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Mutagenesis Suggests Several Roles of Snu114p in Pre-mRNA Splicing*

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Snu114p, a yeast U5 small nuclear ribonucleoprotein (snRNP) homologous to the ribosomal GTPase EF-2, was recently found to play a part in the dissociation of U4 small nuclear RNA (snRNA) from U6 snRNA. Here, we show that purified Snu114p binds GTP specifically. To test the possibility that binding and hydrolysis of GTP by Snu114p are required to stimulate the unwinding of U4 from U6, we produced several mutations of Snu114p. Residues whose mutations led to lethal phenotypes were all clustered in the P loop and in the guanine-ring binding sequence (NKXD) of the G domain, which in elongation factor-G is required for the binding and hydrolysis of GTP. An arginine residue in domain II, which in EF-G forms a salt bridge with a residue of the G domain, when mutated in Snu114p (R487E), led to a temperature-sensitive phenotype. The substitution D271N in the NKXD sequence is predicted to bind XTP instead of GTP. Spliceosomes containing this mutant, isolated by affinity chromatography after heat treatment, retained U4 snRNA paired with the U6 snRNA. U4 snRNA was released efficiently only when these arrested spliceosomes were reactivated by lowering the temperature in the presence of a mixture of ATP and XTP. Because nonhydrolyzable XTP analogues did not consent the release of U4, we conclude that the release requires hydrolysis of XTP. This suggests that Snu114p needs GTP to influence, directly or indirectly, the unwinding of U4 from U6. An additional role for Snu114p is also demonstrated: after growth of the D271N and R487E strains at high temperatures, we observed decreased levels of the U5 and the U4/U6·U5 snRNPs. This indicates that, before splicing, Snu114p plays a part in the assembly of both particles.

Splicing of mRNA precursors (pre-mRNA) proceeds by two consecutive transesterification steps that are catalyzed by the spliceosome. The spliceosome is formed by the ordered interaction of the U1, U2, U5, and U4/U6 snRNPs[1] (in which the U4 and U6 RNAs are base paired) and of several splicing factors, with the pre-mRNA (1). The spliceosome is assembled by the initial interaction of the U1 snRNP with the 5′ splice site, thereafter the U2 snRNP recognizes and binds to the branch site, forming the pre-spliceosome. Spliceosome assembly is completed by the subsequent association of the U4/U6 and U5 snRNPs in the form of a U4/U6·U5 tri-snRNP complex.

Activation of the assembled spliceosome into a catalytically functional machine requires extensive reorganization of the components. Thus, during spliceosome activation, base-pairing between U4 and U6 is disrupted and a new base-pairing between U2 and U6 occurs (reviewed in Ref. 2). Concomitantly with these events, base-pairing of U1 with the 5′ splice site is exchanged for base-pairing between U6 and the 5′ splice site (3, 4). After these rearrangements, U1 and U4 snRNPs are released from the spliceosome prior to catalysis. The conserved loop I of U5 RNA makes contact with exonic sequences at the 5′ and 3′ splice sites, while the splicing reaction proceeds (5).

The unwinding of the U4/U6 snRNA duplex is an important step toward the activation of the spliceosome and depends on the functioning of a large number of protein factors. In addition to the putative U4/U6 helicase Brr2p, five splicing factors in yeast have already been implicated in the release of U4 snRNA during spliceosome activation. These are the U4/U6 snRNP protein Prp4p (6), the non-snRNP protein Prp19p (7), the tri-snRNP protein Prp38p, and the U5 snRNP proteins Prp8p and Snu114p (8–11).

Previously we showed that the G domain-containing protein Snu114p and its human orthologue protein U5–116K are close homologues of the ribosomal elongation factor EF-2 (10). Consistently with this, the U5–116K protein cross-links specifically to GTP in purified U5 snRNP particles, and this cross-linking is stimulated by poly(U) (10). Remarkably, as predicted by its homology with EF-2, we found that Snu114p is involved in rearranging the spliceosomal RNP structure. Indeed, a temperature-sensitive deletion mutant of Snu114p (snu114ΔN), led to the accumulation of arrested, precatalytic spliceosomes in which U4 is still base-paired with the U6 snRNA. This suggested that Snu114p is involved in the transition to an active form of the spliceosome by influencing factors that are directly required for the unwinding of the U4/U6 duplex (11).

As described above, Snu114p is involved in this rearrangement step together with several additional factors, and there is increasing evidence for a link between Snu114p and some of these factors, for example, between Prp8p and the putative U4/U6 helicase Brr2p. The most direct evidence for this link is the strong RNA-independent protein-protein interaction that has been detected in the human system between the proteins 220K/Prp8p, 200K/Brr2p, and 116K/Snu114p after dissociation of these proteins as a stable complex from the U5 snRNP in high salt buffer (12). In yeast, several studies have shown that Snu114p is in contact with Prp8p and that Prp8p interacts with Brr2p (13, 14). Therefore, we had hypothesized (11) that Snu114p is a crucial factor, acting in an earlier step to trigger

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[1] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; XTP, xanthosine triphosphate; XMP-PNP, xanthosine-5′-[(β,γ)-imido]triphosphate; Ni-NTA, nickel-nitrilotriacetic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); EF-G, elongation factor G.

the function of Prp8p/220K and/or of the Brr2p/200K RNA unwindase, in this intricate network of factors involved in the U4/U6 unwinding during spliceosome activation.

To define the details of the reaction that Snu114p catalyzes in concert with other factors, and primarily to test the possibility that binding and hydrolysis of GTP by Snu114p are required for spliceosome activation, we replaced or deleted several amino acids in evolutionarily conserved domains of Snu114p. We found that residues that led to lethal phenotypes are all clustered in the G domain, more precisely, in the P loop and in the guanine-ring-binding sequence (NKXD), which in EF-G are required for GTP binding and hydrolysis (15, 16). We also found that an arginine in domain II, which in EF-G forms a salt bridge with a residue of the G domain, when mutated in Snu114p (R487E), led to a weakly viable, temperature-sensitive phenotype even at 25 °C. Surprisingly, all of the point mutations made in the other domains did not lead to any significant effect, except for a residue in domain IV.

We also replaced aspartic acid 271 with asparagine, in the NKLD sequence of the G domain of Snu114p, to give the mutant snu114-D271N. An analogous substitution in several GTPases changes their specificity of binding and hydrolysis from GTP to xanthosine triphosphate (XTP) (17). Because this mutant does not lead to a lethal phenotype, we tried to address the interesting question of whether GTP hydrolysis (XTP in this case) is required to trigger the unwinding of U4 from U6. This mutant should allow the identification of the XTP-dependent function of mutated Snu114p and the characterization of this function in a crude extract containing a variety of ATPases, some of which are known to bind and hydrolyze GTP as well.

As a prerequisite to these studies, we determined first that Snu114p, similar to its human orthologue, binds GTP *in vitro* in a specific manner, as analyzed by UV cross-linking. Thus, we show that Snu114p is a genuine GTP-binding protein. Next, we studied the D271N mutant in more detail, and found that this mutant leads to a slow growth phenotype that at high temperatures results in arrested splicing *in vivo*. Importantly, we show that heat treatment of the D271N extract leads also to a defect of splicing *in vitro* that is reversed by lowering the assay temperature. We establish that the *in vitro* thermal inactivation results in accumulation of arrested spliceosomes in which U4 is not released, because it is still base-paired with the U6 snRNA. These precatalytic complexes are isolated by affinity chromatography, depleted of residual nucleoside triphosphates (NTPs), and finally reactivated by adding back a mixture of ATP/XTP and by lowering the assay temperature. Using this system, we show that U4 is released efficiently from the spliceosome only when a mixture of ATP (required for the helicases) and hydrolyzable XTP (required by snu114-D271N) is added. Thus, these experiments suggest that snu114-D271N needs hydrolyzable XTP (and wild-type Snu114p needs GTP) to influence the unwinding of U4 from U6.

We also found an additional role for Snu114p. Growth of the D271N and R487E mutant strains at high temperatures leads to extracts that have reduced levels of U5 and tri-snRNP particles, thus suggesting that Snu114p is, directly or indirectly, involved in maintaining proper amounts of U5 and tri-snRNP as well.

EXPERIMENTAL PROCEDURES

Glycerol Gradient Centrifugation—2.5 mg of yeast splicing extract was diluted to reduce the concentration of glycerol from 20% to 8% and loaded on a 10–30% (w/w) glycerol gradient containing 20 mM Hepes (pH 7.9), 200 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 108,000 × *g*, for 18 h at 4 °C, the gradients were fractionated into 24 fractions of 500 μl. Each fraction was phenol-chloroform-extracted, and their RNA composition was analyzed by Northern blotting. The proteins were acetone-precipitated and assayed by Western blotting.

Yeast Strains—Strains were YPF39, *trp1-Δ1*; *his3-Δ*; *ura3–52*; *lys2–801*; *ade2–101*; *snu114Δ::HIS* (*TRP1 pRS424-GPD1/SNU114*, 6his N-terminal, *ARS*, *CEN*); YPF37, *trp1-Δ1*; *his3-Δ*; *ura3–52*; *lys2–801*; *ade2–101*; *snu114Δ::HIS* (*TRP1 pRS314/snu114D271N*, 6his N-terminal, *ARS*, *CEN*); and YCK13, *trp1-Δ1*; *his3-Δ*; *ura3–52*; *lys2–801*; *ade2–101*; *snu114Δ::HIS* (*TRP1 pRS314/snu114R487E*, Flu-tag C-terminal, *ARS*, *CEN*).

Mutagenesis—Most of the mutations in the *SNU114* gene were introduced by the QuikChange site-directed mutagenesis method (Stratagene), as described previously (10). Two oligonucleotide primers, each complementary to opposite strands of *SNU114* and containing the desired substitution, were designed. The PCR cycling parameter suggested by the QuikChange method was employed, using native *Pfu* DNA polymerase. The mutant allele was screened for the desired mutation by sequencing. A PCR-based strategy was used to delete 162 amino acids of domain IV, from position 700 to 862. Plasmids *pRS314/SNU114*, containing the desired substitution or deletion, were sequenced and separately transformed into strain YPF8. The plasmid-shuffling strategy was applied (18). After selection at 25 °C in medium lacking tryptophan, transformants were streaked once on medium lacking tryptophan and grown at 25 °C. Patches were streaked three times on 5-fluoroorotic acid plates to select for cells lacking of the *URA3* plasmid. Cells that survived on 5-fluoroorotic acid plates were streaked on rich medium, and their growth phenotypes were analyzed by incubating cells and dilutions (5×10^4, 5×10^3, and 5×10^2 at 37, 30, 25, and 17 °C for 1–2 days or longer). A good temperature-sensitive strain was one that did not grow in -Ura plates at any of the temperatures and, in addition, did not grow in rich medium at 30 or 37 °C but grew at 25 °C.

Antibody Production—The *SNU114 Bam*HI *SnabI* fragment from plasmid *pBSIISK*(−)*/SNU114*, was cloned into vector *pQE-30* (Qiagen) between *Bam*HI and *Sma*I restriction sites. The resulting plasmid was transformed into *Escherichia coli* strain M15 (pREP4) and used to overexpress a histidine tag containing Snu114p fragment of 59.5 kDa bearing the N-terminal acidic domain, the G domain, and part of domain II. The protein was purified from crude cell lysates by Ni-NTA chelate chromatography, according to the instructions of the manufacturer (Qiagen), and used to immunize a rabbit. The polyclonal antiserum obtained was specific for Snu114p, as tested by Western blot analysis and ECL detection (Amersham Biosciences) of total yeast snRNPs and purified Snu114p. The protein could be clearly detected when the serum was diluted 1:5000.

Purification of Snu114p—His-tagged Snu114p was purified from YPF39 extracts by Ni-NTA chelate chromatography according to the protocol supplied by Novagen. The binding buffer contained 20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole (pH 8.0). In the wash buffer the imidazole concentration was increased to 10 mM. In a second washing step, 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole were used instead. Elution was performed with the latter buffer containing in addition 10% glycerol and 50, 100, 150, 200, or 250 mM imidazole. Most of Snu114p was eluted between 100 and 150 mM imidazole. To the eluted protein, glycerol and β-mercaptoethanol were added to give a final concentration of 20% and 20 mM, respectively. For further purification, the peak fractions of the nickel column eluate were loaded separately on a Superdex G-200 column (Amersham Biosciences). The running buffer contained 50 mM NaH_2PO_4 and 300 mM NaCl. Fractions of 100 μl were collected, and a 30-μl aliquot of each fraction was analyzed by SDS-PAGE.

GTP Cross-linking Assays and Immunoprecipitation—15 pmol of purified Snu114p was incubated in 25 μl of 50 mM Tris-HCl (pH 8.0), 150 mM KCl, 1.3 mM dithiothreitol, 2.5 mM $MgCl_2$, and 0.6 μg/μl poly(U) with 20 μCi (6.6 pmol) of [α-^{32}P]GTP (Amersham Biosciences, specific activity, 3000 Ci/mmol) for 5 min at room temperature. The subsequent UV cross-linking reaction was performed essentially as described previously (19), except that the cross-linking time was extended to 7 min with the sample at a distance of 4.5 cm from the UV lamp. The cross-linked proteins were then immunoprecipitated with the anti-Snu114p antibody or non-immune serum. For the immunoprecipitations, 5–10 μl of anti-Snu114p antisera were coupled to 20 μl of protein A-Sepharose in 500 μl of buffer NET2–150 (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) for 1.5 h at 4 °C and subsequently washed with three times 1 ml of NET2–150. The cross-linked samples or splicing extracts (400 μg of total proteins, see Figs. 3*C* and 5*A*) were added together with 500 μl of NET2–150 buffer to the washed beads, and Snu114p was precipitated from the reactions for 2 h at 4 °C. The beads were subsequently washed four times with 1.5 ml of NET2–150 and

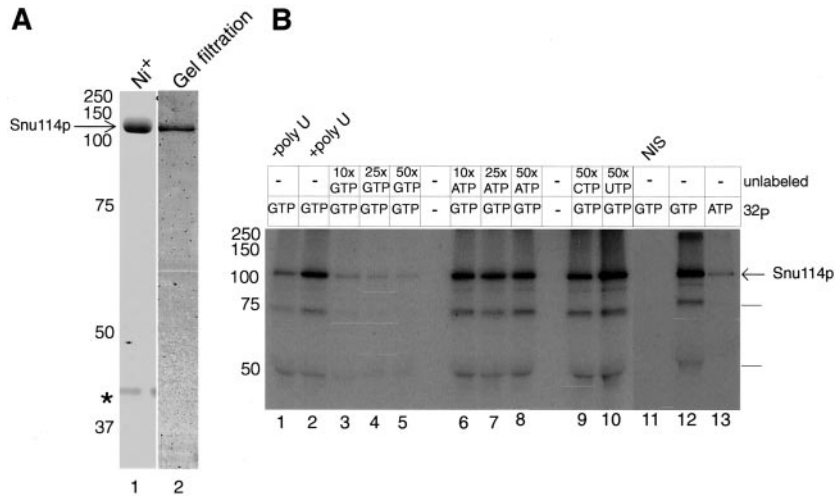

FIG. 1. **Purified Snu114p binds GTP specifically.** *A*, SDS-PAGE of purified Snu114p, which was overexpressed in yeast and purified via Ni-NTA chromatography (Ni^{2+}, *lane 1*) and size-exclusion chromatography (gel filtration, *lane 2*). For visualization the gel was stained with Coomassie Blue. The position of Snu114p is indicated with an *arrow*. The purity of the protein preparation was checked by mass spectrometry, which confirmed that Snu114p is 90–95% pure. The *asterisk* indicates a frequently detected contamination (alcohol dehydrogenase-1-P). In addition, traces of glyceraldehyde-3-phosphate dehydrogenase, cyclin-dependent protein kinase 33, and alkaline phosphatase 8 were found. *B*, binding of GTP to purified Snu114p. Snu114p was incubated with radioactively labeled GTP (*lanes 1–12*) or ATP (*lane 13*) in the absence or presence of 10× (66 pmol), 25× (165 pmol), and 50× (330 pmol) excess of unlabeled nucleotides. After cross-linking Snu114p was immunoprecipitated with anti-Snu114p antibody (*lanes 1–10*, *12*, and *13*) or non-immune serum (*lane 11*). The precipitates were loaded on a 10% SDS-PAGE and radioactive Snu114p (cross-linked to GTP or ATP) was detected by autoradiography. The *lines* indicate degradation products of Snu114p.

precipitated proteins were extracted with phenol/chloroform/isoamyl alcohol (50:49:1, v/v). After acetone precipitation, the proteins were separated by SDS-PAGE. The gel was stained with Coomassie Blue, and cross-linked proteins were detected by autoradiography.

Splicing Extracts, in Vitro and in Vivo Splicing, Temperature Inactivation, and Complementation Assays—Splicing extracts of YPF36 (11), YPF37, YPF39, and YCK13 were prepared by grinding frozen cells under liquid nitrogen (20). For extract preparation the cells were grown at 25 or 30 °C. *In vitro* splicing reactions were performed as described previously (21), generally using actin pre-mRNA transcribed in the presence of [α-^{32}P]UTP. For *in vitro* thermal inactivation according to Ref. 8, the prepared extracts were incubated in 120 mM KPO_4 at 37 °C for the times indicated prior to the splicing reaction. When time-dependent measurements were made, the samples taken at each time point were placed on ice until the splicing reaction was initiated. As a control, the extract was incubated in 120 mM KPO_4 on ice for the whole period of heat treatment. The splicing reactions after thermal inactivation were performed under standard splicing conditions (60 mM KPO_4, 3% polyethylene glycol 8000, 2 mM ATP, 2.5 mM $MgCl_2$, and 2 mM spermidine). Complementation was achieved by mixing equal volumes of the two thermally inactivated extracts before the splicing reaction was carried out. In the experiment shown in the *left panel* of Fig. 3*B*, thermal inactivation was performed by directly incubating the splicing reaction at 32 °C instead of at 25 °C. *In vivo* accumulation of unspliced pre-U3A and pre-U3B RNA transcripts was measured by primer extension as described previously (10).

Affinity Purification of Spliceosomes—For affinity purification of spliceosomes, actin pre-mRNA was transcribed in the presence of biotin-16-UTP (Roche Applied Science) with a UTP/biotin-16-UTP ratio of 20:1 (22). Standard splicing reaction mixtures (100 μl) contained 40 μl of extract and 270 fmol of biotinylated actin pre-mRNA. After incubation for 25 min at 32 °C, ATP was depleted by adding 4 mM glucose and incubating the samples for an additional 10 min. Subsequently, samples were added, together with 500 μl of NET2-75, to 25 μl of washed streptavidin beads (Roche Applied Science) in siliconized tubes. For affinity purification, the samples were rotated for 90 min at 4 °C. The precipitates were washed extensively with NET2-75, and 25 μl of 1× splicing buffer was added together with the indicated concentrations of different nucleotides. Subsequently, the samples were incubated for 25 min at 25 °C, and the precipitates were washed another three times with NET2-75. The precipitates were treated with proteinase K and extracted with phenol/chloroform. The RNAs were ethanol-precipitated and separated on a 10% denaturing gel. The gels were then blotted onto a nylon membrane (Amersham Biosciences), which was UV-irradiated at 120 mJ/cm^2. Northern analyses with uniformly radiolabeled DNA probes specific for snRNAs U1, U2, U4, U5, and U6 was performed.

RESULTS

Binding of GTP by Snu114p—Because Snu114p exhibits the same domain structure as the ribosomal GTPase EF-2, including the GTP-binding and -hydrolyzing G domain (10), we asked whether GTP is required for Snu114p function in splicing. First, we investigated whether Snu114p binds GTP. It was previously shown that the human homologue of Snu114p, the U5–116K protein, binds GTP when it is a part of the U5 snRNP (10), but until now it was unclear whether the yeast protein Snu114p binds GTP independently of the other proteins and RNA present in the U5 snRNP complex. To address this question, we overexpressed Snu114p in yeast and purified it from the extract in two steps, nickel-agarose chromatography and size-exclusion chromatography (Fig. 1*A*).

Fig. 1*B* shows that [α-^{32}P]GTP cross-links to purified Snu114p after UV irradiation (*lanes 1* and *2*, *6–10*, and *12*). Radiolabeled Snu114p, cross-linked to GTP, can be specifically immunoprecipitated with anti-Snu114p antibodies (Fig. 1*B*, *lanes 1–10* and *12*) but not with the non-immune serum (*lane 11*). The observed cross-link is stimulated, as in the case of the human U5 snRNP (10), in the presence of poly(U) (compare *lane 2* with *lane 1*). To determine the specificity of the cross-link obtained with labeled GTP, we performed competition experiments by adding an excess of unlabeled NTPs. Fig. 1*B* (*lanes 3–5*) shows that the [α-^{32}P]GTP cross-linked to Snu114p can be competitively displaced by a 10-, 25-, and 50-fold excess of unlabeled GTP, but not by unlabeled ATP (*lanes 6–8*) or CTP or UTP (*lanes 9* and *10*). This result suggests that purified Snu114p has very high affinity for GTP only.

Some [α-^{32}P]ATP also became cross-linked to Snu114p, although to a lesser extent, corresponding to about 5% or less of the cross-linked [α-^{32}P]GTP (Fig. 1*B*, *lane 13*). This cross-link could reflect a low affinity of Snu114p for ATP. Preliminary results indicate that this cross-link to ATP is prevented by competition from all of the unlabeled NTPs used, suggesting that Snu114p has unspecific affinity for ATP (data not shown).

Mutagenesis of the G Domain of Snu114p—The finding that Snu114p indeed binds GTP in a highly specific manner (see above) and that a point mutation in Snu114p G domain leads to

TABLE I
Summary of all of the mutants of Snu114p

Motif	Mutation	Phenotype	*In vivo* defect	*In vitro* defect	Reference
G1	G145R	Lethal			Fabrizio *et al.* (10)
	K146E	Lethal			This work
	D233K	Viable			This work
G4	N268I	Lethal			This work
	N268D	Viable			This work
	K269I	Viable			This work
	K269E	Viable			This work
	D271N	Slow growth	Splicing defect at 37 °C 60% less tri-snRNP when strain is grown at 30 °C	Splicing inhibition prior to first step at 37 °C U4 unwinding inhibition at 32 °C	This work
Domain II	R487E	ts at 30 and 37 °C and cs at 17 °C	Splicing defect at 37 °C 85% less tri-snRNP when strain is grown at 25 °C	ND[a]	This work
Domain III	E649K	Viable			This work
Domain IV	Deletion (700-862)	Lethal			This work
	G73IS	Viable			This work
	I740D	Viable			This work
	R745W	Viable			This work
	R752W	Viable			This work
	L808A	Viable			This work
	E859I	Lethal			This work
Domain V	R884A	Viable			This work
	916TDLR919 to 916AAAA919	Viable			This work
	F932A + (R973W)	Viable			This work
N-terminal	Deletion (2-128)	ts at 37 °C	Splicing inhibition prior to first step at 36 °C		Bartels *et al.* (11)

[a] Not determined.

a lethal phenotype (10), raised the intriguing question whether Snu114p hydrolyzes GTP, in analogy to the ribosomal translocases, during its function in splicing.

Up to now GTP has never been reported as an essential cofactor for splicing. This could, however, be due to the fact that GTP is already available in the extract, or to the complexity of the splicing process. The non-hydrolyzable analogue of GTP, GTPγS, is not suitable to clarify this question, because it can inhibit splicing by binding not only to Snu114p but also to any of the numerous RNA unwindases that are involved in the splicing process and that are known to bind and hydrolyze promiscuously several of the NTPs (23).

To address the complex question of GTP requirement in splicing, we performed site-directed mutagenesis of Snu114p G domain. This mutagenesis revealed that the G domain is far more sensitive to mutation than the other conserved domains of Snu114p (see below). Several single-residue substitutions in the P loop (G145R and K146E) and in the NKLD (N268I) sequence of Snu114p G domain are lethal, whereas most of the point mutations in domains II–V did not lead to any detectably deviant phenotype (see below and Table I for a summary). The lethal phenotypes observed by mutating specific amino acids of the G domain are consistent with the ones observed in other G proteins. For example, the corresponding N268I substitution in the NK*X*D sequence of the yeast GTPase Ypt1p led to a similar lethal phenotype, with loss of GTP binding (24). These results underscore the possibility that GTP binding, and probably GTP hydrolysis, are important for the function of Snu114p in splicing.

To investigate in more detail the role of GTP in the function of Snu114p, we substituted the aspartic acid at position 271 in the NKLD sequence with an asparagine, to produce the mutant snu114-D271N. The NK*X*D sequence in other G proteins is known to interact with the guanine ring of the bound GTP. The equivalent amino acid replacement in EF-Tu (D138N) and in several other well characterized GTPases, produces an XTP-dependent protein that is converted from a GTPase into a xanthosine triphosphatase (17, 25). In several other systems, this mutation allowed the analysis of the function of individual GTPases in extracts from whole cells. Unexpectedly, the D271N mutation in Snu114p did not lead to a severely impaired phenotype but to a slow growth phenotype at all of the temperatures tested (17, 25, 30, and 37 °C; data not shown).

Mutagenesis of Snu114p Domains II, III, IV, and V—Because the crystal structures of EF-G from *Thermus thermophilus* have been determined (26), a sequence comparison could be made between Snu114p and ribosomal translocases, focusing on the folding domains of EF-G (10). Using these EF-G structural domains for orientation, we have designed and generated substitutions in highly conserved residues of domains II–V.

In domain II, a consensus sequence for translational GTPases was identified by Ævarsson (26). Within this domain, the conserved arginine 329 in EF-G was found to form a salt bridge with an aspartate residue of the G domain. We have exchanged the corresponding arginine of Snu114p with a glutamic acid (R487E). This substitution leads to a temperature-sensitive and a cold-sensitive phenotypes (see Table I).

In the ribosomal elongation factor EF-G, domain IV is crucial for coupling GTP hydrolysis to the translocation of peptidyl-tRNA from the A to the P site of the ribosome (27). Deletion of the equivalent domain IV from Snu114p leads to a lethal phenotype, suggesting that this domain is important for the function of Snu114p as well. Point mutations in this domain have

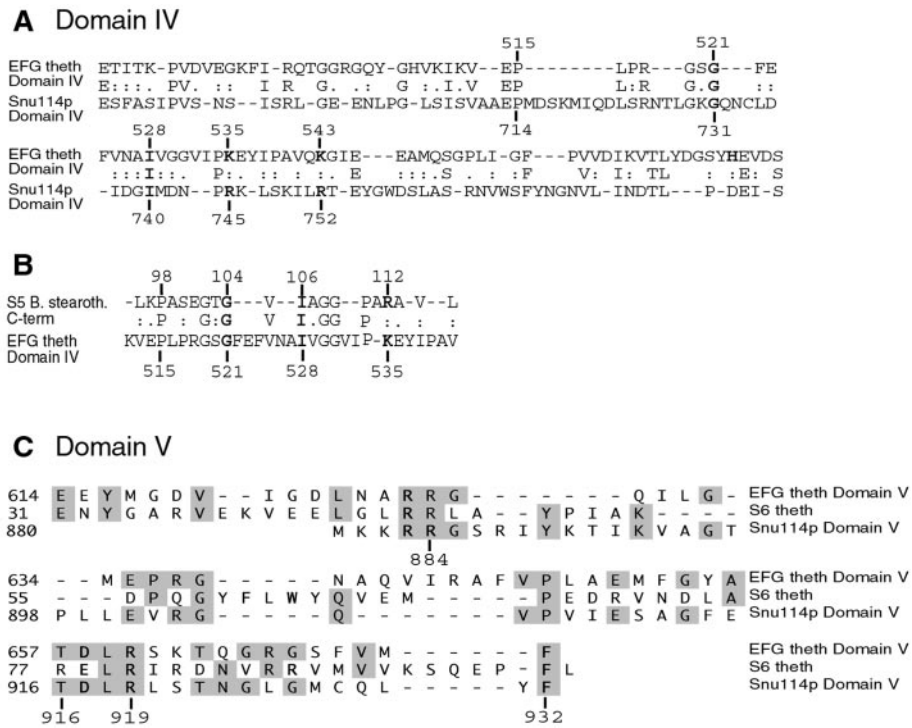

FIG. 2. **Phylogenetic conservation of Snu114p domain IV and V.** *A*, sequence alignment between Snu114p domain IV (amino acids 684–784) and elongation factor EF-G domain IV from *T. thermophilus* (amino acids 484–576), using the ClustalW program. *B*, EF-G domain IV (amino acids 512–541) is aligned with a region of the ribosomal protein S5 from *Bacillus stearothermophilus* (amino acids 96–115), which correspond approximately to the evolutionarily conserved amino acids 714–745 of Snu114p domain IV. *C*, sequence alignment between EF-G domain V (amino acids 614–672), the ribosomal protein S6 from *T. thermophilus* (amino acids 31–98), and Snu114p domain V (amino acids 880–932). Identical amino acids are marked by *gray boxes*. Amino acid positions are indicated at the *left*. *Boldface characters* in *A–C* indicate amino acids that have been substituted in Snu114p (see also Table I).

also been obtained (see Table I). The E859I mutation has a lethal effect. However, several other mutations in domain IV have no effect (Table I). All of these mutations were designed on the basis of the phylogenetic conservation of domain IV between EF-G and Snu114p (Fig. 2*A*) and EF-G and the ribosomal protein S5 (28) (Fig. 2*B*); the most highly conserved sites were chosen for mutation. Two residues, glycine 104 and arginine 112, are highly conserved among S5 proteins and, when mutated in *E. coli* S5, have important effects on translation (29). We changed the corresponding amino acids in Snu114p domain IV as shown in Table I. Surprisingly, these substitutions also failed to produce a clear temperature-sensitive growth phenotype.

Likewise, substitutions of highly conserved amino acids within domains III and V do not exhibit any significant effect (Table I), even when substitutions at highly conserved positions were designed on the basis of the homology of domain V of Snu114p and EF-G to the ribosomal protein S6 (15) (Fig. 2*C*). The crystal structure of S6 showed that this protein has a folding scheme identical to that of the RNA-binding domain of the U1A snRNP protein, which comprises an RNA recognition motif (Ref. 30 and references therein). The amino acids of S6 that correspond to those we have changed in Snu114p are conserved residues suggested to participate in RNA interactions. Thus, these Snu114p residues may similarly be involved in contacting RNA (pre-mRNA or one of the snRNAs).

Characterization of the D271N Mutant—As described above, the D271N mutant may be a unique tool to dissect precisely the one or more steps at which binding and/or hydrolysis of GTP is required in the splicing of pre-mRNA (17). Although this mutation does not lead to a lethal or temperature-sensitive phenotype, we expected that a distinctive phenotype could be revealed. For this reason, we investigated whether an *in vivo* splicing defect could be shown and exacerbated by treatment at high temperatures.

Pre-mRNA splicing was studied *in vivo* by primer-extension analysis of U3 transcripts from wild-type cells and from cells bearing the D271N mutation (Fig. 3*A*). Unspliced U3A and U3B precursors accumulate after shifting the mutant strain to 37 °C for 2 and 4 h, respectively (Fig. 3*A*, *lanes 5* and *6*). Thus, although the D271N substitution does not lead to a discernibly temperature-sensitive phenotype, it does cause a splicing defect, which is revealed by increasing the temperature. No accumulation of unspliced pre-U3A and pre-U3B transcripts could be seen with the wild-type parental control (Fig. 3*A*, *lanes 1–3*).

Because the splicing inhibition *in vivo* may be the result of an indirect effect on splicing, we carried out further characterization of the steps affected by the D271N mutation using an *in vitro* system. We prepared a splicing extract from the mutant strain, which was grown at 23 °C, and analyzed its ability to carry out splicing *in vitro* (Fig. 3*B*). Under standard splicing conditions, the D271N extract was able to splice the actin pre-mRNA as well as the wild-type parental control (*lanes 1*, *2*, and *7*). The observation that an increase in temperature resulted in an accumulation of unspliced precursors *in vivo* led us to analyze the effects of heat on the *in vitro* splicing reaction. The heat treatment was performed according to protocols published by others (Kuhn *et al.* (9) or Xie *et al.* (8), see also Ref. 11).

According to the first protocol, to obtain thermal inactivation, we simply increased the splicing temperature from 25 to 32 °C (*left panel*). According to the second protocol, the splicing extracts are incubated prior to the splicing reaction in 120 mM KPO_4 at 37 °C for different times. The subsequent splicing reactions are then performed for 25 min at 25 °C (*right panel*).

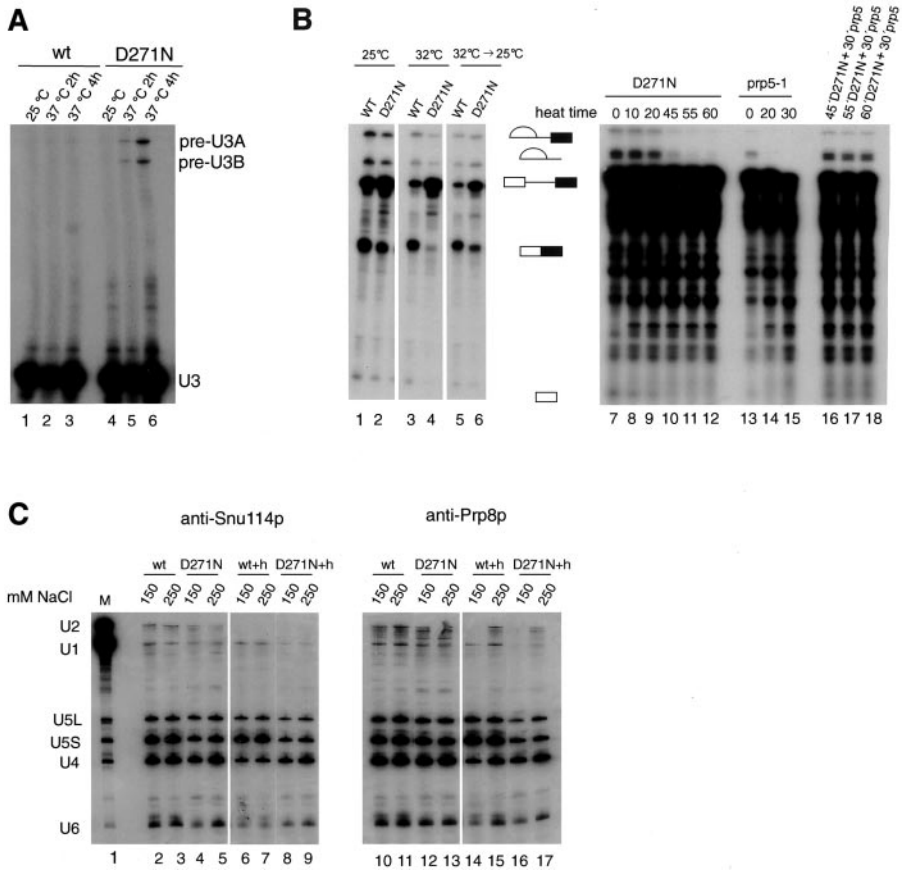

FIG. 3. **The D271N mutation leads to splicing defects *in vivo* and *in vitro*.** *A*, total RNA was extracted from wild-type (*lanes 1–3*) and D271N cells (*lanes 4–6*), which were grown at 25 °C (*lanes 1* and *4*) and afterward shifted to 37 °C for 2 or 4 h (*lanes 2*, *3*, *5*, and *6*). To measure the levels of spliced and unspliced (pre-) U3A and U3B transcripts, primer extension analysis was performed. *B*, the snu114-D271N extract can be heat-inactivated *in vitro* using two different conditions. *Left panel*: splicing activity of wild-type and snu114-D271N extracts at different temperatures. Splicing reactions, including the pre-mRNA, were either performed for 25 min at 25 °C (*lanes 1* and *2*) or at 32 °C (*lanes 3–6*). After 25 min of incubation at 32 °C, aliquots of the reactions shown in *lanes 3* and *4* were shifted back to 25 °C for 25 min (*lanes 5* and *6*). *Right panel*: snu114-D271N or prp5–1 extracts were incubated prior to the splicing reaction at 37 °C for the times indicated above the figure. The following splicing reaction was performed for 25 min at 25 °C. Complementation was achieved by mixing equal volumes of two heat-treated extracts prior to the addition of pre-mRNA (*lanes 16–18*). The identity of the ^{32}P-labeled RNA species (intron-lariat-exon 2 intermediate, excised lariat-intron, pre-mRNA, mature mRNA, and cleaved exon 1 intermediate) are indicated. *C*, heat treatment does not affect the association of snu114-D271N with tri-snRNP particles nor the stability or abundance of the tri-snRNP particles in the extract. Immunoprecipitations of U5 and tri-snRNP particles were performed with anti-Snu114p antibodies (*lanes 2–9*) and anti-Prp8p antibodies (*lanes 10–17*). Prior to immunoprecipitation the extracts were either incubated for 45 min on ice (*lanes 2–5* and *10–13*) or at 37 °C (*lanes 6–9* and *14–17*). After immunoprecipitation the reactions were washed with 150 mM or 250 mM NaCl, and the RNA content of the precipitates was assayed by Northern blotting using U1, U2, U4, U5, and U6 probes indicated on the *left*.

Both methods led to a dramatic decrease of splicing before the first step if the reaction mixture was, or had been, at the non-permissive temperature (Fig. 3*B*, *lanes 4* and *10–12*). The splicing reactions shown in the *left panel* were performed with the wild-type Snu114p (*lanes 1* and *3*) and with D271N extracts (*lanes 2* and *4*) at 25 °C (*lanes 1* and *2*) or at 32 °C (*lanes 3* and *4*). At 25 °C the splicing activity of the mutant extract is only slightly less than that of the wild-type (compare *lanes 1* and *2*). However, although splicing at 32 °C is considerably decreased in the mutant extract, it is only barely reduced in the wild-type (compare *lanes 3* and *4*). In addition, we investigated whether the observed inhibition of splicing at 32 °C can be reversed by shifting the reaction back to 25 °C (*lanes 5* and *6*). Aliquots of the reactions shown in *lane 3* and *4* were afterward incubated for an additional 20 min at 25 °C. Indeed, the blockage of splicing was reversed efficiently in the mutant extract. This indicates that the high temperature leads not to a dead-end splicing reaction but, rather, to a reaction that is inhibited at 32 °C and can be reactivated, at least in part, by lowering the temperature.

In the experiment shown in Fig. 3*B* (*right panel*) during heat inactivation in the absence of precursor, aliquots of the reaction were withdrawn at the times indicated (heat time) and kept on ice. The precursor was then added, and splicing reactions were performed for 25 min at 25 °C. After 45 min or more of heat treatment, the mutant showed a significant decrease in splicing activity before the first step (*lanes 10–12*). To determine whether this inhibition was due to a specific inactivation of snu114-D271N, we tested whether splicing activity could be restored by complementing two heat-inactivated extracts. The combination of the thermally inactivated prp5–1 extract (31, 32) with heat-inactivated snu114-D271N extract, efficiently reversed the splicing defect (Fig. 3*B*, *right-hand panel*, *lanes 16–18*). We conclude that the splicing defect observed *in vitro* is due to the specific inactivation of the snu114-D271N mutant and not to an accidental inactivation of other factors. Again, we investigated whether the achieved inactivation is reversible. After heat treatment, but before the addition of precursor, the mutant extract was incubated at the permissive temperature. The inactivation was found to be irreversible (data not shown), most probably because these conditions damage irreversibly the structure of the D271N mutant.

In summary, we found two independent conditions for the specific inactivation of snu114-D271N *in vitro*: incubation at

37 °C before splicing or at 32 °C during the splicing reaction. One possible explanation for the two forms of inactivation is that the mutant protein is structurally damaged such that lowering the temperature after thermal inactivation only allows it to recover if mild inactivation conditions have been used. The observation that the D271N extract is splicing-competent at 23 °C, even without the addition of exogenous XTP, may be explained with the fact that there are low levels of XTP in living cells, and it is therefore likely that enough XTP is present in yeast extracts to complete at least one round of splicing.

To characterize the D271N tri-snRNP particle, immunoprecipitations were performed. Fig. 3*C* shows immunoprecipitation experiments carried out with the active extracts utilized above (wild-type and D271N), using anti-Snu114p and anti-Prp8p antibodies (Fig. 3*C*, *lanes 2–9* and *10–17*, respectively). The D271N tri-snRNP particle is efficiently immunoprecipitated, and the U5 (L and S), U4, and U6 snRNAs are clearly detected by Northern analysis even after heat treatment of the extract for 45 min at 37 °C prior to immunoprecipitation (Fig. 3*C*, compare *lanes 8–9* with *6–7*, and *lanes 16–17* with *14–15*). Glycerol gradient centrifugation confirmed this result (data not shown). This demonstrates that the abundance and stability of U5 and tri-snRNP particles (containing snu114-D271N), in extracts heat-treated *in vitro*, are substantially similar to those of comparable wild-type extracts.

The D271N Mutation Leads to Accumulation of Precatalytic Spliceosomes That Can Be Activated, at Least in Part, by the Addition of a Combination of ATP and XTP—We demonstrated recently that Snu114p is involved in the unwinding of U4/U6 during catalytic activation of the spliceosome (11). Although the mutant that we have used in our previous work had an intact G domain, the complexity of the system prevented us from finding out whether GTP is required for the U4/U6 unwinding *in vitro*. In the present work, we use the D271N XTP mutant as a tool to dissect this intricate system.

We anticipated that the splicing defect observed *in vitro* by treating the D271N extract at high temperatures (32–37 °C) could be due to the inhibition of the unwinding of U4/U6. To prove this, we assembled spliceosomes by using biotinylated actin pre-mRNA in a standard *in vitro* splicing reaction performed at 32 °C instead of 25 °C. Assembled splicing complexes of the D271N mutant were subsequently affinity-purified with streptavidin and analyzed by denaturing gel electrophoresis. These experiments show that the tri-snRNP particles of the D271N mutant join the pre-spliceosomes as expected, but that the dissociation of U4 from U6 (the subsequent step toward spliceosome activation) is inhibited, and the U4 snRNA is no longer efficiently released from the spliceosome in the mutant extract (Fig. 4*A*, *lane 1*, see also the working model above the figure). Analysis of the purified snRNAs in a non-denaturing gel shows clearly that this was due to inhibition of the U4/U6 unwinding (data not shown). Thus, by using non-permissive conditions, it is possible to inhibit the catalytic activation of the spliceosome, which leads to the accumulation of splicing complexes from which U4 has not been released.

Most importantly, because the splicing defect observed *in vitro* is reversible upon shifting the reaction back to 25 °C when "mild" heat treatment was used (Fig. 2*B*, *left panel*), we could test the possibility of reactivating these assembled and arrested splicing complexes. In addition, this system allowed us to investigate the nucleotide dependence of this reactivation. After 25 min of splicing at 32 °C (see working model), ATP was removed by adding glucose, and the assembled complexes were bound to streptavidin beads. The beads were washed thoroughly to remove all of the remaining NTPs, and the isolated splicing complexes were incubated for a further 20 min at 25 °C in the presence of 1× splicing mixture containing the desired NTPs. The reactions were then stopped and the snRNA content was analyzed by using Northern blots.

In the absence of nucleotides, the shift back to 25 °C did not lead to any change in the snRNA composition of the assembled splicing complexes (Fig. 4*A*, compare *lanes 1* and *2*). In contrast, the addition of 1 mM ATP led to a partial release of the U4 snRNA from the precatalytic splicing complexes (*lane 3*). Exactly the same effect was observed even when much higher concentrations of ATP were used (2–4 mM, data not shown). Remarkably, the combination of 1 mM ATP with 1 mM XTP led to a small, but reproducible, enhancement of the release of U4 (*lane 4*), whereas the addition of XTP alone had no effect (*lane 5*). The combination of ATP and GTP resulted merely in the same effect that had been observed following the addition of ATP alone (compare *lanes 6* and *3*). We quantified the U4 signals in each lane, and a graphical representation of the results is shown next to the figure.

In the reactions in which U4 dissociates from the purified complexes, a small loss of U6 snRNA was also detected in all of the experiments performed. Furthermore, in none of the reactions was the expected release of U1 (simultaneous with the dissociation of U4) observed. The same observations were described previously (9). Kuhn *et al.* (9) hypothesized that factors required for a stable incorporation of unwound U6 into the RNA structure essential for catalysis were removed by the extensive washing steps. Likewise, factors required for the release of U1 may be removed by washing. Another explanation for the retention of U1 could be that pre-spliceosomes are co-isolated that contain U1 and/or U2 (11).

To verify that the addition of ATP and ATP/XTP leads to a predominant release of the U4 snRNA and not to dissociation of the complete U4/U6 di-snRNP from the precatalytic splicing complexes, we also calculated the U4:U6 ratios in each lane (Fig. 4*A*). Indeed, this ratio decreases only in the reactions in which ATP or ATP and XTP are added. This suggests that much more U4 than U6 is released.

In addition, we investigated the effects of other nucleotides: CTP, UTP, and xanthosine diphosphate. None of these, when added, resulted in a significant reactivation of the purified complexes, and when they are combined with ATP no enhancement of the U4 dissociation appeared (data not shown). This indicates that the observed enhancement of U4 release is due specifically to the addition of XTP. Moreover, because non-hydrolyzable XMP-PNP did not lead to enhanced dissociation of U4 (data not shown), we hypothesize that the enhancement of the U4 release after addition of a combination of ATP and XTP implies hydrolysis of XTP; this indicates that the mutant snu114-D271N takes part in the process, which in turn implicates GTP hydrolysis by Snu114p in the wild-type spliceosome. Taken together, our results show that the stalled precatalytic spliceosome can be reactivated and that this reactivation is dependent only in part on the lowering of the temperature in the presence of ATP. Most importantly, an additional activation is observed if (and only if) hydrolyzable XTP is mixed with ATP.

To exclude the possibility that an independent protein, in addition to snu114-D271N, is present in the purified complexes and utilizes XTP to enhance the U4/U6 unwinding, we used another mutant of Snu114p, the N-terminal deletion mutant snu114ΔN. This mutant lacks about 130 amino acids at its N terminus, but it possesses an intact G domain, so it should still retain its GTP-binding activity. We showed recently that heat treatment of snu114ΔN extracts also prevents the unwinding of U4/U6, which is in this case most probably due to a loss of

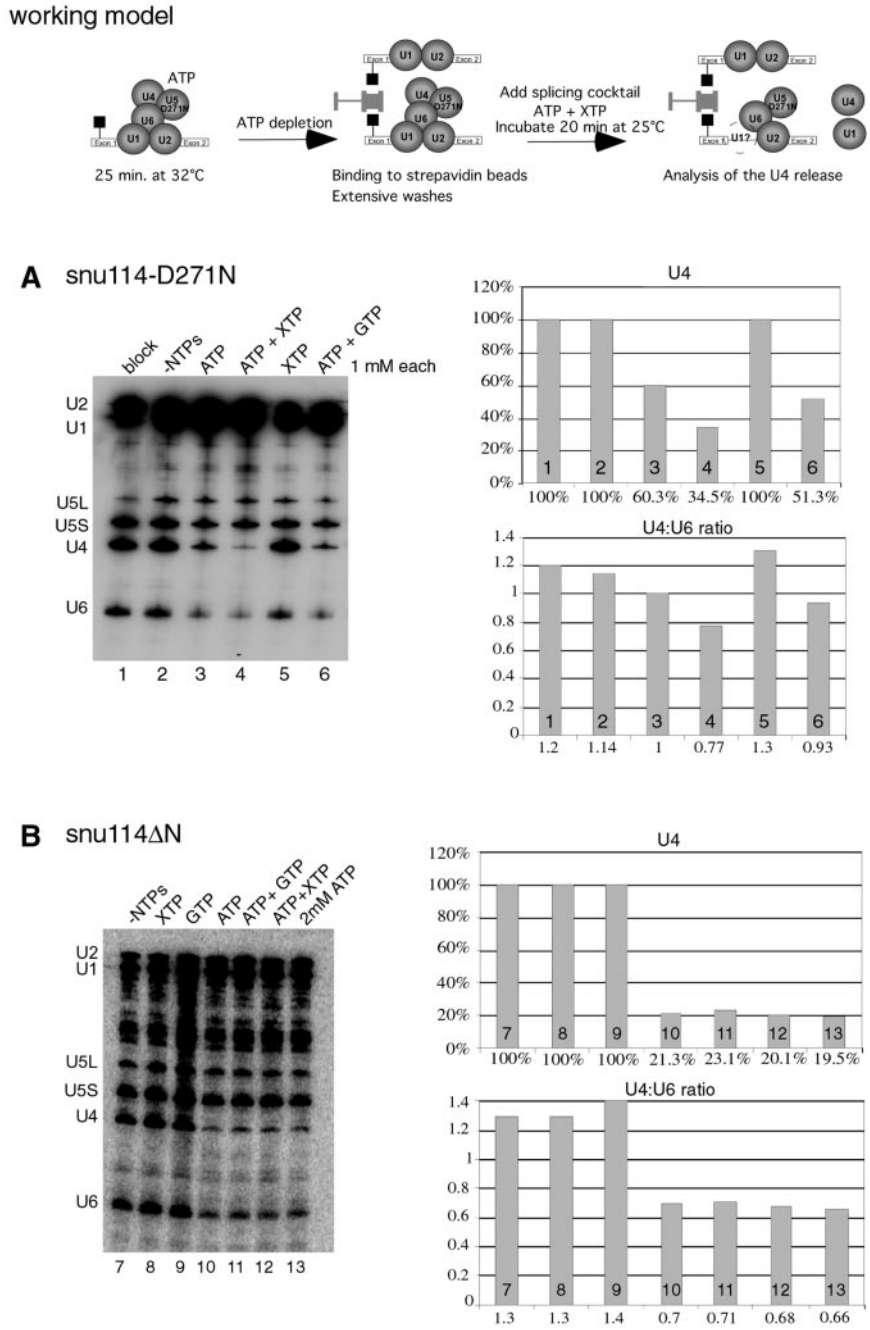

FIG. 4. **Reactivation of stalled precatalytic spliceosomes of the D271N XTP mutant of Snu114p.** *A*, spliceosomes were assembled in snu114-D271N extracts on biotinylated pre-mRNA at 32 °C and affinity-purified via streptavidin beads (*lane 1*). After affinity purification, the stalled precatalytic complexes were incubated again under splicing conditions at 25 °C in the absence (*lane 2*) or presence of the indicated nucleotides (*lanes 3–6*) (see also the working model above the figure). The RNA content was analyzed by Northern blotting. *B*, the same experiment was performed with snu114ΔN extracts as a control (11). Next to the figure a graphical representation of the U4 release and the U4:U6 ratio in each lane is shown. The U4 and U6 snRNA bands were quantified by PhosphorImager analysis.

crucial protein-protein interaction between the N terminus of Snu114p and other factors that regulate the this unwinding (11).

Fig. 4*B*, *lane 10*, shows that the corresponding precatalytic complexes purified from snu114ΔN extracts could be reactivated only in part by the addition of ATP. No enhancement of the U4 dissociation was observed, either when both ATP and GTP (see "Discussion") or both ATP and XTP (*lane 11* and *12*) were used. Taken together, these data support the earlier finding that Snu114p is involved in the U4/U6 unwinding during catalytic activation of the spliceosome (11), and they strongly link snu114-D271N to the effect of XTP that we observe on the release of U4.

Requirement for Snu114p in the Assembly of Stable U5 and Tri-snRNP Particles—Because in earlier studies the strain carrying the D271N mutation exhibited a slow growth phenotype at all of the temperatures tested, it was routinely grown at 30 °C instead of at 25 °C before extract preparation. We observed that extracts prepared from these strains were consistently inactive in splicing *in vitro* (data not shown). Fig. 5*A* shows the immunoprecipitation of tri-snRNP particles obtained from these extracts (400 μg of proteins), using anti-Snu114p and anti-Prp8p antibodies. The reactions were washed at various salt concentrations, and the precipitated snRNAs were analyzed by Northern blotting.

In comparison with wild-type extracts only 40% of tri-snRNP was present in the D271N mutant extract (Fig. 5*A*, compare *lanes 3* and *4* with *lanes 1* and *2*). An even more dramatic effect was observed when the tri-snRNP of the domain II mutant R487E was analyzed (Fig. 5*A*, *lanes 5* and *6*). To exclude the possibility that the decreased levels of tri-snRNP were due to a reduced association of the mutant proteins snu114-D271N and R487E with the particle, or to inadequate recognition of the mutants by the anti-Snu114p antibody, we also precipitated the tri-snRNPs with anti-Prp8p antibodies (Fig. 5*A*); here too, a similar effect was observed. In summary, Fig. 5*A* shows that

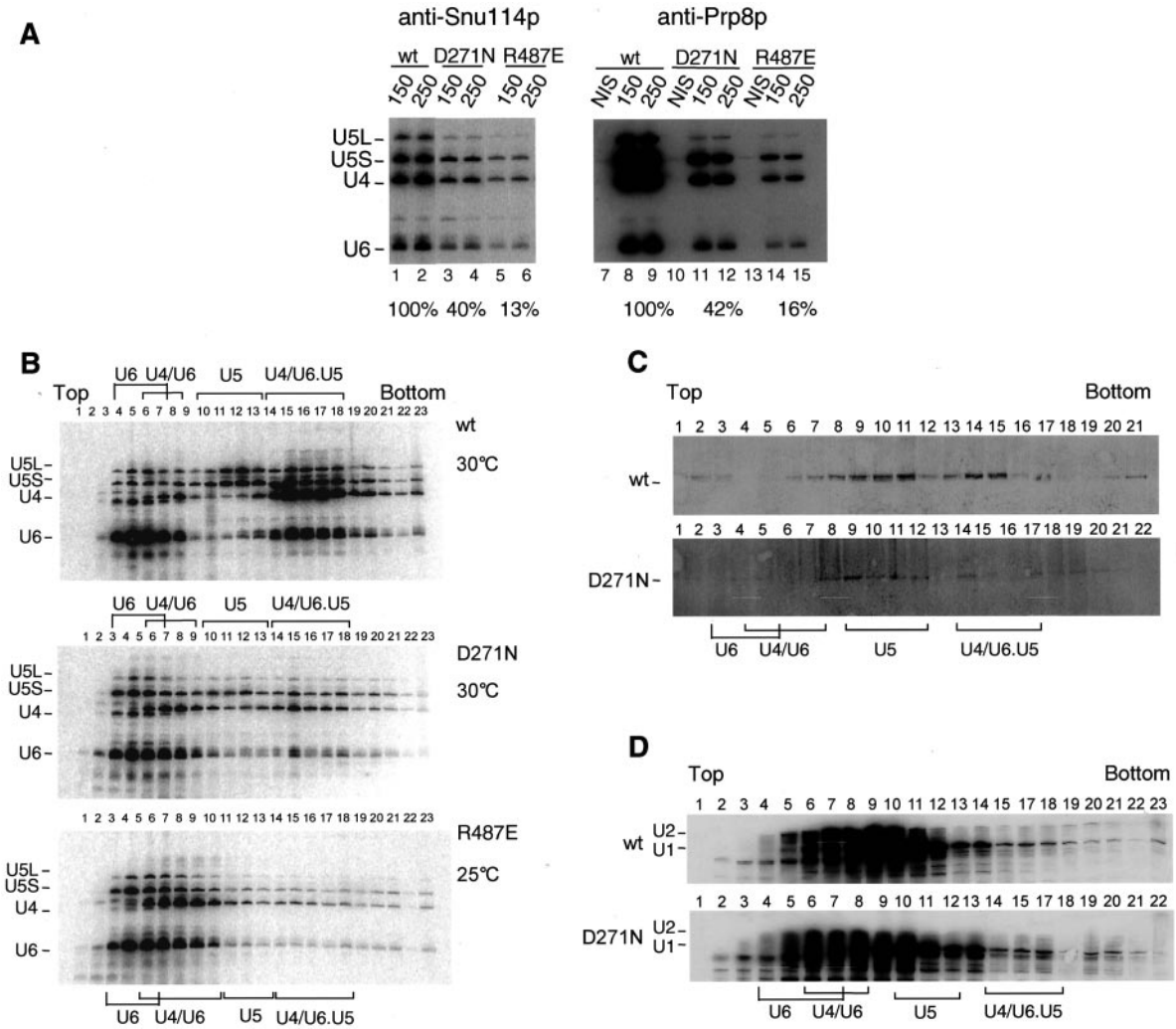

FIG. 5. **Strains containing the D271N and R487E snu114p mutants lead to low levels of U5 and U4/U6·U5 tri-snRNP particles when grown at high temperatures.** *A*, immunoprecipitation of U5 and tri-snRNP particles obtained from wild-type, D271N, and R487E strains grown at 30 or 25 °C, respectively. Extracts (400 μg) were used in immunoprecipitation experiments using anti-Snu114p antibodies (*lanes 1–6*), anti-Prp8p antibodies (*lanes 8*, *9*, *11*, *12*, *14*, and *15*) or non-immune serum (*lanes 7*, *10*, and *13*). After immunoprecipitation the reactions were washed with different salt concentrations, and the RNA content of the precipitates was analyzed by Northern blotting. The bands were quantified by PhosphorImager analysis. *B*, wild-type, D271N, and R487E extracts (2.5 mg) obtained from the corresponding strains grown at 30 or 25 °C were sedimented on a 10–30% glycerol gradient. The fractions were phenol-chloroform-extracted, and their snRNA content was analyzed by Northern blotting using U4, U5, and U6 snRNA probes or U1 and U2 snRNA probes (see *D*). *C*, Western blot of the gradient fractions from wild-type and D271N extracts using anti-Snu114p antibodies. The Western blot of the gradient fractions obtained from the R487E mutant extract showed very low levels of protein, and it is not shown.

the D271N and R487E extracts have about 60 and 85% less tri-snRNP than the wild-type extract. This result suggests that Snu114p is required to maintain proper levels of the tri-snRNP particle.

A similar or even more pronounced effect was obtained when U5 and tri-snRNP particles were examined after loading equivalent amounts of extracts, 2.5 mg of proteins, on each glycerol gradient (Fig. 5*B*). Tri-snRNP particles and free 16 S U5 snRNPs are clearly less abundant in the D271N extract than in those from the wild-type. Western blot analysis of these gradients revealed that there was no free protein at the top of the gradients, suggesting that most of the snu114-D271N present in the extract was associated with the U5 and tri-snRNP particles (Fig. 5*C*). However, Fig. 5*B* also shows that the reduced levels of U5 and tri-snRNP particles in the D271N extracts correlated with a reduced amount of mutant protein, which appeared at about 40% of the wild-type level. To verify whether this extract also contained reduced levels of U1 and U2 snRNPs, a Northern blot analysis was performed by using probes that recognized the U1 and U2 snRNAs. Fig. 5*D* shows that the levels of the U1 and U2 snRNPs were not affected in the mutant extract. In addition, because the levels of U1 and U2 snRNPs are similar in both gradients, this experiment provides an internal control that confirms that equivalent amounts of extracts were used.

Similar observations were made with the R487E extract prepared from strains grown at 25 °C. Fig. 5*B* (*lower panel*) shows that the effect due to this mutation is even more clearly seen than with the mutant D271N; in fact, only minor amounts of U5 and tri-snRNP particles were detected. Also with this mutant, no effect on the level of U1 and U2 snRNAs, could be seen (data not shown). Altogether, these results show that Snu114p is required to assemble 16S U5 and tri-snRNP particles prior to splicing.

DISCUSSION

Dissociation of the U4/U6 snRNA duplex during spliceosome activation allows the subsequent base-pairing of U6 with U2 snRNA to form the catalytic core. Snu114p, the spliceosomal homologue of the ribosomal GTPase EF-2, is involved in the

dissociation of U4 from U6 snRNA. To understand the function of Snu114p in more detail, we performed mutagenesis of highly evolutionarily conserved residues and domains. This brought to light several previously unknown and potentially important features of Snu114p function, including a possible involvement of GTP in spliceosome activation and a role of Snu114p in building the U5 and U4/U6.U5 tri-snRNP particles. The possible functional role of Snu114p as a GTPase involved in the disruption of the U4/U6 RNA duplex during activation of the spliceosome is presented and discussed.

Purified Snu114p Binds GTP Specifically—We have shown that purified Snu114p, as predicted by its sequence, binds GTP *in vitro* independently of the other splicing factors of the U5 snRNP. However, this activity is stimulated by RNA cofactors, *e.g.* poly(U), and may be enhanced by protein cofactors. This is reminiscent of the spliceosomal ATPases, the function of which *in vitro* is also stimulated by RNA, indicating that all of these spliceosomal enzymes interact with RNA. It is not known whether Snu114p binds specifically and directly to RNA. As observed for domains III, IV, and V in EF-G, the corresponding domains in Snu114p probably also share structural homology with RNA-binding proteins (15, 16) and may therefore interact directly with RNA.

As expected for a GTP-binding protein containing a canonical G domain, Snu114p has high affinity for GTP only. We think that the modest amount of ATP binding observed in our UV cross-linking studies is irrelevant and unspecific, because it can be reversed by adding any of the unlabeled NTPs tested. In addition, we investigated whether purified Snu114p not only binds but also catalyzes the hydrolysis of GTP. Using the purified Snu114p shown in Fig. 1*A* in a GTPase assay, we reproducibly observed some GTP hydrolysis (data not shown). Although this hydrolysis was slightly stimulated by the addition of poly(U), and competition experiments suggested some specificity for GTP, we cannot rule out the possibility that this activity is due in part to a contaminating protein. As determined by mass spectrometry, small amounts of other proteins were detected together with Snu114p, such as the phosphatase PHO8, and all the attempts to separate the two proteins completely from each other failed. Therefore, we cannot exclude the possibility that this phosphatase was responsible for, or contributed to, the observed GTPase activity. To circumvent these technical difficulties, we pursued the question of whether Snu114p hydrolyzes GTP in splicing, by performing site-directed mutagenesis of the G domain.

New Insights into Snu114p Function Are Revealed by Mutagenesis—Because sequence motifs provided clues to the functions of some of the spliceosomal proteins, *e.g.* the DE*X*(D/H) box family members, we probed the function of Snu114p by replacing or deleting several amino acids in evolutionarily conserved domains of Snu114p. Using the N-terminal deletion mutant of Snu114p (snu114ΔN), we showed that Snu114p is involved in the dissociation of U4 from U6 snRNA during spliceosomal activation (11). However, we did not demonstrate whether the binding and hydrolysis GTP are needed to trigger spliceosomal activation. For this reason, we generated numerous mutants of the G domain of Snu114p with the aim of finding conditionally lethal mutants that might answer the question of whether GTP hydrolysis is required during spliceosome activation.

In addition, we replaced some of the residues of the other domains of Snu114p, especially residues that have been highly conserved during evolution and that perform important tasks in other systems. Domains IV and V of Snu114p are evolutionarily highly conserved between EF-2 and EF-G. The fold of EF-G domain IV shares a surprising similarity with the ribosomal protein S5, whereas domain V has an RNA recognition motif and is very similar to the ribosomal protein S6 (15, 28). We expected that changing identical residues in Snu114p would reveal important aspects of the function of Snu114p. However, from all these data we conclude that only the G domain is very sensitive to mutagenesis, and indeed, several substitutions in this domain led to lethal or conditional lethal phenotypes.

There are ten distinct examples in the literature of GTPases that have been successfully engineered to accept XTP, and in most of these cases the specificity was clearly shown to switch from GTP to XTP (17). By making the corresponding substitution (D271N) in Snu114p we likewise generated an XTP mutant (see below).

The Spliceosomal D271N XTP Mutant as a Tool to Understand Whether XTP Hydrolysis Is Involved in Spliceosomal Activation—We show that the D271N substitution in the guanine-ring binding sequence of the G domain leads to a splicing defect that is manifested *in vivo* and *in vitro* at high temperatures and is associated with U4 snRNA remaining paired with the U6 snRNA. We could further establish a specific requirement for XTP by isolating these stalled precatalytic spliceosomes from which U4 is not released, at high temperatures, by isolating and freeing them completely from residual NTPs. We show that U4 is released efficiently from these spliceosomes only when a mixture of ATP and XTP is added and the assay temperature is lowered, suggesting that Snu114p uses GTP to activate the unwinding of U4 from U6. Because a non-hydrolyzable XTP analogue (XMP-PNP) did not lead to the enhancement of the U4 dissociation, we speculate that the enhancement of the U4 release after addition of a combination of ATP and XTP implies hydrolysis of XTP (and therefore GTP for wild-type Snu114p) and implicates snu114-D271N in this process.

The requirement for ATP in the activation of the precatalytic spliceosome was expected, because it is plausible that the U4/U6 unwinding during spliceosome activation is catalyzed by one of the spliceosomal DE*X*(D/H) box proteins. Results from Lauber *et al.* (33) and Raghunathan *et al.* (34) implicate the U5 snRNP-specific protein U5–200K/Brr2p in the U4/U6 unwinding. This is supported by the result of Laggerbauer *et al.*, (35), who showed that protein U5–200K unwinds U4/U6 duplexes *in vitro* and that its RNA unwindase activity is only observed in the presence of hydrolyzable ATP or dATP. Moreover, protein U5–200K works less efficiently with CTP and dCTP and not at all with GTP or UTP. In our experiments we could successfully replace ATP by dATP but not with one of the other deoxynucleotides or with ATPγS (data not shown). Thus, the NTP specificity pattern observed for the U4/U6 unwinding resembles that previously observed for the human homologue of Brr2p and confirms an involvement of Brr2p in the described reactivation (35). Similar observations were described by Kuhn *et al.* (9), who found, in addition, that Prp8p also participated in the U4/U6 unwinding.

The fact that XTP does not lead to an increase in the dissociation of U4 when it is combined with ATP in the snu114ΔN mutant extract underscores the specificity of XTP for the D271N extract. The unexpected lack of enhanced U4 dissociation after the addition of a combination of ATP and GTP in the snu114ΔN mutant extract may be due to the large size of the snu114ΔN deletion, which compromises the structure to such an extent that lowering the assay temperature can no longer induce recovery. It could well be that its structure remains too compromised in these purified precatalytic complexes, even when the temperature is decreased.

We tried to overexpress the snu114p XTP mutant in yeast

with the aim of studying the binding and hydrolysis of XTP *in vitro* using purified mutant protein. These studies would have been of primary importance to demonstrate directly whether snu114-D271N binds and more importantly, whether it hydrolyzes XTP. Moreover, we would have circumvented the problem that contaminating proteins would be responsible of the enzymatic activity, because additional xanthosine triphosphatases, besides snu114-D271N, should not be present in the yeast extract. However, we could not overexpress snu114-D271N in yeast, because it is mostly unstable when overexpressed and most of it is quickly degraded, at both 23 and 30 °C. It is thought that nucleotide-free guanine nucleotide-binding proteins are usually unstable for extended periods of time (36), furthermore, we expect that the intracellular levels of XTP are low when compared with GTP.

An Additional Role of Snu114p: Building the U5 and U4/U6.U5 Particles—As has been shown in the past for Prp8p (37), we show here that Snu114p is required for the stable formation of the tri-snRNP. In addition, growth at high temperatures of mutant strains containing snu114-D271N (30 °C) and snu114-R487E (25 °C) causes the levels of the 16 S U5 snRNP to decline dramatically, while a U5 snRNP with a reduced sedimentation velocity appears, the majority of these particles migrating in approximately the same position as free U6 particles (Fig. 5*B*). Very similar results were obtained *in vivo* by depleting cells of Prp8p (37). This indicates that, independently of its role in splicing, Snu114p plays a part in building both the U5 and the tri-snRNP particles, perhaps again in concert with Prp8p.

Although substantial amounts (40%) of assembled tri-snRNP particles are present in the D271N extract prepared following growth of the strain at 30 °C, the extract is reproducibly inactive in splicing *in vitro*. This suggests that when snu114-D271N is heated *in vivo* it suffers a structural or conformational defect that may impair not only its association with the U5 and tri-snRNP particles but also its overall function within these particles. At the growth temperature of 23 °C, snu114-D271N is expressed and assembled in U5 and tri-snRNP particles as efficiently as in wild-type strains (Fig. 3*C*). Most probably, the incorporation of snu114-D271N into an snRNP particle protects it from degradation. However, at the growth temperature of 30 °C, snu114-D271N is expressed, but not completely assembled, in U5 and tri-snRNP particles, most probably because it is damaged. This damage may be induced by the combination of the high temperature (which loosens the protein structure) with the insufficient amount of XTP (an insufficient amount would make the protein unstable, provided that it is not immediately included in a particle). Indeed, we show that the fraction of the snu114-D271N mutant that is not incorporated into U5 and tri-snRNP particles is most probably degraded, because no free protein is visible at the top of the gradient (Fig. 5*C*).

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