient formation of interspecies complexes (Fig. 2A). In the light of this result, it is also not surprising that we fail to complement the lethal phenotype of YAB8 disruption with human SMN (S. Hannus and U. Fischer, unpublished data).

In vitro binding studies have previously shown that the C-terminal sequence is important for SMN oligomerization. Moreover, most intragenic mutations linked to SMA locate within this sequence and interfere with oligomerization (23,24). Interestingly, the C-terminus of Yab8p is highly homologous to the corresponding region in SMN, and most residues found to be mutated in SMA patients are conserved in Yab8p. This suggests that the C-termini of Yab8p and SMN fulfill similar functions.
Surprisingly, *in vitro* binding assays indicated that the C-terminus is dispensable for Yab8p oligomerization (39). It is interesting to note, however, that in our hands not only Yab8p, but also SMN, failed to oligomerize through its C-terminus. Hence, it is currently unclear whether the C-terminal sequences of Yab8p and SMN are engaged in similar interactions. Clearly, a more detailed functional mapping of the C-termini of both proteins is required to solve this issue.

Although Yab8p and SMN share N- and C-terminal similarity, their central regions are divergent. Interestingly, this sequence comprises a tudor domain in SMN and has been shown to mediate direct binding to spliceosomal Sm proteins. Moreover, this interaction is crucial for the cytosolic function of SMN in U snRNP assembly (27). It is reasonable to speculate that Yab8p does not bind to Sm proteins *in vitro* because it lacks a tudor domain. However, at present, the alternative, that

---

*Figure 6.* Depletion of Yab8p causes accumulation of poly(A) mRNA in the nucleus. Analysis of poly(A) mRNA export localization by *in situ* hybridization with Cy3-labelled oligo-dT oligonucleotide. Overview representation of poly(A) mRNA distribution visualized by fluorescence microscopy in wild-type (A) and mutant cells (F). The corresponding DAPI stains are shown in (B) and (G), respectively. Higher magnification of wild-type and mutant cells as seen by fluorescence microscopy (C and H) and Normarsky optics (D and I). Overlay of corresponding fluorescence and Normarsky images [(C)/(D) and (H)/(I)] reveals homogeneous distribution of poly(A) mRNA in wild-type cells (E) and nuclear accumulation in mutant cells (K).
additional factors mediate association of Sm proteins with Yab8p in vivo, cannot be excluded.

Given that the tudor domain in SMN is essential for its cytoplasmic function in higher eukaryotes, it is currently unclear whether Yab8p participates in U snRNP assembly in a similar manner in *S. pombe*. In contrast, our results support the notion that Yab8p (and possibly also Yip1p) fulfill a function that is related to the proposed nuclear role of SMN. Two lines of evidence support this conclusion. First, Yab8p is an essential nuclear protein, and suppression of Yab8p expression results in poly(A) mRNA accumulation in the nucleus. This is consistent with a splicing defect, since previous studies have shown that unspliced and partially spliced mRNAs accumulate in the nucleus (36). Secondly, and most importantly, splicing of U6
snRNA is inhibited in cells depleted of Yab8p. Since this RNA, although not belonging to the mRNA class, is spliced by the pre-mRNA splicing machinery (38,39), we conclude that Yab8p is an essential pre-mRNA splicing factor in *S. pombe*. Interestingly, in vitro splicing studies indicated a role for SMN in pre-mRNA processing, probably for the recycling of factors after pre-mRNA splicing (22). Thus, based on these data together with its overall similarity to SMN, it is tempting to speculate that Yab8p may be functionally related to the nuclear pool of human SMN. Further studies will be needed to evaluate whether Yab8p fulfills a similar role in recycling of splicing factors as has been proposed for human SMN.

To date, computer searches failed to identify a gene with homology to SMN in the baker’s yeast *Saccharomyces cerevisiae*. Whether this organism contains a protein that is related to human SMN or to Yab8p remains to be shown. On the other hand, the similarity between SIP1 and Brrlp indicated that at least some proteins found in the SMN complex may be conserved (11). However, sequence alignments revealed that Brrlp has no significant homology to Yip1p (data not shown). It is, therefore, currently unclear whether Brrlp is indeed the functional homologue of SIP1. The identification of Brrlp-interacting proteins may help to resolve this issue.

We have presented a model system in yeast that may help to characterize by genetic approaches the SMA-associated proteins SMN and SIP1. Thus, the combination of yeast genetics with biochemical studies in higher eukaryotes is likely to accelerate significantly our understanding of the cellular function of SMN and its role in this disease.

**MATERIALS AND METHODS**

**Yeast strains and genetic procedures**

All *Schizosaccharomyces pombe* strains used in this study were derived from the wild-type strains 972 h− and 975 h+. Yeast was grown in complete medium (YES medium: 0.5% yeast extract, 2% bactopeptone, and 2% glucose) supplemented with amino acids (250 mg/l) as required. All strains were grown at 30°C. Yeast strains and genetic procedures described above and purified as described in the user manual (Qiagen, Hilden, Germany).

**Cloning of YAB8 and YIP1 constructs into *S. pombe* expression vectors**

Full-length YAB8 cDNA was amplified from an *S. pombe* cDNA library using primers YABspfw (GCT ACT AGT ATG GAC CAG AGC CAA AAA G) and YABnotv (GCT GCG GCC GCG CAA TA CAA GTA GGA TAA C), introducing an SpeI site preceding the start codon and a NotI site 3′ of the stop codon, thereby including 80 nucleotides of 3′-UTR in the PCR product. The SpeI–NotI fragment was cloned into *S. pombe* expression vector pNWl (kindly provided by G. Superti Furga, EMBL, Heidelberg, Germany) under the control of the nmt41 promoter (33–35). EGFP (Clontech, Palo Alto, CA)-tagged YAB8 was cloned by triple ligation of YAB8 and EGFP fragments, which were amplified by PCR using primers: YABspfw (see above), YABbamrv (5′-TTT GGA TCC ATC TTT AGG TTG CTC AC-3′), GFP-Lbamfw (5′-TTT GGA TCC CCA TGG CCA TGG CCA TGG ATG GTG AGC AAG GGC GAG GAG GATG-3′) and GFPxhorv (5′-TTT CTC GAG CTA CTT GTA CAG CTC GTC CAT GCC G-3′). YIP1-EGFP was similarly cloned using primers: YIP1spfw (5′-TTT ACT AGT ATG ATG CCC TCG AAA AGA AAA AGA AAT-3′) and YIP1notv (5′-CGC GCG GCC GCA TAG ACT CTA TGT TTG AAA GAG AT-3′), GFPspfw (5′-CGG ACT AGT ATG CTA TGG TGA CCA AGG GCG AG-3′) and GFPspwy (5′-TTT ACT AGT AGA TCT GAG TCC GGA CTT GTA CAG-3′).

**Expression of recombinant proteins**

DNA fragments corresponding to the ORFs of YAB8, YAB8ΔN, YIP1, SMNΔN and *S. pombe* SmB (SpB) were generated by PCR. YAB8, YIP1 and SpB were amplified from an *S. pombe* cDNA library (Clontech), whereas the other constructs were generated from master plasmids encoding the respective full-length cDNAs. YAB8 and YIP1 constructs were cloned in-frame into vectors pET28a and pET22Za1a (Novagen, Madison, WI), the latter containing a ZZ-tag that was inserted into the Nhel and BamHI sites of the polylinker of pET21a (Novagen).

For expression of recombinant proteins, plasmids were transformed in *E.coli* BL21(DE3) cells, grown to mid-logarithmic phase and induced with 1 mM IPTG for 6 h. Subsequently, the cells were pelleted by centrifugation, resuspended in 500 mM NaCl, 5 mM MgCl₂ and 50 mM Tris–HCl pH 7.4 and lysed by sonification. After centrifugation, the soluble supernatant was bound directly to IgG–Sepharose and used for binding studies. His-tagged YAB8 was expressed as described above and purified as described in the user manual (Qiagen, Hilden, Germany). *In vitro* translation of 35S-labelled protein was carried out following the manufacturer’s protocol using a Tnt-kit from Promega (Madison, WI). Translated protein (1–3 µl) was included in each binding assay, depending on the efficiency of translation.

**In vitro binding assays**

For *in vitro* binding assays, ~0.5 µg of ZZ-tagged recombinant protein was bound to 50 µl of IgG–Sepharose by incubation at 4°C for 30 min. Proteins that did not bind were removed by repeated washing steps with phosphate-buffered saline (PBS). *In vitro* translated and 35S-labelled proteins (1–3 µl) were added to the Sepharose and incubated for 30 min on ice. Thereafter, the Sepharose beads were washed five times with 250 mM NaCl, 5 mM MgCl₂ and 50 mM Tris–HCl pH 7.4. After the fourth wash, the Sepharose beads were transferred to a new reaction tube in order to minimize background. A 100 µl volume of protein sample buffer without dithiothreitol (DTT) was then added to the drained Sepharose, heated for 10 min at 95°C and analysed by SDS–PAGE. The gel was then fixed in 40% methanol/10% acetic acid for 30 min, followed by a 20 min incubation in amplify solution (Amersham, Uppsala, Sweden). The dried gel was then analysed by fluorography. All binding assays shown were reproduced at least three times and found to be highly reproducible.
Two-hybrid screening

An NcoI–PstI fragment containing full-length cDNA of YAB8 was inserted into the bait plasmid pAS2ΔΔ and transformed into Saccharomyces cerevisiae strain CG1945 (Clontech) by using the lithium acetate method as described previously (42), creating the ‘bait’ strain. The ‘bait’ strain was then transformed with a Schizosaccharomyces pombe cDNA library cloned into pGADGH (Clontech). Double transformants were plated on selective medium lacking tryptophan, leucine and histidine in the presence of 10 mM 3-amino-1,2,4-triazole (3AT; Sigma, Denver, CO). Clones growing under these conditions were tested for β-galactosidase activity in a colony-lift assay as described in the Clontech user manual. The interactions were then verified by in vitro binding assays. The screen was co-transformation of two plasmids coding for two strongly interacting proteins. As a negative control, the ‘bait’ strain was transformed with a plasmid coding for an unrelated protein. The ‘prey’ plasmids of positive clones were isolated (43), and amplified for further analysis.

Cloning of complete YIP1 cDNA by 5’ RACE

A partial ORF lacking the first 21 nucleotides of full-length YIP1 was identified in the two-hybrid screen using YAB8 as a bait. The full-length YIP1 sequence was obtained by 5’ RACE of an S. pombe cDNA library as template, using the vector-specific primer pACTIIfw (5’-GGG GAA TTC CAA CGC AGC GCT TTT CC-3’) and YIP1 sequence-specific primer YIP1rv (5’-GGG CTC GAG CGC CAA GAT TTA TAG ACT C-3’). The PCR product was subcloned EcoRI/XhoI into the multiple cloning site of pBluescript KS+ (Stratagene, La Jolla, CA) and sequenced.

Gene disruption and complementation

The one-step disruption was performed as described (41). For disruption of YAB8, the two primers YAB8disfw (5’-CTA CTG TGT TAA TAA AGG TTT TTG ATG GTA CAA AGA AAG ACA TAC AGG AAT AGA GTA TCT ACA ACT TGT TAC ACA AAA AGC GAT CCC CGG GGT ATT TAA-3’) and YAB8disrv (5’-GAA GAA TTC GAT GAT AAT AAA TCT TAC CAT CAA TAG CAA GAA GTA GTA GTA GAA GTA CAA TTA TAA CCA CAT CAA CAA TGA AGG AAT TCG AGC TCG TTT AAA-3’) were designed to amplify a 1.6 kb fragment containing the kanamycin resistance cassette from plasmid pFA6αKanMX6 (41), thereby introducing 75 and 76 nucleotides complementary to 5’- and 3’-UTR of YAB8 ORF, respectively (sequences matching the kanamycin cassette are underlined). Ten micrograms of the PCR product were directly transformed into the diploid wild-type strain ZP94. Homologous recombination of the kanamycin cassette into the YAB8 locus generated diploid mutant strain SP94-D1.

To control correct integration of the cassette into the genome, analytical PCR of genomic DNA was carried out using primers directed against sequences upstream of the disrupted gene and within the integrated cassette. The expected 1.3 kb band indicative of homologous recombination was found in five of six independent clones. Tetrad analysis was performed as described by Talbot et al. (26). Briefly, cells were sporulated for 3 days on malt extract medium [3% malt extract (Difco, Augsburg, Germany), supplements as required except lysine, adjusted to pH 5.5]. Ascii were placed on YES plates using a Singer micromanipulator, and incubated at 30°C until the ascus wall was broken. Spores were segregated, incubated for 3 days at 30°C and tetrads examined using a light microscope.

For complementation of heterozygous diploids bearing yab8::kanres, S. pombe was transformed with expression plasmid pNWL-YAB8. Plasmid pNWL carries the LEU2 marker and the thiamine-repressible promoter nmt1 (34,35). Transformants were submitted to random spore analysis. Haploids carrying the yab8::kanres allele and the YAB8-encoding plasmid were selected by replica plating. Sporulated cells were transferred to 1 ml of YES medium to promote germination of ascospores, and incubated overnight at 30°C. Cytohelicase was added to the mixture to kill vegetative cells. Release of spores was supported by vigorous vortexing. A dilution series was streaked on selective medium and colonies of different sizes were restreaked on both EMM2-Leu masterplates and YES plates, the latter containing 2.5 mg/l Phloxin B. Phloxin B is used to distinguish between the ploidy states since it stains diploid cells dark red whereas haploids remain light pink. Ploidy was reconfirmed by analytical PCR using the primers MT1, MP and MM (see http://www.bio.uva.nl/pombe/handbook/section1/section1-3.html).

The EMM2-Leu masterplates were then replicated on YES-G418 plates to select for cells lacking the genomic copy of YAB8. Cells growing on plates containing geneticin are dependent on plasmid-borne YAB8 as the only source to complement the lethal phenotype of YAB8 disruption.

Extraction of total RNA and primer extension analysis

To prepare total RNA from mutant and wild-type strains, cells were grown to midlog phase (OD₅₉₅ 0.5–0.7). Fifty millilitres of cultures were harvested, washed once with distilled water and resuspended in 5 ml of RNA extraction buffer (20 mM Tris–HCl pH 8.5, 10 mM EDTA, 1% SDS). After addition of 5 ml of phenol/chloroform/isoamylalcohol (P/C/I) and 1 ml of acid-washed RNase-free glass beads, the suspension was submitted three times to 1 min of vigorous vortexing alternating with incubations on ice for 5 min. The aqueous phase was repeatedly extracted with P/C/I until the supernatant remained clear. RNA was precipitated by the addition of 2 vol of ethanol (EtOH) and ammonium acetate (final concentration 1 M), and pelleted by centrifugation at 10 000 g for 15 min. The pellet was resuspended in 1 ml of TE buffer and ethanol precipitated. After centrifugation, the RNA pellet was washed with 70% EtOH and resuspended in 200 μl of TE buffer. The concentration of the extracted RNA was determined spectrophotometrically.

Primer extension analysis was performed as described by Seraphin (37). Briefly, 10 pmol of primer were radiolabelled by kinasing with T4-oligonucleotide kinase (MBI Fermentas, St Leon-Roth, Germany). Labelled primer was hybridized to 4 μg of total RNA for 1 h at 42°C. After the addition of 10 mM dNTPs 0.25 μl of actinomycin D (5 mg/ml), 0.5 μl of DT1 (20 mM), 0.5 μl of MgCl₂ (200 mM), 0.5 μl of 5X reaction buffer and 0.5 μl of AMV reverse transcriptase (Stratagene), extension was carried out for 30 min at 37°C. Extension products were loaded and separated on denaturing 6% gels containing 7.5 M urea as described (20).
Fixation of cells was by adding 5 ml of 30% paraformaldehyde and 1 ml of 2% glutaraldehyde to 45 ml of culture. Cells were then incubated at 30°C in a rotary shaker for 90 min. After centrifugation, the cells were washed twice with 0.1 M KPO₄/1.2 M sorbitol (wash buffer) and finally resuspended in 1 ml of wash buffer. Then 0.5 mg of 100T zymolyase (ICN, Costa Mesa, CA) was added and incubated for 40 min at 30°C. Permeabilized cells were then extensively washed and dehydrated by sequential incubations in –80°C methanol and acetone for 6 and 1 min, respectively. After extensive washing in 2× SSC, cells were first resuspended and incubated for 1 h in a pre-hybridization buffer (50% formamide, 10% dextran sulphate, 4× SSC, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll 400, 125 µg/ml E.coli tRNA and 500 µM dNTPs). Cy3-labelled oligo-dT nucleotide was added to a final concentration of 0.75 pmol/µl, and incubation continued for 12 h. Subsequently, excess oligonucleotide was removed by consecutive wash steps with 2×, 1× and 0.5× SSC. The distribution of Cy3-coupled oligo-dT nucleotide and DAPI was investigated using a Zeiss Axioshot fluorescence microscope and a Zeiss confocal scanning microscope. DAPI staining was carried out as described (44). For localization of EGFP-tagged Yab8p and Yip1p, S.pombe strains were transformed with expression vectors and localization was examined using the GFP channel of a Zeiss Axioshot fluorescence microscope.

**REFERENCES**


37. Seraphin, B. (1995) Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *EMBO J.*, 14, 2089–2098.


