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The yeast U5 snRNP coisolated with the U1 snRNP has an unexpected protein composition and includes the splicing factor Aar2p

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ABSTRACT

We describe the purification and characterization of a 16S U5 snRNP from the yeast *Saccharomyces cerevisiae* and the identification of its proteins. In contrast to the human 20S U5 snRNP, it has a comparatively simple protein composition. In addition to the Sm core proteins, it contains only two of the U5 snRNP specific proteins, Prp8p and Snu114p. Interestingly, the 16S U5 snRNP contains also Aar2p, a protein that was previously implicated in splicing of the two introns of the *MATa1* pre-mRNA. Here, we demonstrate that Aar2p is essential and required for in vivo splicing of U3 precursors. However, it is not required for splicing in vitro. Aar2p is associated exclusively with this simple form of the U5 snRNP (Aar2-U5), but not with the [U4/U6.U5] tri-snRNP or spliceosomal complexes. Consistent with this, we show that depletion of Aar2p interferes with later rounds of splicing, suggesting that it has an effect when splicing depends on snRNP recycling. Remarkably, the Aar2-U5 snRNP is invariably coisolated with the U1 snRNP regardless of the purification protocol used. This is consistent with the previously suggested cooperation between the U1 and U5 snRNPs prior to the catalytic steps of splicing. Electron microscopy of the Aar2-U5 snRNP revealed that, despite the comparatively simple protein composition, the yeast Aar2-U5 snRNP appears structurally similar to the human 20S U5 snRNP. Thus, the basic structural scaffold of the Aar2-U5 snRNP seems to be essentially determined by Prp8p, Snu114p, and the Sm proteins.

Keywords: Aar2-U5 snRNP; electron microscopy; pre-mRNA splicing; Prp8p; Snu114p

INTRODUCTION

Nuclear pre-mRNA splicing involves two catalytic steps that take place in the spliceosome (Moore et al., 1993; Burge et al., 1999). The spliceosome is a multisubunit ribonucleoprotein complex that is formed by the ordered interaction of the four snRNPs, U1, U2, U4/U6, and U5, and an unknown number of splicing factors, with conserved sequence elements within the pre-mRNA. Each of the snRNPs contains one molecule of RNA (the U4/U6 snRNP contains two RNAs that are extensively base paired) and several proteins, which fall into two classes. U1, U2, U4, and U5 share a set of seven common Sm proteins, whereas the U6 snRNP contains a unique set of seven Sm-like proteins (Lsm2p–Lsm8p). Additionally, each snRNP contains a set of particle specific proteins (Krämer, 1996; Will & Lührmann, 1997).

Spliceosome assembly is initiated by interactions of the U1 and U2 snRNPs with the pre-mRNA 5′ splice site and branchpoint sequence, respectively, generating the prespliceosome (reviewed by Moore et al., 1993). This complex then recruits the preformed [U4/U6.U5] tri-snRNP to form the spliceosome. Major structural rearrangements of the RNAs then take place that activate the spliceosome for the two catalytic steps of splicing. For example, the U1 and U4 snRNA are dissociated from the 5′ splice site and U6 snRNA, respectively (reviewed by Nilsen, 1994; Burge et al., 1999). Thus, the catalytically active spliceosome contains only the U2, U5, and U6 snRNPs. Following catalysis, the postspliceosomal complex, which contains the lariat intron and the U2, U5, and U6 snRNPs, disassembles, the lariat is degraded, and the snRNPs are then re-

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cycled for a next round of splicing (Burge et al., 1999). Because snRNPs must be reused (Moore et al., 1993), a recycling process must exist to rebuild the tri-snRNP from free U5 and U4/U6 snRNPs. In yeast, the spliceosomal protein Prp24p is the recycling factor that reanneals U4 and U6 snRNAs and allows regeneration of duplex U4/U6 snRNPs (Raghunathan & Guthrie, 1998). However, whether factors are also needed to build U5 snRNPs has not been determined.

The U5 snRNP plays a central role in pre-mRNA splicing (Newman, 1997). The U5 snRNA interacts with exon nucleotides at the 5′ splice site and, in addition, prior to the second step of splicing, with exon nucleotides at the 3′ splice site. Initially the 5′ splice site and, later, the 3′ splice site are contacted by the U5-Prp8p protein (U5-220 kDa in man; Teigelkamp et al., 1995; Siatecka et al., 1999). The Prp8/5′ splice site interaction may occur, in addition to the U1 snRNP, when the U5 snRNP is a constituent of tri-snRNPs (Maroney et al., 2000). For this reason, it has been suggested that the U5 snRNP may participate in early steps of spliceosome assembly such as recognition and definition of the 5′ splice site (Maroney et al., 2000). In support of this later finding, earlier studies documented a physical U1/U5 snRNP interaction. In yeast, a U1/U5 snRNP complex was identified, which suggested a collaboration of the U5 with the U1 snRNP early during spliceosome assembly (Neubauer et al., 1997; Ruby, 1997). Moreover, an interaction between the U5 snRNP protein Prp8p and the U1 snRNP protein Prp40p was demonstrated (Abovich & Rosbash, 1997; Gottschalk et al., 1998). Notably, this interaction may be evolutionarily conserved. The fact that in human splicing extracts U1 and U5 snRNPs can be crosslinked before the first catalytic splicing step indicated that this interaction plays a role in directing the U5 snRNP to the 5′ splice site (Ast & Weiner, 1997b).

U5 snRNPs exist as free particles in yeast and human extracts. It is currently not clear whether they represent the pool of U5 snRNPs that originate from the tri-snRNP after the spliceosomal cycle, or whether they represent distinct forms of U5 snRNPs that may function independently of the tri-snRNP. Recently, it has been established that the yeast [U4/U6.U5] tri-snRNP contains at least 28 distinct proteins (Gottschalk et al., 1999; Stevens & Abelson, 1999); seven canonical Sm proteins, seven Sm-like proteins, Lsm2p–Lsm8p, and 14 tri-snRNP specific proteins. However, an independent U5 snRNP, not associated with the tri-snRNP complex, has not been isolated from yeast cells yet.

The human 20S U5 snRNP has been thoroughly studied. It contains, in addition to the seven common Sm proteins, eight particle-specific proteins with molecular masses of 220 (yeast Prp8p), 200 (yeast Brr2p), 116 (yeast Snu114p), 102 (yeast Prp6p), 100 (yeast Prp28p), 52, 40, and 15 kDa (yeast Dib1p), which are stably bound (Bach et al., 1989; Bach & Lührmann, 1991). Most of these proteins are evolutionarily conserved between yeast and mammals and appear to perform similar essential functions. For example, the U5-220/Prp8p protein, as mentioned above, is believed to function at the heart of the spliceosome as cofactor to an RNA enzyme (Collins & Guthrie, 2000). In addition, the U5-116/Snu114p kDa protein, which is homologous to the ribosomal translocase EF-2, may be a driving force behind the many RNA conformational changes within the spliceosome (Fabrizio et al., 1997; Staley & Guthrie, 1998).

To learn more about the life cycle and biogenesis of the U5 snRNP, additional information about the protein composition of the U5 snRNP is needed. In a previous study, we obtained preliminary data about the protein organization of a U5 snRNP form that was coisolated with the yeast U1 snRNP (Neubauer et al., 1997). In this work, we have purified this species of U5 snRNP particle to homogeneity and we show that it contains Prp8p, Snu114p, and the Sm core proteins. Surprisingly, it contains also Aar2p, a protein that was previously implicated in splicing of the *MATa1* pre-mRNA, but not in splicing of actin pre-mRNA (Nakazawa et al., 1991). Here, we demonstrate that Aar2p is essential for growth and its genetic depletion leads to a splicing defect in vivo of additional precursors such as U3A and B. However, it is not essential for conventional in vitro splicing, indicating that it may be required for snRNP biogenesis. In support of this possibility, we found that Aar2p is not part of the tri-snRNP or the spliceosome and we provide evidence for an involvement of Aar2p in later rounds of splicing. The Aar2p-U5 snRNP, thus may be a core particle from which the mature U5 snRNP and subsequently the [U4/U6.U5] tri-snRNP are assembled. In addition or alternatively, because the Aar2-U5 snRNP is consistently coisolated together with the U1 snRNP, Aar2p, as part of the U5 snRNP, may play a role in early spliceosome assembly events or in 5′ splice site recognition and selection of a specific subset of precursors. Because the Aar2-U5 snRNP is biochemically much simpler than the human 20S U5 snRNP, we have investigated by electron microscopy whether this results in a different structure. Interestingly, we show that the two particles have a very similar shape, providing evidence that Prp8p, Snu114p, and the Sm proteins are sufficient to form a scaffold that is equivalent between yeast and man and on which all the additional factors may be incorporated.

RESULTS

Aar2p is specifically associated with the U5 snRNP but not with the [U4/U6.U5] tri-snRNP

We have previously reported the purification of the yeast U1 snRNP from a strain expressing a polyhistidine-

tagged U1 snRNP specific protein (Snp1p), utilizing a two step affinity purification procedure. We observed that the U5 snRNP consistently copurified with the U1 snRNP. Following separation of the two particles by glycerol gradient centrifugation, we determined that the U5 snRNP sedimented at about 15–16S and seemed to contain, in addition to the Sm core proteins, only two highly conserved U5 snRNP-specific proteins, Prp8p and Snu114p (Neubauer et al., 1997; Gottschalk et al., 1998). However, none of the other specific proteins associated with the [U4/U6.U5] tri-snRNP appeared to be present (Gottschalk et al., 1999; Stevens & Abelson, 1999). In addition to Prp8p and Snu114p, a novel protein with an apparent molecular weight of 42 kDa also cosedimented with the U5 snRNA (Gottschalk et al., 1998). The peptide sequences obtained revealed that this protein is Aar2p (Nakazawa et al., 1991).

Interestingly, Aar2p was previously shown to be required for splicing of the two short introns of the *MATa1* pre-mRNA, but apparently not for splicing of actin pre-mRNA (Nakazawa et al., 1991). We have now investigated in more detail which role Aar2p plays in pre-mRNA splicing. To investigate whether Aar2p, in our U1/U5 snRNP coisolate, is strictly and exclusively associated with the U5 snRNP, we raised antibodies against Aar2p expressed in *Escherichia coli*. The polyclonal rabbit antiserum specifically recognized a single band of 42 kDa in yeast extract (Fig. 1C and data not shown). We used these antibodies to perform immunoprecipitation experiments from yeast total cell extracts. Northern analysis of the coprecipitated snRNAs (Fig. 1A) demonstrated that Aar2p is associated with U5L and U5S snRNAs at 150 mM NaCl, whereas many less U5 snRNPs were coprecipitated with Aar2p at higher salt concentrations (400 and 700 mM NaCl). The association of Aar2p with the U5 snRNP was specific, as neither U1, U2, nor U4 and U6 snRNAs were coprecipitated with anti-Aar2p antibodies and none of the snRNPs were coprecipitated with the nonimmune serum (Fig. 1A, lane 4). This indicates that Aar2p is a component of a free form of the U5 snRNP that is not associated with the U4/U6 snRNP. However, Aar2p could also be part of the [U4/U6.U5] tri-snRNP but inaccessible to the antibody. Therefore, we performed the following complementary experiment. We fractionated a wild-type whole cell extract by glycerol gradient centrifugation and then assayed each fraction separately for its snRNA (by northern analysis; Fig. 1B) and protein content (by western analysis using the anti-Aar2p antiserum; Fig. 1C). The free U5 snRNP sedimented between fractions 11 and 17 with a peak in fractions 14 and 15, corresponding to 16S, whereas fractions 19 to 23 contained the peak of the 25S tri-snRNP, as indicated by the presence of U4, U6, and U5 snRNAs. We observed a cosedimentation of Aar2p with free U5 snRNPs between fractions 12 and 17. Aar2p was not seen in high molecular weight complexes, confirming that it is associated only with free U5 snRNPs and not with the tri-snRNP.

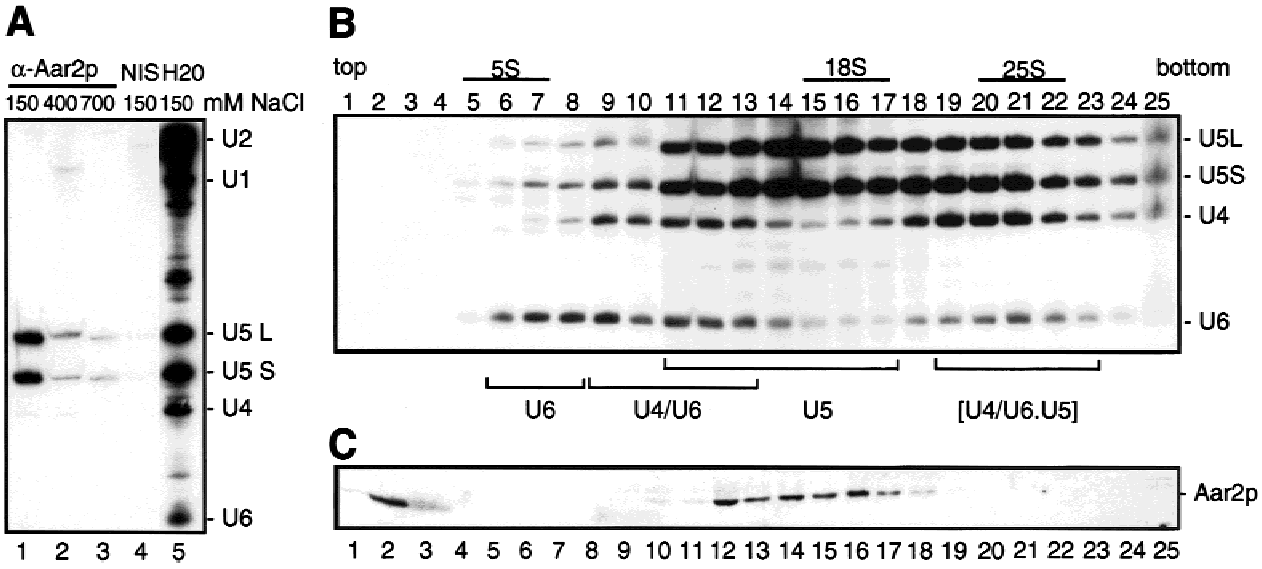

FIGURE 1. Aar2p is exclusively associated with U5 snRNPs but not with [U4/U6.U5] snRNPs in extracts. **A:** The U5 snRNP was immunoprecipitated with polyclonal antibodies raised against recombinant GST-Aar2p fusion protein. The precipitate was washed with buffers containing the salt concentration indicated above each lane. snRNAs were analyzed by northern blot analysis. NIS: nonimmune serum; H20: monoclonal antibody that binds the 5′ m_3G-cap of snRNAs. **B:** A yeast total-cell extract was sedimented on a 10–30% glycerol gradient and 25 fractions were taken from the top and phenol-extracted for RNA analysis by northern blot. **C:** Proteins obtained from the gradient described in **B** were separated on a SDS 13% polyacrylamide gel, blotted to nitrocellulose, probed with primary antibodies specific for Aar2p and secondary antibodies coupled with horseradish peroxidase, and visualized by chemiluminescence. An estimated 20% of the soluble Aar2p was present as free protein at the top of the gradient.

However, this experiment does not exclude that other species of U5 snRNP, with a different protein composition, exist in the extract.

Purification of the Aar2-U5 snRNP

To specifically purify this form of the yeast U5 snRNP, we created a yeast strain that expressed Aar2p with a deca-histidine tag at its C-terminus. The lethality of the *aar2* deletion could be reversed by expression of Aar2p-10xHis, confirming that it was functional (see Material and Methods).

A total-cell extract was prepared, which was then subjected to a two-step affinity purification (Neubauer et al., 1997). Briefly, a mixture of all snRNPs was isolated by immunoaffinity chromatography, utilizing antibodies specific for the tri-methylguanosine (m_3G) cap of the snRNAs. The isolated snRNPs were then applied to the second affinity (Nickel-NTA) column and the U5 snRNP was eluted with 50 mM imidazole. Analysis of the RNAs extracted from the eluate of the nickel column showed that the U5 snRNP was again coisolated with the U1 snRNP (Fig. 2A, lane 2). The U1 snRNA can be clearly seen together with the U5L and U5S snRNA, although the U5L form of U5 snRNA seems less abundant. Figure 2A, lane 1, shows, for comparison, the RNAs isolated after purification of the U1 snRNP containing a histidine-tagged Snp1p. As was shown previously, the U1 snRNA copurifies with the U5 snRNA (Neubauer et al., 1997; Gottschalk et al., 1998). Thus, under both conditions tested, the two snRNAs coisolate.

To further purify the U5 snRNPs present in the U1/U5 mixture, the U1/U5-containing fractions were separated by 10–30% glycerol gradient centrifugation. The

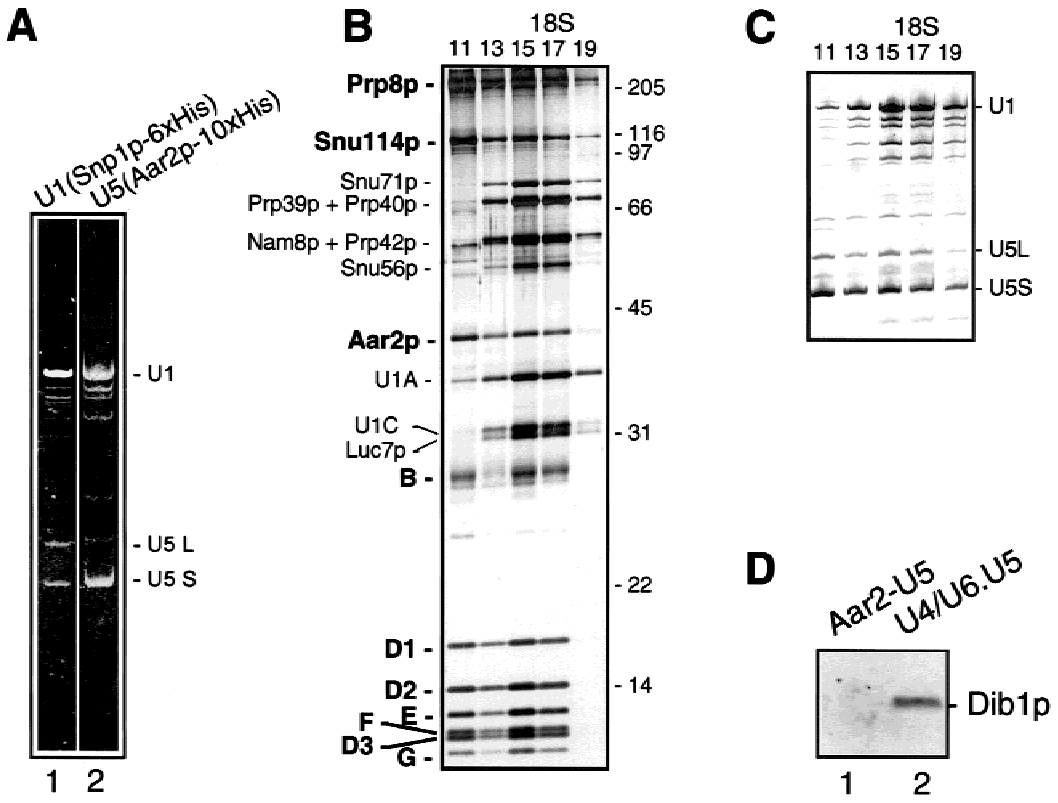

FIGURE 2. Aar2-U5 snRNPs, coisolated with U1 snRNPs, can be separated by glycerol gradient centrifugation and comprise Prp8p, Snu114p, Aar2p, and the Sm proteins. **A:** The U5 snRNP was purified from yeast total-cell extract containing a histidine-tagged Aar2p (lane 2) or a histidine-tagged U1 Snp1p (lane 1; Neubauer et al., 1997). The U5 snRNP was eluted from the second affinity (Ni(II)-NTA) column together with U1 snRNPs. The eluate was subjected to glycerol gradient centrifugation at 200 mM KCl in a Beckman ultracentrifuge in a 70Ti rotor for 20 h at 29,000 rpm. **B:** The gradient was analyzed by denaturing gel electrophoresis and subsequent silver staining to visualize the proteins. The identity of the proteins is indicated on the left and the position of molecular weight markers on the right. **C:** The same gradient was analyzed for the snRNAs composition. The identity of the RNAs is indicated on the right. The U5 snRNA-containing fractions contained Prp8p, Snu114p, Aar2p, and the Sm proteins B, D1, D2, D3, E, F, and G. A weak protein band of 25 kDa in fraction 11 is most likely a degradation product. This protein is not Prp18p (Horowitz & Abelson, 1993), as determined by western blot (not shown). **D:** Dib1p is not present in purified free U5 snRNPs. Equal amounts of purified Aar2-U5 snRNPs (lane 1) and [U4/U6.U5] tri-snRNPs (lane 2) were phenol extracted and the respective proteins were separated by gel electrophoresis, the gel was blotted, and Dib1p was visualized using an antiserum raised against the human ortholog of Dib1p, the U5-15kD protein (Reuter et al., 1999).

odd fractions were analyzed for proteins (Fig. 2B) and RNAs (Fig. 2C) by silver staining. The U5 and U1 snRNPs were partially separated from each other. U5 sedimented in fractions 11–17 with a major peak in fraction 11 and a second peak in fraction 15. Although the U5 snRNP sedimented in two peaks, both peaks contained only Prp8p, Snu114p, Aar2p, and the Sm proteins (Fig. 2B). We named this particle Aar2-U5 snRNP. One reason for this bimodal sedimentation could be that the U1/U5-complex dissociated during centrifugation and, as a consequence, some of the U5 snRNPs, free of U1 snRNPs, sedimented in an uppermost fraction of the gradient. The U1/U5 snRNP, however, sedimented in fractions 13–19, with only one peak in fraction 15. As shown in Figure 2B, we found all of the U1 snRNP associated proteins (Neubauer et al., 1997; Gottschalk et al., 1998). However, none of the other proteins known to be part of the [U4/U6.U5] tri-snRNP or the proteins of the U2 snRNP were identified. Only the presence or absence of one tri-snRNP protein, Dib1p, could not be judged from the gel, since it comigrates with SmD2 (Gottschalk et al., 1999; Reuter et al., 1999). The human ortholog of Dib1p, the U5-15kDa protein, is part of the U5 snRNP and shares 65% identity with Dib1p. To clarify whether Dib1p is associated with the Aar2-U5 snRNP, we used an antiserum raised against the human U5-15kDa protein to detect Dib1p by western blot analysis. The Aar2-U5 and [U4/U6.U5] tri-snRNPs were phenol extracted and the proteins analyzed by western blot (Fig. 2D, lanes 1 and 2). A protein of 14 kDa could be specifically detected in the [U4/U6.U5] tri-snRNP (Fig. 2D, lane 2), but not in the U5 snRNP (Fig. 2D, lane 1). This experiment shows that Dib1p is not stably associated with the free Aar2-U5 snRNP.

The yeast Aar2-U5 snRNP, although biochemically different, is structurally similar to the human 20S U5 snRNP

Because the yeast Aar2-U5 snRNP is biochemically much simpler than the human U5 snRNP, we have examined by electron microscopy whether this different organization leads to a different structure. To obtain pure Aar2-U5 snRNPs suitable for electron microscopy analysis, we needed to completely separate the U5 and U1 snRNPs. We tried to affect differentially the solubility of the two snRNP particles in glycerol gradients by using several buffer conditions. In a buffer containing 50 mM KCl, we observed that the U1 snRNP aggregated during the sedimentation process, whereas the U5 snRNP sedimented unaffected and in a single peak corresponding to 16S (Fig. 3A, lanes 8 and 9; analysis of the RNAs). The particle did not contain any proteins other than Prp8p, Snu114p, Aar2p, and the Sm proteins (Fig. 3B, silver-stained proteins). Sm B could not be detected in this gel as it stains poorly and is only seen when larger amounts are present. The purified yeast U5 snRNP has essentially the same sedimentation behavior as the free U5 snRNPs present in a whole-cell extract (compare the sedimentation of the U5 snRNA shown in Fig. 3A to Fig. 1B).

Pure Aar2-U5 snRNPs, taken from fraction 8 of the glycerol gradient shown in Figure 3A,B, were used to prepare negatively stained carbon-film samples for transmission electron microscopy (TEM). Figure 3C shows a typical overview of a small area on the carbon film. The Aar2-U5 snRNP is pure and homogenous, as there are only particles visible that have a similar size and shape. Images of U5 particles have been picked from several micrographs and subsequently aligned in similar orientation. Figure 3D shows some typical examples. The Aar2-U5 snRNP appears a well-defined particle with an elongated shape and a size of 12 by 25 nm. Most views show particles that can be divided into three domains: a central body, a broader upper head, and a smaller lower foot. In some views, small protrusions can be seen extruding from the body domain. As in the case of the human 20S U5 snRNP (Fig. 3E), a doughnut shaped structure characteristic of the Sm core domain, cannot be recognized in these images (Kastner et al., 1990, 1992). Surprisingly, the main features of the yeast Aar2-U5 snRNP closely resemble those of the human 20S U5 snRNP, although the latter contains many more proteins. However, on close inspection, there are many details that differ (Fig. 3, compare D with E). The head and the main body of the Aar2-U5 snRNP appear thinner in many views and some protrusions are missing. For example, the characteristic tip located at the bottom of the human 20S particle is lacking (Kastner et al., 1990). Because Prp8p/U5-220, Snu114p/U5-116, and the Sm core are highly conserved entities found in both the yeast and the human U5 snRNPs, it is expected that their structure is also evolutionarily conserved. Therefore, these proteins may provide a basic scaffold that accommodates additional proteins without changing its overall appearance.

Aar2p is required for pre-mRNA splicing in vivo but not in vitro

Previously it was reported that a mutation in the *AAR2* gene led to a severely reduced splicing efficiency of the *MATa1* pre-mRNA. In the same work, it was also indicated that *AAR2* was not involved in splicing of actin pre-mRNA. However, because the growth defect caused by the *aar2* mutation was not suppressed by the intronless *MATa1* gene, it was proposed that *AAR2* is involved in splicing of some unidentified genes that are important for cell growth (Nakazawa et al., 1991). Because here we have shown that Aar2p is clearly associated with a particular form of the U5 snRNP and because disruption of *AAR2* leads to a lethal phenotype, we analyzed whether Aar2p is required for splic-

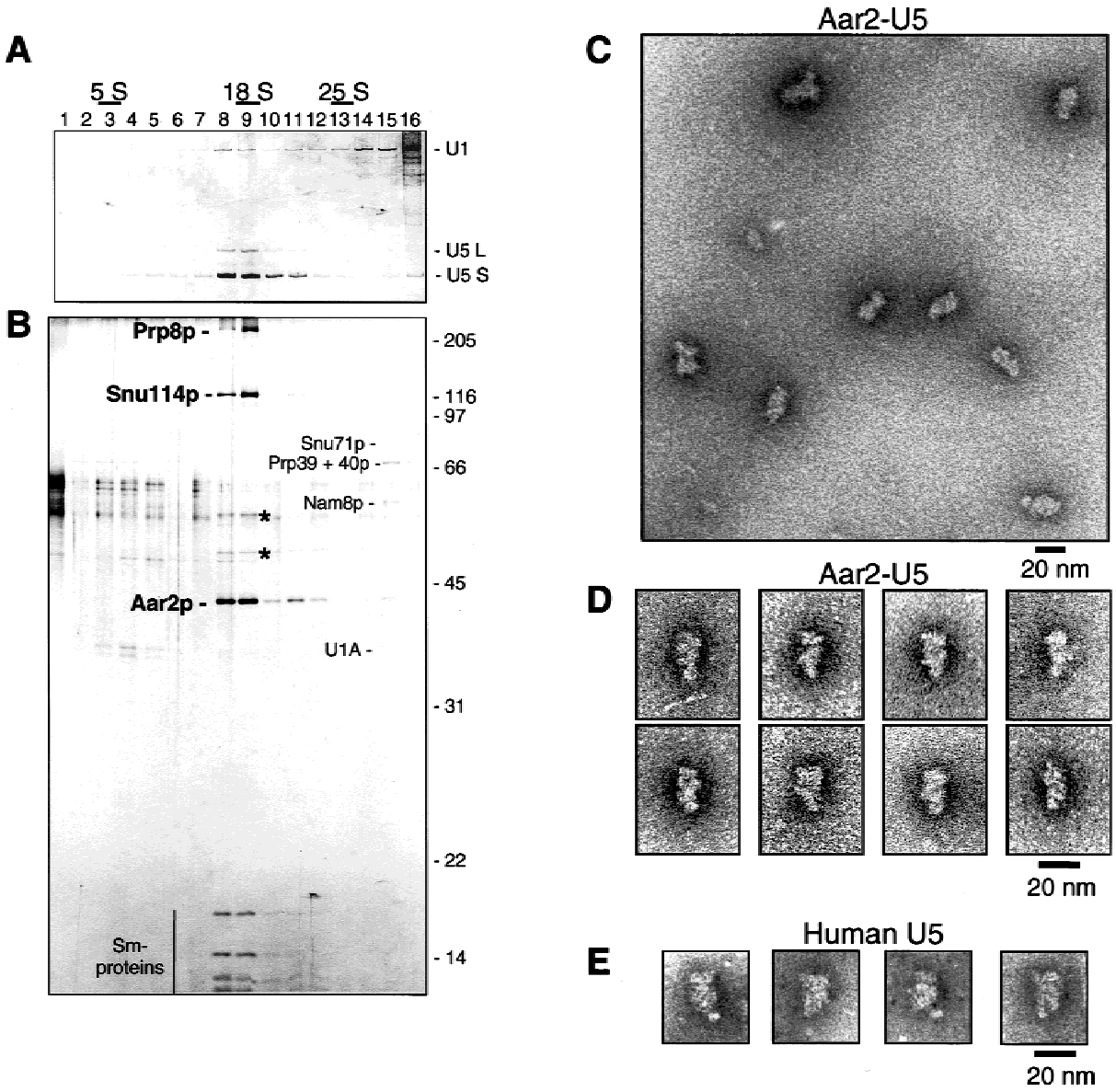

FIGURE 3. Electron microscopy of purified 16S yeast Aar2-U5 snRNPs. A U5/U1 snRNP containing fraction from the Nickel column was loaded onto a 10–30% glycerol gradient containing 50 mM KCl and separated in a Beckman TL-100 analytical ultracentrifuge in a TLS-55 rotor at 55,000 rpm for 3 h 40 min. RNAs (**A**) and proteins (**B**) present in each fraction were phenol extracted and analyzed. The identity of the snRNAs and proteins is indicated. Marked with asterisks are two faint protein bands that are most likely degradation products of Snu114p. We observed that the strain overexpressing histidine-tagged Aar2p from the *GAL1* promoter contained more U5S than U5L snRNA. Purified Aar2-U5 snRNPs obtained from fraction 8 of the gradient shown in **A** and **B** were adsorbed on carbon film, negatively stained, and used as samples for transmission electron microscopy. **C:** Typical overview of an area of the carbon film showing pure Aar2-U5 snRNPs in various projection classes. The size bar corresponds to 20 nm. **D:** Eight examples of Aar2-U5 snRNPs were picked and aligned in similar orientation for better comparison. The particles appear to consist of a head and a body domain that approximately correspond to one-third and two-thirds of the total mass of the particle. Several protrusions are apparent on the body domain, depending on the rotational orientation of the particle along its longitudinal axis. The size bar corresponds to 20 nm. **E:** Four panels showing the human 20S U5 snRNP (see text for details).

ing in vivo of additional precursors, that is, pre-U3A and B. We transformed in the Δ*aar2* background, an extra-chromosomal inducible allele of *AAR2* that was placed under the control of the *GAL1* promoter. Transcription from this promoter is repressed when the cells are grown in glucose and induced in galactose. We measured the levels of unspliced U3A and U3B transcripts by primer extension in cells grown in galactose and after the shift to glucose for 10 and 12 h, respectively. Figure 4A shows that unspliced pre-U3A and pre-U3B transcripts accumulate over background in cells grown in glucose for 10 h, but not in cells grown in galactose (Fig. 4A, lane 5 compare with the control, lane 2). Thus, deletion of *AAR2* leads to an in vivo splicing defect of at least

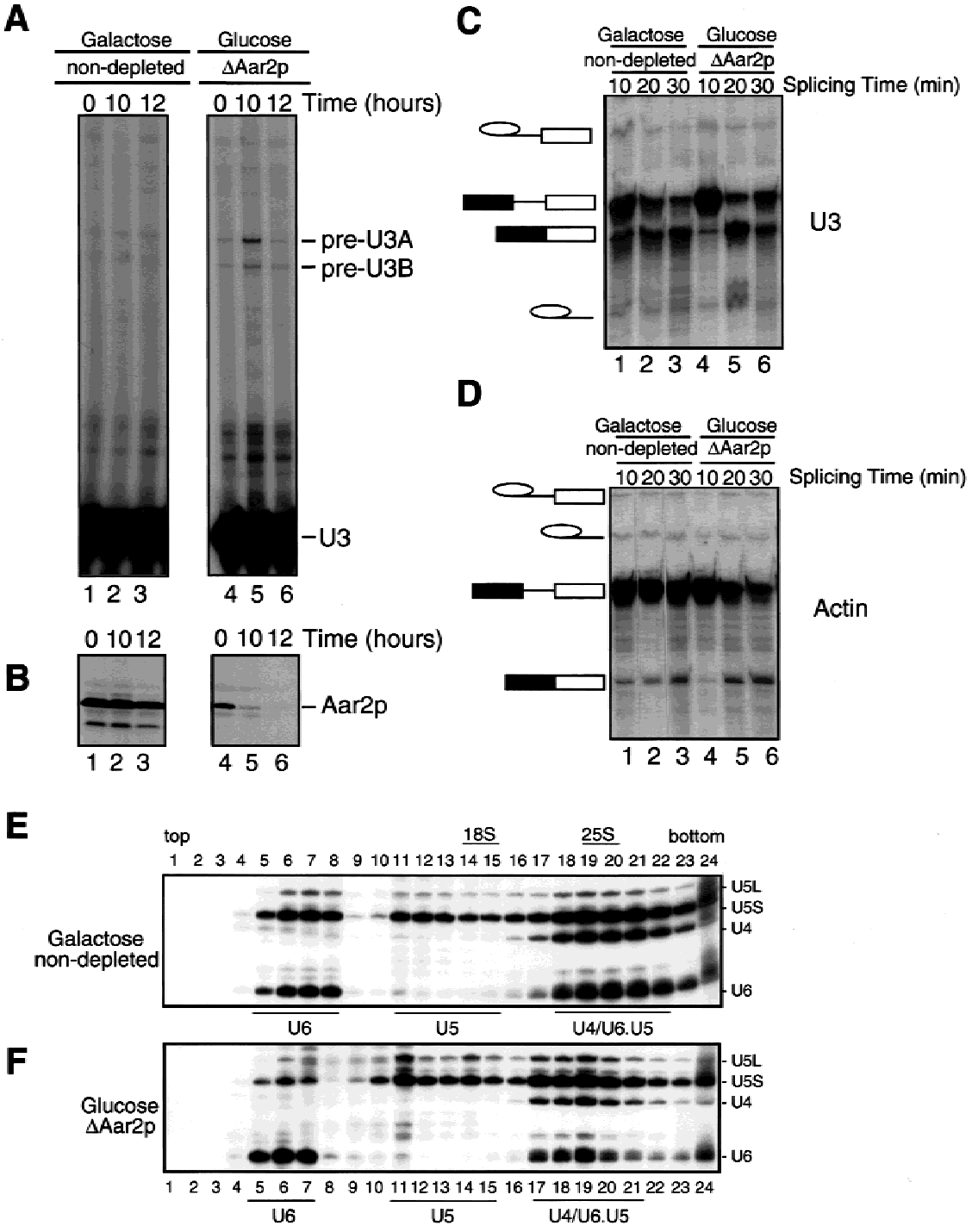

FIGURE 4. Depletion of Aar2p leads to a splicing defect in vivo but not in vitro. **A:** A yeast strain containing an inducible allele of *AAR2* was grown in galactose- or glucose-containing medium for the times indicated. Total RNA was prepared from the cells and assayed for the presence of pre-U3A, pre-U3B, and mature U3 transcripts by primer extension using a ^{32}P-labeled primer complementary to exon 2 of the U3A and B RNAs. The identity of the reverse transcripts is indicated on the right. The decrease in the level of pre-U3A and B transcripts in lane 6 is most likely due to their instability. **B:** Proteins were prepared from the same culture described in **A** and analyzed by western blot, using an antibody directed against Aar2p. **C** and **D:** Splicing reactions were performed at 25 °C using nondepleted (lanes 1–3) and ΔAar2p extracts (lanes 4–6) for the time indicated above each lane. Indicated on the left is the identity of the ^{32}P-labeled RNA-species for the U3 transcript, from top to bottom: intron-lariat-exon 2 intermediate, pre-mRNA, mature mRNA, and excised lariat-intron intermediate (**C**). For the actin transcript, from top to bottom: intron-lariat-exon 2 intermediate, excised lariat-intron, pre-mRNA, mature mRNA (**D**). **E** and **F:** To examine the profile of tri-snRNPs, nondepleted and ΔAar2p extracts were analyzed after glycerol gradient centrifugation for their RNA contents by northern blots, which were hybridized with probes for U4, U5, and U6 snRNAs. The sedimentation of U6, U5, and [U4/U6.U5] snRNPs is indicated. The U4/U6 snRNP is not well visible, most probably due to instability of its RNAs.

two additional precursors, pre-U3A and B. Western blot analysis using anti-Aar2p antibodies showed that the splicing defect correlated with the efficient depletion of the Aar2p protein (Fig. 4B, lanes 5 and 6, compare with lanes 2 and 3).

To study whether depletion of Aar2p had an influence on the splicing reaction in vitro, we prepared splicing extracts from the ΔAar2p strain 14 h after depletion and compared its ability to carry out splicing in vitro with that of the control extract for the time indicated in Figure 4C,D. We used two precursor substrates, U3 and actin, respectively. As can be seen in Figure 4C,D, depletion of Aar2p did not lead to a detectable decrease of splicing of either precursor (Fig. 4C,D, compare lanes 4 and 5 to 1–3). This result suggests that Aar2p is not required for conventional in vitro splicing of at least two precursors, U3 and actin, respectively. Because depletion of Aar2p does not inhibit in vitro splicing, tri-snRNP levels must be satisfactory in ΔAar2p extracts. To analyze this, we compared the amount of tri-snRNP in nondepleted and ΔAar2p extracts by glycerol gradient sedimentation (Fig. 4E,F). Although removal of Aar2p resulted in a reduction of 25S tri-snRNP (Fig. 4F), however, tri-snRNP levels are sufficient to support in vitro splicing, as shown in Figure 4C,D.

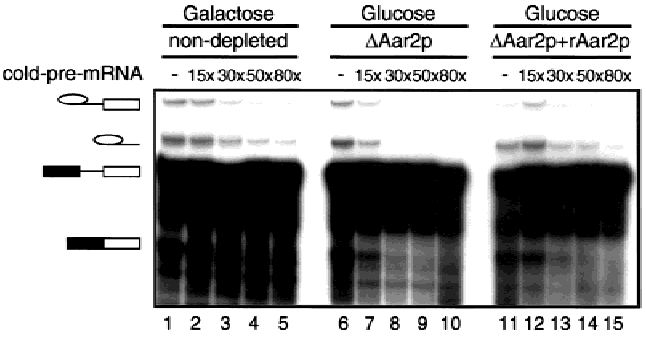

FIGURE 5. Depletion of Aar2p prevents later rounds of splicing. **A:** Time course of standard in vitro splicing reactions in nondepleted (lanes 1–5) and Aar2p-depleted extract, ΔAar2p (lanes 6–10). Nondepleted extract was prepared from strain AGY8 grown in galactose and ΔAar2p extract by shifting AGY8 to glucose for 14 h. To consume tri-snRNPs, splicing reaction mixtures contained various amounts of unlabeled actin pre-mRNA during the first incubation (15 min) and were supplemented with 0.3 nM high specific activity actin pre-mRNA during the second incubation (30 min). Concentrations of unlabeled pre-mRNA in reaction mixtures are indicated as fold excess cold pre-mRNA over 0.3 nM labeled pre-mRNA. Complementation of ΔAar2p extract splicing defect was obtained with 50 ng of recombinant Aar2p protein purified from yeast, rAar2p (lanes 11 to 15). Intermediates and products of splicing reaction from top to bottom are as follows: lariat-exon 2, excised lariat-intron, pre-mRNA, mature mRNA.

Depletion of Aar2p interferes with later rounds of splicing

Because Aar2p is not required for in vitro splicing and is not present in [U4/U6.U5] tri-snRNPs, we asked whether Aar2p could be a spliceosomal recycling factor involved in [U4/U6.U5] tri-snRNP formation from its U5 and U4/U6 components. If one function of Aar2p is to refurnish U5 snRNPs for spliceosome assembly, then Aar2p should be dispensable for in vitro splicing as long as [U4/U6.U5] tri-snRNP levels are satisfactory. Thus, the recycling function of Aar2p may be hidden in the typical assay. As shown above, in vitro splicing reactions progress normally in ΔAar2p extracts. Usually snRNP proteins that are essential for splicing in vivo are essential for splicing in vitro, with the exception of Prp24p, which is required for snRNP recycling (Raghunathan & Guthrie, 1998). To test Aar2p function in snRNP recycling competence, [U4/U6.U5] tri-snRNPs in the extract must be consumed. We used an in vitro splicing assay that is sensitive to a recycling defect (Fig. 5; Raghunathan & Guthrie, 1998). To drive most of the [U4/U6.U5] snRNPs through a round of splicing, we added unlabeled pre-mRNA to splicing reactions. Eventual splicing activity was monitored by adding labeled pre-mRNA in a subsequent incubation. When Aar2p was present, even an 80-fold excess of unlabeled pre-mRNA did not fully impede later rounds of splicing (Fig. 5, lane 5). In contrast, incubation of ΔAar2p extract with a 30-fold excess of unlabeled pre-mRNA significantly decreased subsequent rounds of splicing (Fig. 5, lane 8). Aar2p was the crucial component because recombinant Aar2p partially complemented the ΔAar2p splicing defect (Fig. 5, lanes 13–15). Thus, although Aar2p is not required for conventional in vitro splicing, it has an effect when splicing depends on snRNP recycling.

DISCUSSION

In this work, we describe the isolation and characterization of a 16S form of the yeast U5 snRNP, named Aar2-U5, which is consistently coisolated with the U1 snRNP. This work provides several interesting insights into the function and significance of this form of U5 snRNP.

Database searches using the Aar2p sequence identified likely orthologs in a wide variety of organisms, including *Homo sapiens* (gb NP056326), *Arabidopsis thaliana* (gb AC074025), *Schizosaccharomyces pombe* (pir T38750), *Caenorhabditis elegans* (pir T20689), and *Drosophila melanogaster* (gb AAF55521). Aar2p is evolutionarily conserved between humans, plants, fission yeast, nematodes, and flies, with approximately 25% identity and 40% similarity (data not shown). The conservation of Aar2p, Prp8p, and Snu114p among species suggests that a U5 form similar to the yeast Aar2-U5 snRNP is evolutionarily conserved as well and plays a conserved role in splicing.

One point to consider is whether the interaction between the Aar2-U5 and U1 snRNPs is functionally significant. The literature provides several well-documented examples in support of such an interaction, although it

was never determined or examined which form of U5 interacts with the U1 snRNP. An interaction between U1 and U5 snRNPs has been observed early during formation of the mammalian spliceosome in vitro and the U1 and U5 snRNAs can even be crosslinked to each other (Ast & Weiner, 1997b). In addition, it is also known that the U1 snRNP proteins Prp40p, Prp39p, and the U5 snRNP protein Prp8p interact (see Abovich & Rosbash, 1997; Gottschalk et al., 1998; van Nues & Beggs, 2001). In both nematode and HeLa cell extracts, an early interaction was discovered between the U5 protein Prp8p and the 5′ splice site, in addition to the U1 snRNP, which was not dependent on prior binding of the U2 snRNP to the branch point (Maroney et al., 2000). Although the Prp8/5′ splice interaction occurred when the U5 snRNP was a constituent of the tri-snRNP, these findings led to the conclusion that the U1 and U5 snRNPs functionally collaborate to recognize and define the 5′ splice site (Maroney et al., 2000). Taken together, these data are consistent with a functional significance of the physical U1/U5 interaction at early steps of spliceosome assembly and in 5′ splice site recognition. This interaction may be strong enough to exist in the absence of pre-mRNA and spliceosomes, thus leading to the coisolation of U1 and U5 snRNPs. Surprisingly, we could not see this interaction by coimmunoprecipitation experiments using antibodies directed either against U1 or U5 snRNP proteins (see Fig. 1 and Gottschalk et al., 1999, and data not shown). One possible explanation is the presence of detergent in the buffers used for immunoprecipitation or the antibody itself, which may disrupt the contact between the U1 and U5 snRNPs. This contact may also be destroyed during gradient centrifugation, as we have not detected a U1/U5 di-snRNP with a sedimentation coefficient above 18S, which would be expected combining two particles with S values of 16 and 18, respectively.

Is the Aar2-U5 a specialized form of the U5 snRNP? The Aar2-U5 has a simpler protein composition than the human U5 snRNP. However, it contains in addition to Aar2p, two evolutionarily highly conserved proteins, Prp8p and Snu114p, which are both of critical importance for several steps of the splicing pathway. One possibility is that the Aar2-U5 snRNP functions exclusively together with the U1 snRNP to regulate splicing of a subset of pre-mRNAs. If this is correct, it would mean that the only specific proteins that are needed to accomplish this regulation are Prp8p, Snu114p, and Aar2p. Aar2p was originally identified in a screen for mutants affecting the repression of haploid-specific genes in diploid cells, which resulted in a defect in splicing of the two short introns of the *MATa1* pre-mRNA (Nakazawa et al., 1991). Because not all phenotypes of the *aar2* mutant could be repressed by the introduction of an intronless *MATa1* gene, it was assumed that Aar2p might have additional functions other than facilitating proper splicing of the *MATa1* pre-mRNA. Nakazawa et al. (1991) have also shown that the *aar2* mutant does not exhibit a defect in splicing of actin pre-mRNA. However, here we have shown that genetic depletion of Aar2p leads to an in vivo splicing inhibition of the pre-U3A and B introns. Although we did not analyze whether splicing of additional introns is inhibited upon depletion of Aar2p, in yeast there are plenty of genes known to have one or more introns (Ares et al., 1999). Some of these genes may be selectively affected by depletion of Aar2p. Splicing regulation generally takes place at the level of splice site recognition by the U1 and perhaps the U5 snRNPs. As discussed above, there is growing evidence that the early interaction between the U1 and U5 snRNPs may play a role in directing U5 snRNPs to the 5′ splice sites (Ast & Weiner, 1997a, 1997b; Maroney et al., 2000). This might explain how the Aar2p protein (most probably in the context of the U5 snRNP) could specifically regulate splicing of the *MATa1* pre-mRNA or of additional selected introns. Another means by which Aar2p could influence splicing of mating-type-dependent pre-mRNAs would be a mating-type-regulated expression of Aar2p, as it could then differentially affect the composition of U5 snRNPs in a mating-type-specific manner. However, we can most likely rule out this possibility. Aar2p is expressed equally in cells of all three mating types, as judged by western analysis (data not shown). Also, equal amounts of U5 snRNPs were coprecipitated from extracts made from a- or α-mating-type cells (data not shown).

An additional possibility is that the Aar2-U5 snRNP is just a “core” particle. Because only two of the tri-snRNP proteins appear to be stably associated with the Aar2-U5 (Prp8p, Snu114p), we may consider it a core or precursor particle to the mature U5 snRNP. In this scenario, some of the remaining yeast tri-snRNP proteins (for example, Brr2p and Dib1p) most likely must associate with the core Aar2-U5 to form a mature yeast U5 snRNP (similar to the human U5 snRNP), which subsequently assembles with the U4/U6 snRNPs and with additional specific proteins to form the tri-snRNP. Interestingly, Stevens et al. have isolated U5 snRNPs with a protein composition remarkably similar to the 20S human snRNP, by tagging Brr2p (S.W. Stevens, I. Barta, H.Y. Ge, R.E. Moore, M.K. Young, T.D. Lee, & J. Abelson, pers. comm.). These data suggest that the U5 snRNP exists in at least two forms in yeast cells.

Aar2p is present in the 16S U5 snRNP, but not in the “mature” [U4/U6.U5] tri-snRNP. As discussed above, one possibility is that the Aar2p-U5 snRNP is an intermediate particle during U5 snRNP biogenesis. As a consequence, Aar2p may be involved, directly or indirectly, in the in vivo formation of the U5 snRNP prior to its engagement to the spliceosome. Subsequently, Aar2p would then leave U5 when additional protein factors join to form a canonical U5 snRNP. In support of this

latter possibility, we found that Aar2p, although essential for splicing in vivo, is not essential in vitro. That Aar2p is not essential in vitro has been observed reproducibly with both actin and U3 precursors. In fact, Aar2p is not associated with the spliceosome, the pre-mRNA, splicing intermediates, and the resulting products, as we could not coprecipitate any of these molecules from splicing reactions using anti-Aar2p antibodies (data not shown). Aar2p was also not detected in spliceosomal complexes that were affinity purified together with biotinylated pre-mRNA, ruling out the possibility that Aar2p may be inaccessible to antibodies in the spliceosome (data not shown). Consistent with these findings, depletion of Aar2p does not lead to an in vitro splicing defect (Fig. 4C,D). The splicing activity of an extract containing Aar2p did not differ from that of the Aar2p-depleted extract, unless "multiple-turnover" conditions were chosen. Only in the latter case, the Aar2p-depleted extract exhibited an impaired splicing activity (in comparison to the extract containing Aar2p) that could be reversed, at least in part, by the addition of recombinant Aar2p (Fig. 5). Thus, Aar2p was the critical component and, although it is not required for traditional in vitro splicing, it may be necessary when splicing depends on snRNP recycling.

The simple composition of the yeast Aar2-U5 snRNP together with the electron microscopic structure allow us to draw some conclusions about the structural organization of this particle. It is known that the core proteins bind to the Sm site of U5 snRNA; however the characteristic ring-shaped structure of the core domain is not visible by electron microscopy of the particle. The lack of visible core structures in the yeast U5 particle is in good agreement with the ultrastructure of human 20S U5 snRNPs, where the Sm core domain is also not detected (Kastner et al., 1990). This suggests that the Sm core must be hidden in the inner part of the particle and most probably is in close contact with other proteins in the U5 snRNP. As our biochemical analysis has demonstrated, these proteins must be Prp8p and Snu114p. This is consistent with findings from other studies. Dix and collaborators (1998), showed that Prp8p and Snu114p can be site-specifically crosslinked to the U5 snRNA at the conserved loop I, internal loop 1 (Prp8p), and at the internal loop 2 (Prp8p and Snu114p). In addition, they found three crosslinks to small proteins of 30, 16, and 10 kDa that required the presence of an intact Sm site; therefore, these proteins may be Sm proteins. Because the internal loop 1 is near to the Sm site and Prp8p binds this loop, it is possible that Prp8p contacts also the Sm core. It seems likely that these two specific proteins found in the 16S Aar2-U5 snRNP are the only ones that directly bind the U5 RNA. Aar2p most likely does not contact the U5 snRNA directly or extensively, as recombinant GST-Aar2p is unable to pull down in vitro-transcribed U5 snRNA (data not shown).

It is also remarkable that the electron microscopic structure of the yeast Aar2-U5 snRNP appears similar to that of the human 20S U5 snRNP (compare to Kastner et al., 1990). Both particles show a head/body structure of similar appearance. This is interesting, because the human U5 snRNP contains several additional proteins. Thus, the U5 RNA, Prp8p, Snu114p, and the Sm proteins alone are capable of forming the main scaffold of the yeast Aar2-U5 particle, accounting for the typical U5 snRNP shape. This scaffold may contain several cavities that accommodate the additional homologous proteins present in the human U5 snRNP without changing the overall appearance of the basic scaffold.

MATERIALS AND METHODS

Yeast strains, oligonucleotides, and plasmids used in this study

To purify Aar2p and the associated U5 snRNP, we created a strain expressing Aar2p from the *GAL1*-promoter with a C-terminal deca-histidine tag. First, the *AAR2* gene including the flanking regions between the stop and start codons of the upstream and downstream genes, respectively, was amplified from yeast genomic DNA by PCR using two primers (AAR2-gen-up: 5′-GGCCCCTCGAGTAGATGAAATGAAAATAC-3′; AAR2-gen-low: 5′-GGCCCGAGCTCTTTTCTTTGTAGCGTTTA-3′) and subsequently cloned into vector pRS306 between the *Xho*I and *Sac*I restriction sites. From the coding region of *AAR2*, 616 nt were removed (between unique *Sph*I and *Eco*47III restriction sites) and substituted with the *URA3* marker gene, which was amplified from genomic DNA using two primers (URA3-up-SphI: 5′-GGCGGGCATGCACGCTTTTCAATTCAATT-3′; URA3-low-Eco47III: 5′-GGCGGAGCGCTAGGGTAATAACTGATATA-3′). DNA containing the *aar2*Δ*::URA3* deletion allele was cut from the resulting plasmid and transformed into strain TR1 (*MATa/MATα, trp1*-Δ*/trp1*-Δ*, his3*-Δ*/his3*-Δ*, ura3-52/ura3-52, lys2-801/lys2-801, ade2-101/ade2-101*). Sporulation and tetrad dissection of the resulting strain AGY5 (genotype as TR1, but containing *AAR2/aar2*Δ*::URA3*) resulted in the formation of only two viable spores per tetrad. The coding region of *AAR2* was amplified by PCR from genomic DNA using a 5′ primer that includes a *Bam*HI restriction site and the start codon (AAR2-codreg-up: 5′-GGCCGGGATCCATGAATACTGTACCATTT-3′), as well as a 3′ primer that omits the *AAR2* stop codon and encodes 10 histidines followed by a stop codon and a *Xho*I restriction site (5′-GCCGCCTCGAGTTAGTGATGGTGATGGTGATGGTGATGGTGATGTGGCCTTTGGTAATAGAG-3′). This PCR product was cloned into a vector originating from pRS314 (*ARS, CEN, TRP1*), but containing one copy of the *GAL1* gene in front of a polylinker and a terminator region, between *Bam*HI and *Xho*I sites. The resulting plasmid was transformed into strain AGY5, the cells were sporulated and tetrads dissected on plates containing galactose as the only carbon source. Some of the tetrads gave rise to four viable colonies. Replica plating showed cosegregation of the *URA3* and *TRP1* marker genes, demonstrating that the plasmid-encoded Aar2p-10xHis protein could rescue the lethality of the *aar2*Δ*::URA3* knockout allele. This strain, AGY8 (*trp1*-Δ*, his3*-Δ*, ura3-52, lys2-*

801, ade2-101, aar2Δ::URA3+pRS314-GAL1::AAR2-10xHis, ARS, CEN, TRP1), was isolated and used for the subsequent purification of the U5 snRNP. In vivo accumulation of unspliced pre-U3A and pre-U3B RNA transcripts was assayed by primer extension as described previously (Fabrizio et al., 1997).

Production of antibodies against Aar2p and coprecipitation experiments

The *AAR2* coding region was amplified from genomic DNA by primers AAR2-codreg-up (see above) and AAR2-codreg-low (5′-GGCCGCTCGAGTTATGGCCTTTGGTAATA-3′) and cloned into vector pGEX-4T1 (Amersham-Pharmacia) between *Bam*HI and *Xho*I restriction sites. The resulting plasmid was transformed into *E. coli* strain BL21 and used to overexpress a fusion protein of glutathione-S-transferase (GST) and Aar2p. The GST-Aar2p fusion protein was purified from crude cell lysates according to the instructions of the manufacturer and used for the immunization of a rabbit. The polyclonal antiserum obtained was specific for Aar2p, as tested by western analysis and ECL detection (Amersham-Pharmacia) of total yeast proteins. The protein could be well detected when the serum was diluted 1:4,000. For immunoprecipitation experiments, 5 μL of anti-Aar2p antiserum was coupled to 20 μL of protein-A sepharose in 500 μL of buffer NET-2 150 (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40) for 1.5 h at 4 °C and subsequently washed with 3 times 1 mL of NET-2 150. Then, 20 μL of splicing extract from a wild-type strain, prepared by combining the methods of Lin et al. (1985) and Umen and Guthrie (1995), were added in 500 μL NET-2 150. Aar2p and associated snRNPs were precipitated for 2 h at 4 °C, the beads were subsequently washed three times with 1.5 mL of NET-2 buffer containing 150, 450, or 700 mM NaCl, proteinase K digested, and extracted with phenol/chloroform/isoamylalcohol (50/49/1). Preimmuneserum of the same rabbit was used for precipitation as a negative control, and 10 μg of H20 antibodies as a positive control. The isolated RNAs were separated on an 8% polyacrylamide gel containing 6 M urea, blotted, and UV crosslinked for 3 min to nylon membrane (Quiagen) and then probed with uniformly labeled DNA probes specific for the U1, U2, U4, U5, and U6 snRNAs. Glycerol gradient centrifugation of total yeast extract and analysis of the sedimentation profile of Aar2p was performed as described in Gottschalk et al. (1998).

Purification of U5 snRNPs, glycerol gradient centrifugation, mass spectrometric identification of Aar2p, and immunological detection of Dib1p

The U5 snRNP was purified from AGY8 cell extracts, as previously described (Neubauer et al., 1997). Briefly, total-cell extract was prepared, which was then subjected to the two-step affinity purification in buffers containing 200 mM KCl. First, a mixture of all snRNPs was obtained from an immunoaffinity column containing antibodies specific for the m_3G cap present on the snRNAs. Depending on the salt concentration, different sets of snRNPs bind to this column. The free U5 snRNP binds better in the presence of 200 mM KCl, whereas the tri-snRNP is isolated with higher yields in 100 mM KCl. The isolated snRNPs were then applied to the second affinity (Nickel-NTA) column and the U5 snRNP was eluted with 50 mM imidazole. The U5/U1 particles were further separated by glycerol gradient centrifugation and analyzed as previously described (Gottschalk et al., 1999). The mass spectrometric analysis of the proteins of the U5 snRNP was as previously described (Neubauer et al., 1997). The Aar2p peptides sequenced are the following: NINLTGSFYLPKNIETGR, AYNEISGLQGDQFPR. Dib1p was detected by western analysis performed with equal amounts of purified Aar2-U5 snRNP and [U4/U6.U5] tri-snRNP proteins, respectively, using an antiserum raised against the human U5-15kD protein at a dilution of 1:2,500 (Reuter et al., 1999).

Electron microscopic analysis of purified Aar2-U5 snRNPs

Samples of the Aar2-U5 snRNP were prepared as previously described in Fabrizio et al. (1994). Purified Aar2-U5 snRNPs obtained from fraction 8 of the gradient shown in Figure 3A,B were adsorbed on carbon film, negatively stained with 25% uranyl-formate, covered with a second carbon film, mounted on a copper microgrid and used as samples for TEM. Micrographs were obtained on a Philips CM 120 electron microscope at a magnification of 80,000-fold.

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The yeast U5 snRNP coisolated with the U1 snRNP has an unexpected protein composition and includes the splicing factor Aar2p.

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