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An evolutionarily conserved U5 snRNP-specific protein is a GTP-binding factor closely related to the ribosomal translocase EF-2

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The driving forces behind the many RNA conformational changes occurring in the spliceosome are not well understood. Here we characterize an evolutionarily conserved human U5 small nuclear ribonucleoprotein (snRNP) protein (U5-116kD) that is strikingly homologous to the ribosomal elongation factor EF-2 (ribosomal translocase). A 114 kDa protein (Snu114p) homologous to U5-116kD was identified in *Saccharomyces cerevisiae* and was shown to be essential for yeast cell viability. Genetic depletion of Snu114p results in accumulation of unspliced pre-mRNA, indicating that Snu114p is essential for splicing *in vivo*. Antibodies specific for U5-116kD inhibit pre-mRNA splicing in a HeLa nuclear extract *in vitro*. In HeLa cells, U5-116kD is located in the nucleus and co-localizes with snRNP-containing subnuclear structures referred to as speckles. The G domain of U5-116kD/Snu114p contains the consensus sequence elements G1–G5 important for binding and hydrolyzing GTP. Consistent with this, U5-116kD can be cross-linked specifically to GTP by UV irradiation of U5 snRNPs. Moreover, a single amino acid substitution in the G1 sequence motif of Snu114p, expected to abolish GTP-binding activity, is lethal, suggesting that GTP binding and probably GTP hydrolysis is important for the function of U5-116kD/Snu114p. This is to date the first evidence that a G domain-containing protein plays an essential role in the pre-mRNA splicing process.
Keywords: elongation factor EF-2/GTP binding/GTPase superfamily/pre-mRNA splicing/U5 snRNP

Introduction

Splicing of nuclear mRNA precursors (pre-mRNA) proceeds via two consecutive transesterification steps. In the first step, the 2′ hydroxyl group of the branch-point adenosine attacks the 5′ splice site, generating the splicing intermediates, exon 1 and lariat-exon 2. The second step involves nucleophilic attack by the 3′ hydroxyl group of exon 1 at the 3′ splice site, producing ligated exons 1 and 2 and excised intron in the form of a lariat. Splicing requires a large number of *trans*-acting factors that assemble in an orderly manner on the pre-mRNA, thereby forming the catalytic splicing machinery known as the spliceosome. Despite the fact that exogenous phosphates are not incorporated into the pre-mRNA during splicing, ATP is an essential cofactor (Hardy *et al.*, 1984; Frendewey and Keller, 1985) and has been shown to be involved in several steps from spliceosome assembly to product release (reviewed in Guthrie, 1991; Moore *et al.*, 1993).

Two classes of splicing factors are distinguished currently. The first class comprises four evolutionarily conserved small nuclear ribonucleoprotein (snRNP) particles, U1, U2, U4/U6 and U5, that contain either one (U1, U2, U5) or two (U4/U6) snRNA components (for review, see Green, 1991; Guthrie, 1991; Rymond and Rosbash, 1992; Moore *et al.*, 1993); the second class consists of an as yet unknown number of proteins that are not tightly bound to snRNPs and are therefore termed non-snRNP splicing factors (see Lamm and Lamond, 1993; Beggs, 1995; Krämer, 1995).

The composition of the U snRNPs has been studied most extensively in HeLa cells (Will *et al.*, 1995). At low salt concentrations (up to 100 mM), where HeLa nuclear extracts support pre-mRNA splicing *in vitro*, a 12S U1 snRNP, 17S U2 snRNP and a 25S [U4/U6·U5] tri-snRNP complex are found. At high salt concentrations (350–450 mM), the tri-snRNP complex dissociates into a 20S U5 and a 12S U4/U6 particle. In the U4/U6 snRNP, the U4 and U6 snRNAs interact through extensive sequence complementarity (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985; Brow and Guthrie, 1988).

The proteins of the snRNPs fall into two groups, the common proteins (B/B′, D1, D2, D3, E, F and G), which are present in each snRNP, and the particle-specific proteins. While U1 and U2 snRNPs contain three (70K, A and C) and 11 specific proteins respectively, the tri-snRNP has an even more complex protein composition. The 20S U5 snRNP component contains nine specific proteins with apparent mol. wts of 15, 40, 52, 100, 102, 110, 116, 200 and 220 kDa (Behrens and Lührmann, 1991), while two proteins with apparent mol. wts of 60 and 90 kDa are associated with the 12S U4/U6 snRNP (Gozani *et al.*, 1994; Lauber *et al.*, in preparation). The 25S [U4/U6·U5] tri-snRNP complex contains five additional proteins with apparent mol. wts of 15.5, 20, 27, 61 and 63 kDa, which are required for tri-snRNP formation (Behrens and Lührmann, 1991; Utans *et al.*, 1992; Lauber *et al.*, in preparation; reviewed in Will *et al.*, 1995). The protein composition of the snRNPs of *Saccharomyces cerevisiae* has not yet been studied in detail, but recent genetic and biochemical results strongly indicate that not only the snRNA components, but also the snRNP proteins are evolutionarily highly conserved (Fabrizio *et al.*, 1994; Lauber *et al.*, 1996; Neubauer *et al.*, 1997; for review, see Beggs, 1995; Krämer, 1995).

The spliceosome is formed by the ordered, stepwise assembly of both snRNPs and other splicing factors with the pre-mRNA. A striking feature of the spliceosome assembly pathway is the formation of a dynamic RNA network which not only involves interactions between the pre-mRNA and the snRNAs but also among the snRNAs themselves (Moore *et al.*, 1993; Madhani and Guthrie, 1994; Nilsen, 1994). In the early phase of spliceosome formation, U1 snRNA base-pairs with the 5′ splice site, while U2 snRNA interacts with the branch site. At this stage, several non-snRNP splicing factors, such as SF2/ASF, U2AF, SC35 and SF1, cooperate with U1 and U2 to form the mammalian pre-spliceosome (Krämer and Utans, 1991; Fu and Maniatis, 1992; Krämer, 1992; Zamore *et al.*, 1992; Eperon *et al.*, 1993; Zuo and Manley, 1994; reviewed in Hodges and Beggs, 1994; Reed, 1996). In the final step of spliceosome assembly, the 25S [U4/U6·U5] tri-snRNP complex and an as yet unknown number of non-snRNP splicing factors interact with the pre-spliceosome to form the mature spliceosome (reviewed in Moore *et al.*, 1993). Before (or concomitantly with) the first step of splicing, the two helices of the U4/U6 interaction domain dissociate, and a new base-pairing interaction is formed between U2 and U6 (Datta and Weiner, 1991; Wu and Manley, 1991; Madhani and Guthrie, 1992; Sun and Manley, 1995). In addition, the U1 snRNA dissociates from the 5′ splice site which is then recognized by the conserved ACAGAG sequence of the U6 snRNA (Fabrizio and Abelson, 1990; Sawa and Abelson, 1992; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993; Sontheimer and Steitz, 1993). The conserved loop I of U5 snRNA also contacts exon sequences at the 5′ and 3′ splice sites, probably in a sequential manner, while the splicing reaction proceeds from step I to step II (Newman and Norman, 1991; Wyatt *et al.*, 1992; Cortes *et al.*, 1993; Sontheimer and Steitz, 1993). Upon completion of the splicing reaction, the spliceosome dissociates, after which the snRNPs are thought to undergo a recycling process. The interaction between the U2 and U6 snRNA and that between U6 snRNA and the pre-mRNA must revert to the earlier U4/U6 snRNA interaction, a process that is not yet well understood (reviewed in Moore *et al.*, 1993).

A major goal of current spliceosome research is to understand the driving forces behind the many conformational changes occurring within the spliceosome and during the recycling of the spliceosomal subunits. A key to understanding these processes may lie in the protein moiety of the spliceosome. In yeast, several non-snRNP proteins have been shown to be essential for splicing; their sequences designate them as putative ATP-dependent RNA helicases of the DEAD-box family or its DEAH subgroup. These include Prp5p (Dalbadie-McFarland and Abelson, 1990) and Prp28p (Strauss and Guthrie, 1991), which belong to the DEAD-box family, and Prp2p (Chen and Lin, 1990; King and Beggs, 1990), Prp16p (Burgess *et al.*, 1990) and Prp22p (Company *et al.*, 1991), which are members of the DEAH-box subgroup (Wassarman and Steitz, 1991; Schmid and Linder, 1992). The participation of these essential proteins also explains, at least in part, the requirement for ATP in the splicing process. We recently have identified a novel putative RNA helicase in purified human 20S U5 snRNPs, the first intrinsic snRNP protein in this category (Lauber *et al.*, 1996). Interestingly, an evolutionarily conserved homolog of this protein has also been found in yeast. The yeast homolog Snu246p is essential for cell viability and is also an integral component of yeast [U4/U6·U5] tri-snRNPs (Lauber *et al.*, 1996; Lin and Rossi, 1996; Noble and Guthrie, 1996; Xu *et al.*, 1996).

The helicases described above are thought to affect the conformation of the spliceosome through a direct interaction with RNA. Although ATP-dependent RNA helicases appear to play a major role in the structural rearrangements of the spliceosome, structural changes in other ribonucleoprotein complexes, such as the ribosome, are mediated by a different class of enzymes. In particular, conformational changes within the ribosome are dependent upon the GTPases EF-1α and EF-2 (EF-Tu and EF-G in procaryotes) rather than ATP-dependent helicases. These proteins interact transiently with the ribosome: their GTPase activity induces dissociation from the ribosome before a new cycle of translation can begin. EF-G becomes activated upon association with the ribosome and induces a transition of the ribosome from a pre- to a post-translocational state (reviewed in Noller *et al.*, 1990; Nierhaus *et al.*, 1993; Abel and Jurnack, 1996). The other ribosomal GTPase, EF-Tu, is thought to play a critical role in the fidelity of the translation process (reviewed in Noller *et al.*, 1990; Nierhaus *et al.*, 1993; Abel and Jurnack, 1996). As pre-mRNA splicing also requires a precise chemistry, it is conceivable that the spliceosome utilizes similar mechanisms to provide fidelity (Burgess and Guthrie, 1993).

In search of additional snRNP proteins that might play a role in regulating spliceosomal conformational changes, we initially microsequenced proteins present in the U5 and [U4/U6·U5] snRNPs. Strikingly, we discovered that the 116 kDa protein of human U5 snRNPs (U5-116kD) is closely related to the eukaryotic ribosomal translocase (elongation factor EF-2). U5-116kD contains the consensus sequence elements typical of the GTPase superfamily of proteins that bind and hydrolyze GTP. We demonstrate that this protein indeed binds GTP specifically. Homologs of U5-116kD were also identified in yeast (Snu114p) and nematodes and mouse. In yeast, Snu114p is essential for cell viability and splicing *in vivo*. In addition, a point mutation in the P-loop of the putative GTP-binding site of Snu114p is lethal. Taken together, the data presented here suggest that the GTP-binding domain of the U5-116kD protein plays an important role in either the splicing process itself or the recycling of spliceosomal snRNPs.

Results

Identification and characterization of a cDNA coding for the human U5-specific 116 kDa protein

Peptide sequences obtained by microsequencing the purified human U5 snRNP-specific 116 kDa protein (hereafter termed U5-116kD protein) allowed a database search for corresponding open reading frames (ORFs). The three peptide sequences obtained (see Materials and methods) were 100% identical to a human gene isolated from a myeloblast KG-1 cDNA library (Nomura *et al.*, 1994). The ORF of the cDNA encodes a protein that is 972 amino acids long, with a predicted mol. wt of 109.4 kDa and a calculated isoelectric point of 4.74 (Figure 1A).

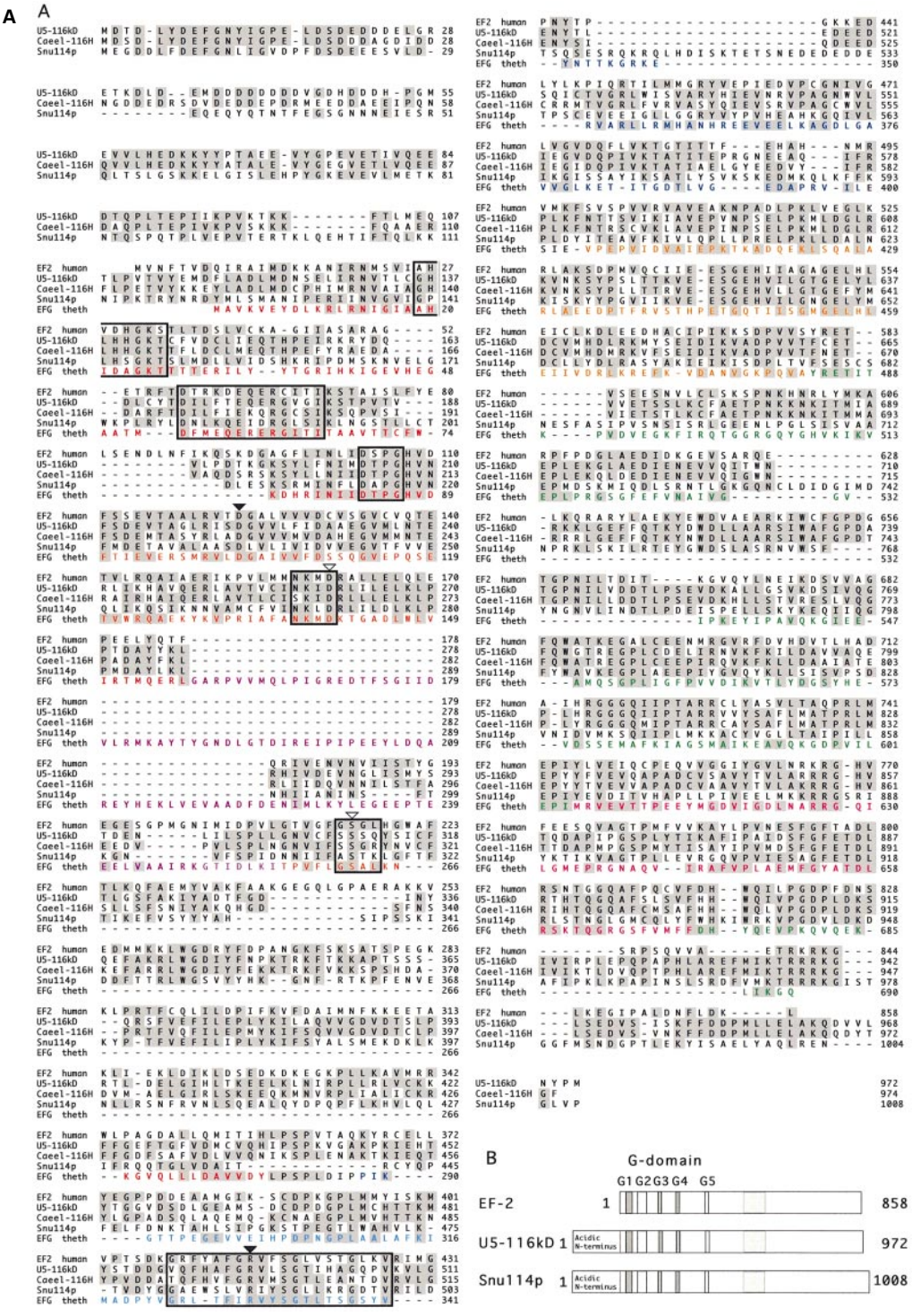


Fig. 1. Comparison of the primary structure of the human U5-specific 116 kDa snRNP protein with that of the yeast U5 protein Snu114p, the nematode Caeel-116H homolog and the ribosomal translocases EF-2 and EF-G. (**A**) Amino acid sequence alignment of the human elongation factor EF-2 (Swiss-Prot accession no. P13639), the U5-specific 116 kDa protein (U5–116kD), *C.elegans* 116 kDa homolog (Caeel-116H, GenBank U50193), the yeast U5-specific protein Snu114p (Swiss-Prot P36048) and *T.thermophilus* elongation factor EF-G (Swiss-Prot P13551). Gray boxes indicate identical amino acids. The conserved motifs G1, G2, G3, G4 and G5 of the G domain and the domain II conserved element are boxed (Dever, 1987; Bourne *et al.*, 1991; Ævarsson, 1995). The amino acid sequences corresponding to the five domains defined by the crystal structure of EF-G from *T.thermophilus* (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994), are color-coded as follows: G domain (red), G′ subdomain (purple), domain II (blue), domain III (orange), domain IV (green) and domain V (pink). Closed arrowheads indicate conserved amino acids involved in salt bridge formation in EF-G and EF-Tu (Ævarsson, 1995 and references therein), and open arrowheads indicate amino acids involved in the recognition of the guanine base in ribosomal elongation factors (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993; Czworkowski *et al.*, 1994). The three peptide sequences obtained by microsequencing were found in the predicted amino acid sequence at positions 65–91 (116 b), 326–340 (116 a) and 722–754 (116 c) (see also Materials and methods). (**B**) Diagram of the primary structure of EF-2, U5-116kD and the yeast U5-specific protein Snu114p. The conserved motifs G1, G3 and G4 of the G domain (Dever, 1987) are shown as shaded boxes, G2 and G5 (Bourne *et al.*, 1991; Ævarsson, 1995) as open boxes and the domain II conserved element (Ævarsson, 1995) as a hatched box. The mouse U5-116kD protein (GenBank U97079) is identical to the human protein, except for a methionine at position 36 which is missing in the mouse protein and a serine instead of threonine at position 362. Some of the aspartic acids found at positions 37–49 of the human U5-116kD are replaced by glutamic acid in the mouse protein (data not shown).

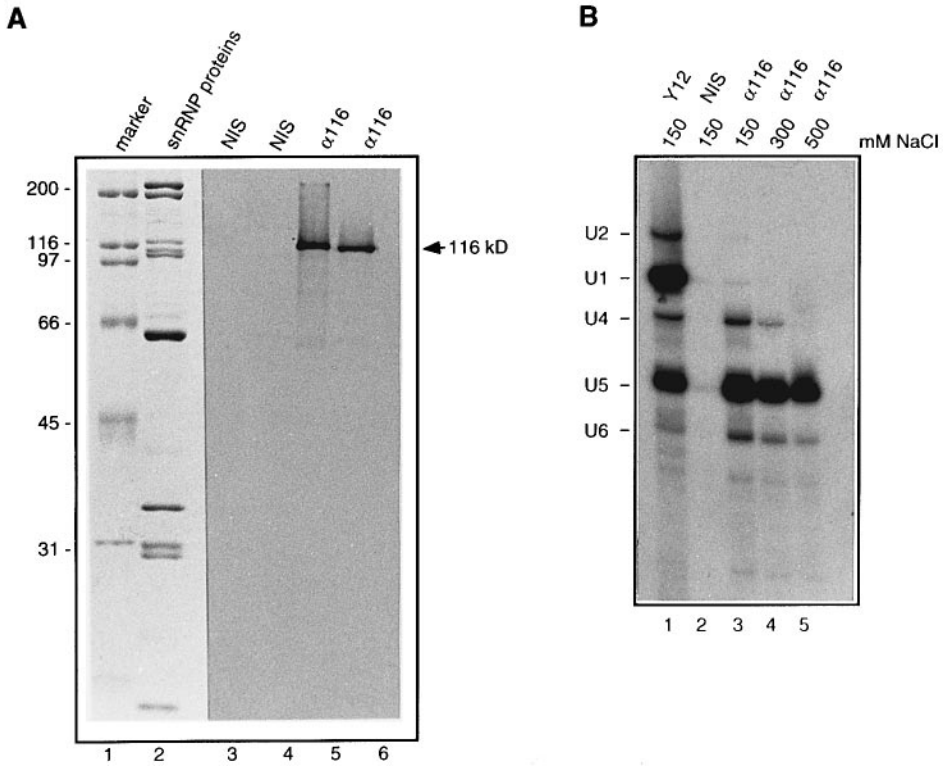


Fig. 2. Immunological characterization of the U5-116kD protein. (**A**) Anti-U5-116kD antibodies react specifically with U5-116kD on immunoblots. Total snRNP proteins, affinity-purified with monoclonal antibody H20, (10 μg/lane) or HeLa nuclear extract (10 μg/lane) were separated on a 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose. Lane 1, size markers (molecular weights are given on the left); lane 2, total snRNP proteins stained with Coomassie blue; lanes 3 and 5, total snRNP proteins probed respectively with non-immune serum and with antibody against the U5-116kD protein; lanes 4 and 6, HeLa nuclear extract probed respectively with non-immune serum and with antibody against the U5-116kD protein. The position of the U5-116kD protein is indicated on the right. (**B**) Specific precipitation of U4, U5 and U6 snRNPs by anti-U5-116kD antibodies. Immunoprecipitations were performed with anti-m_3G affinity-purified snRNP particles and various antibodies as described in Materials and methods. Immunoprecipitated snRNAs were detected by 3′ end-labeling with [^{32}P]pCp and subsequent separation on a denaturing 10% polyacrylamide gel. Immunoprecipitations were performed with the monoclonal antibody Y12 which reacts with all snRNPs (lane 1), non-immune serum (lane 2) and anti-U5-116kD antibodies (lanes 3–5). The concentration of NaCl employed during immunoprecipitation is indicated above each lane. The identities of the snRNAs are indicated on the left.

Three criteria were used to demonstrate that the protein product encoded by this clone is the U5-116kD protein. First, as stated above, all three peptide sequences obtained by microsequencing were found in the predicted amino acid sequence of the protein. One peptide was located in the amino-terminal portion of the protein. The other two were found in the central and carboxy-terminal portions, respectively (legend to Figure 1 and Materials and methods). Secondly, antibodies raised against the recombinant amino-terminal acidic region of the cloned U5-116kD protein reacted on immunoblots specifically with the native U5-116kD when proteins from purified snRNPs or HeLa cell nuclear extract were used as a source of antigen (Figure 2A, lanes 5 and 6, respectively). Finally, the anti-U5-116kD rabbit antiserum precipitated specifically the [U4/U6·U5] tri-snRNP complex from a mixture of total spliceosomal snRNPs when the immunoprecipitation assay was carried out at 150 mM NaCl (Figure 2B). At 500 mM NaCl, where the tri-snRNP complex dissociates into U5 and U4/U6 particles, anti-U5-116kD antibodies precipitated predominantly the U5 snRNP (Figure 2B), consistent with our previous biochemical observation that the U5-116kD protein is strongly associated with the U5 snRNP.

The U5-116kD protein is structurally closely related to the ribosomal translocases EF-2 and EF-G

A database search with the human U5-116kD protein sequence revealed that it is strikingly homologous to the eukaryotic translation elongation factor (ribosomal translocase) EF-2. With the exception of an amino-terminal acidic domain in U5-116kD (pI of this domain: 3.6), which comprises 109 residues rich in aspartic and glutamic acids (Figure 1A), the remainder of U5-116kD exhibits extensive phylogenetic conservation with respect to EF-2. Since crystal structures of EF-G from *Thermus thermophilus*, in both the GDP-bound and the nucleotide-free states, have recently been obtained (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994), a sequence comparison could be made between U5-116kD and ribosomal translocases with respect to the folding domains of EF-G. For clarity, the amino acid sequences corresponding to the five structural domains of EF-G, i.e. domains I (G domain) to V, are color-coded (Figure 1A) (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994). Using these EF-G structural domains for orientation, we have compared the sequences of U5-116kD and the phylogenetically conserved human ribosomal translocase EF-2. Upon close inspection, we find an overall 27–50% identity between U5-116kD and

the human EF-2 sequence (Figure 1A), depending on the region examined. There are only a few sites in U5-116kD at which amino acids have been inserted or deleted relative to EF-2. For example, positions 78–88, 198–202 and 239–250 in EF-2 or the carboxy-terminal positions 916–922 and 931–936 of U5-116kD represent such differences.

The G domain (Figure 1A, red), which is known to bind and hydrolyze GTP in both EF-2 and EF-G (reviewed by Bourne *et al.*, 1990, 1991), contains three well characterized consensus motifs, G1, G3 and G4 (Figure 1A and B), whose sequences and relative positions in U5-116kD, EF-2 and EF-G are strikingly similar (Figure 1A, sequences indicated by boxes) (Bourne *et al.*, 1991). G1 is the ATP/GTP-binding motif, or P-loop ([A,G]X_4GK[S,T]) (Walker *et al.*, 1982; Saraste *et al.*, 1990), and is found at positions 136–143 of U5-116kD. The G3 element (DXXG) spans positions 204–207, and G4 (NKXD) positions 258–261 (Figure 1A). A sequence corresponding to the G2 consensus motif (DX_nRGITI) that is often found in ribosomal GTPases (Bourne *et al.*, 1991) is located at positions 169–181 (Figure 1A); however, the consensus sequence IT has been replaced by VG at positions 179 and 180 in U5-116kD (Figure 1A). Moreover, the U5-116kD G domain sequence SSSQ (residues 310–313) matches reasonably well the G5 consensus sequence element (GSA[K,L]) and is found at the same position as the G5 element of ribosomal GTPases (Bourne *et al.*, 1991; Kjeldgaard *et al.*, 1993). The conservation of the serine residue at position 311 of U5-116kD is most significant, since the side chain of this serine and the main chain NH group of the downstream amino acid interact with the guanine base, as revealed by the three-dimensional structure of bacterial ribosomal elongation factors (Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993; Czworkowski *et al.*, 1994).

Ribosomal translocases from all species analyzed to date contain an insert in the G domain designated the G′ subdomain. Recently, based on the three-dimensional structure of EF-G from *T.thermophilus*, Ævarsson (1995) (see also Ævarsson *et al.*, 1994) re-evaluated previously published sequence alignments of the G domain of bacterial, archaeal and eukaryotic ribosomal translocases. According to the refined alignment, the G′ subdomain of EF-G (residues 158–255, purple in Figure 1A) is considered to be unique to bacteria, whereas in EF-2 there is only a small insert of 30 amino acids at this position (residues 179–209, Figure 1A). Moreover, following a short stretch of 11 amino acids (residues 210–220) which harbor the G5 consensus element of the G domain (Figure 1A), eukaryotic EF-2 contains another insert, called the G″ subdomain (residues 221–344, Figure 1A), which is not found in bacterial EF-G proteins. Like the ribosomal translocases, U5-116kD also contains a large insert in the G domain. Importantly, when this insert is aligned with those of EF-2 and EF-G according to Ævarsson (1995), a strong sequence homology between U5-116kD and EF-2 is observed (Figure 1A). Like EF-2, U5-116kD also contains a short G′ (residues 279–304) and a long G″ (residues 316–424) subdomain at equivalent positions, which share significant homology with the corresponding regions of EF-2 (35% identity and 70% similarity between G′, 28% identity and 55% similarity between G″ of EF-2 and U5-116kD).

As compared with the G domain (35% identity and 60% similarity), the region of EF-2 and U5-116kD that corresponds to domain II of EF-G (blue in Figure 1A) is less highly conserved (30% identity). Using the structure-based sequence alignment of translation factors, Ævarsson (1995) identified in domain II a single conserved sequence motif (GX[V,I,L,F]$X_{(3–4)}$[R,K][V,I,L,A]XXGX[V,I,L]XX-GXX[V,I,L]) that is found in all members of this family. This consensus sequence element is located in EF-G at positions 323–341, and in EF-2 at positions 408–427. Significantly, this sequence motif is also conserved in domain II of U5-116kD (positions 488–507, boxed in Figure 1A).

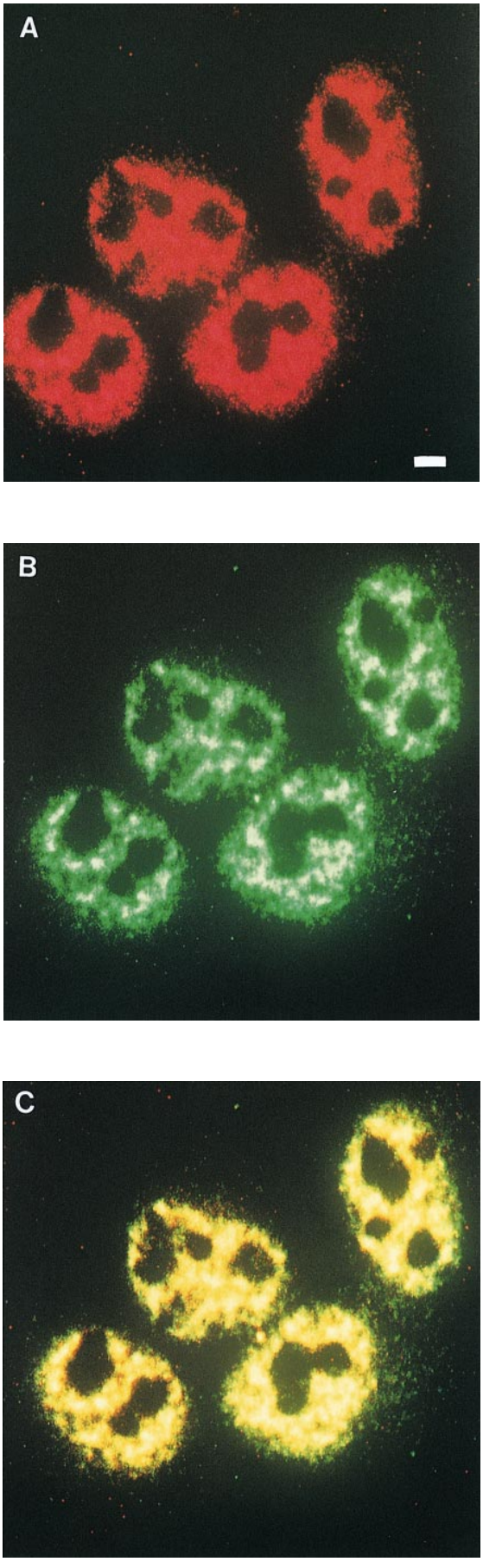
Finally, domain III of EF-G (orange in Figure 1A) aligns with sequences of EF-2 and U5-116kD, which share 45% identity and 66% similarity. The 160 carboxy-terminal amino acids of EF-2 and U5-116kD, which partially correspond to domains IV and V of EF-G, exhibit ~50% identity and 70% similarity (Figure 1A, green and pink, respectively). The pronounced evolutionary conservation between domain IV and V sequences of U5-116kD and the ribosomal translocase is of particular interest in view of the recently observed structural similarity between this region of EF-G and the tRNA anticodon stem–loop region (see Discussion).

Counterparts of U5-116kD in yeast, nematode and mouse

A database search with the amino acid sequence of the U5-116kD protein revealed close homologs in the yeast *S.cerevisiae* and the nematode *Caenorhabditis elegans*. The ORF of the yeast protein, previously designated Gin10, is encoded by the gene *YKL173w* on chromosome XI. Since Gin10 is an snRNP component (see below), we propose re-naming this protein Snu114p (Snurp 114 kDa protein) (Figure 1A and B). U5-116kD shares an overall 32% identity with Snu114p, and 74% identity and 86% similarity with its *C.elegans* counterpart (proposed name Caeel-116H for *Caenorhabditis elegans* U5-116kDa homolog; Figure 1A). Snu114p and Caeel-116H exhibit the same domain structure as discussed for U5-116kD, including an additional amino-terminal region that is rich in acidic residues (Figure 1A); this region of Snu114p and Caeel-116H exhibits 28 and 54% identity, respectively, with that of U5-116kD. Thus, this supports the notion that the *S.cerevisiae* and *C.elegans* proteins are functional counterparts of the U5-116kD and not of elongation factor EF-2. Moreover, both proteins possess the same evolutionarily conserved sequence motifs in their domains I, II and III (including the G″ subdomain), which exhibit spacing similar to those found in the U5-116kD protein (see Figure 1 and preceding discussion) and are essential structural pre-requisites for the proteins to function as GTPases. Finally, we have characterized a full-length cDNA encoding the U5-116kD counterpart from mouse (GenBank accession number U97079). The amino acid sequence of the mouse U5-116kD is strikingly homologous to the human U5-116kD (99% identity) (see legend of Figure 1).

Subcellular localization of U5-116kD in HeLa cells

Data presented in Figure 2 demonstrated that U5-116kD is tightly associated with the U5 and [U4/U6·U5] snRNPs



and would, therefore, predict a predominantly nuclear localization of this protein. In view of the unexpected high degree of overall homology with the ribosomal elongation factor EF-2, which is a cytoplasmic protein, we have investigated the subcellular distribution of U5-116kD by immunofluorescence microscopy. Immunolocalization of U5-116kD was carried out with affinity-purified antibody raised against the amino-terminal acidic domain of U5-116kD which is absent from EF-2 (Figure 1; for specificity of the antibody see Figure 2). As shown in Figure 3A, *in situ* labeling of HeLa cells with the anti-U5-116kD antibody resulted in a predominantly nuclear staining pattern, whereby the nucleoli and cytoplasm remained largely unstained. Since we were particularly interested in determining whether U5-116kD co-localizes *in situ* with nuclear speckles, which have been identified as typical snRNP-containing structures (for a review see Lamond and Carmo-Fonseca, 1993), HeLa cells were double-stained with anti-U5-116kD (Figure 3A) and monoclonal antibody Y12 (Figure 3B), which reacts with the snRNP Sm proteins (Lerner *et al.*, 1981). Consistent with the biochemical association of U5-116kD with U5 snRNPs, both antibodies exhibit a very similar nuclear staining pattern, in which 30–40 'speckles' are observed above a less intense general staining of the nucleoplasm (Figure 3). As seen in the confocal overlay, snRNPs and U5-116kD predominantly co-localize in the speckles (Figure 3C). Taken together, our data suggest that U5-116kD is associated predominantly with snRNP-containing structures *in vivo*.

Antibodies raised against the human U5-116kD inhibit pre-mRNA splicing in vitro

A possible role for the U5-specific 116 kDa protein in pre-mRNA splicing was investigated initially in HeLa nuclear extract. As a first approach, we performed splicing inhibition studies with affinity-purified antibodies raised against the amino-terminal region of the U5-116kD protein. The addition of these antibodies led to a block in the second step of the splicing reaction, as evidenced by the accumulation of splicing intermediates (Figure 4, lanes 6–10). Identical concentrations of non-specific immunoglobulin (Figure 4, lanes 2–4) or changes in the buffer conditions due to antibody addition (data not shown) had no effect. While these results suggest a role for U5-116kD in the second step of splicing, they do not exclude the possibility that U5-116kD is also involved in the first step, i.e. the anti-U5-116kD antibody might, for example, only partially inhibit the function of the 116kD protein.

U5-116kD binds GTP specifically, as shown by UV cross-linking

The presence of a G domain in U5-116kD with homology to EF-2 raised the intriguing question as to whether the

Fig. 3. Subcellular localization of U5-116kD in HeLa cells as investigated by immunofluorenscence microscopy. HeLa cells were double stained with affinity-purified antibodies specific for U5-116kD (**A**) and monoclonal anti-Sm antibody Y12 (**B**). (**C**) Virtual confocal overlays of HeLa cells double stained with anti-U5-116kD and Y12 antibodies. The yellow color highlights those nuclear structures which are decorated by both antibodies. U5-116kD is localized predominantly in the nucleus but not in the nucleoli or the cytoplasm. In the nucleus, U5-116kD clearly co-localizes with typical, snRNP-containing subnuclear domains, denoted speckles. The bar indicates 2 μm.

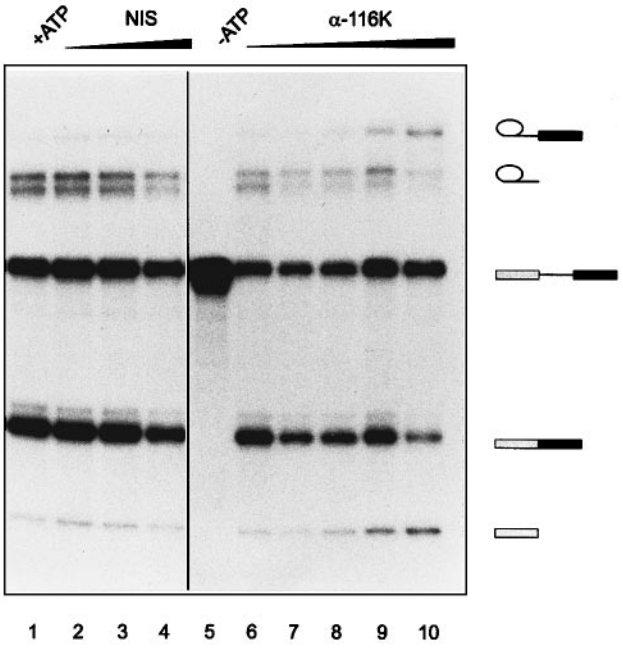


Fig. 4. Inhibition of step 2 of splicing by antibodies against the U5-116kD protein. Standard *in vitro* splicing assays (12.5 µl) performed with HeLa nuclear extract were incubated for 30 min on ice with various amounts of immunoglobulin purified on protein A–Sepharose. As controls, reactions without serum (with and without ATP) and reactions with non-immune serum were also performed. Lane 1: standard assay, no antibody. Lanes 2–4: standard assay plus 0.5, 1.0 or 2.0 µl respectively of the non-immune immunoglobulin preparation (5.3 µg/µl). Lane 5: standard assay omitting ATP; no immunoglobulin. Lanes 6–10: standard assay plus 0.5, 1.0, 2.0, 4.0 or 6.3 µl respectively of affinity-purified antibody against the amino-terminus of U5-116kD (0.1 µg/µl). The reactions were incubated for 60 min at 30°C, and the RNA subsequently was recovered by phenol extraction and ethanol precipitation and separated on a 10% denaturing polyacrylamide gel. The positions of the pre-mRNA, splicing intermediates and products are indicated on the right. From top to bottom: lariat-exon 2, excised lariat-intron, pre-mRNA, spliced mRNA, free exon 1.

U5-116kD protein is capable of binding GTP specifically. Since U5-116kD is complexed with the U5 snRNP, and may be functional solely in the context of an RNP particle, GTP binding was investigated with purified U5 snRNPs. UV cross-linking of radiolabeled GTP to purified U5 snRNPs was performed since it, in contrast to filter binding assays, allows the identification of the GTP-binding protein. As shown in Figure 5A (lane 1), only weak GTP–protein cross-links with apparent mol. wts of 92, 100, 116 and 200 kDa were visible above a reproducible smear of radioactivity in the gel. While the latter three signals superimposed with Coomassie-stained U5 snRNP proteins, a corresponding 92 kDa protein was present only in sub-stoichiometric amounts (Figure 5B, lane 1).

Next, we investigated the GTP cross-linking pattern upon the addition of the polyribonucleotide poly(U). This was done under the assumption that poly(U), either directly by binding to U5-116kD (for possible RNA-binding properties of U5-116kD see above and Discussion) or indirectly by binding to other U5 proteins such as the 200 kDa putative RNA helicase (Laggenbauer *et al*., 1996; Lauber *et al.*, 1996), might influence the GTP-binding properties of U5-116kD. Indeed, in the presence of poly(U), the U5-116kD cross-link was the predominant signal, while the intensity of the 92 kDa cross-link decreased and another band of ~94 kDa appeared (Figure 5B, lane 2). The 94 kDa band does not superimpose with any of the major snRNP proteins (Figure 5B) and must, therefore, be present in sub-stoichiometric amounts. The specificity of the GTP cross-links was investigated by a competition experiment with non-labeled NTP added in 75-fold excess over labeled GTP (Figure 5A, lanes 3–6). In the presence of poly(U), the U5-116kD protein binds GTP specifically, since only an excess of cold GTP reduced the cross-link signal significantly (Figure 5A, lane 4); neither ATP, CTP nor UTP competed to a significant extent (Figure 4A, lanes 3, 5 and 6). The ~94 kDa protein also binds GTP in a specific manner, while the U5-200kD protein appears to interact non-specifically with GTP (Figure 5A, lanes 3–6). Interestingly, preliminary data (not shown here) indicate that other polynucleotides, such as poly(A), stimulate the formation of the U5-116kD protein cross-link less efficiently.

In order to demonstrate that the U5-116kD cross-linked protein corresponds to the cloned 116 kDa U5-specific protein and not to a contaminant of the same molecular weight, subsequent to cross-linking, immunoprecipitations were performed with an antibody that was raised against the recombinant U5-116kD protein. Prior to the addition of the anti-U5-116kD antiserum, the U5 snRNPs were dissociated by the addition of detergents to avoid the co-immunoprecipitation of other proteins by means of their association with U5-116kD. Under these conditions, the anti-U5-116kD antiserum efficiently and exclusively precipitated the cross-linked U5-116kD protein (lanes 9, 10, 12 and 13), while no significant precipitation was observed with the non-immune serum (Figure 5A, lane 7). The specificity of the antibody reaction was supported further by the fact that immunoprecipitation of the cross-link signal was only observed in those reactions that yielded a 116kD–GTP cross-link; i.e. while the anti-U5-116kD antibody precipitated the U5-116kD protein in all reactions, as demonstrated by Coomassie staining of the immunoprecipitates (Figure 5B, lanes 8–13), the cross-link signal was only precipitated from reactions that contained poly(U) and where no GTP competitor was added (Figure 5A). The above results thus demonstrate that the native 116 kDa U5 snRNP protein binds GTP as part of the intact U5 snRNP.

The yeast counterpart of U5-116kD is essential for cell viability and pre-mRNA splicing in vivo

The identification of a yeast counterpart of U5-116kD allowed us to study the function of this protein *in vivo*. Prior to these studies, we established that Snu114p is indeed an intrinsic protein of the yeast U5 snRNP. This conclusion is based on the following observations: (i) Snu114p co-purifies with yeast U5 snRNA during stringent purification of U5 snRNPs by anti-m_3G-cap immunoaffinity chromatography followed by Ni-NTA chromatography and glycerol gradient centrifugation, and (ii) fractionation of purified yeast U5 snRNP proteins on an SDS gel, followed by sequencing of the peptides by mass spectrometry, clearly demonstrated the presence of Snu114p in the U5 snRNP (Neubauer *et al.*, 1997 and data not shown).

Using yeast genetics, we first determined whether the

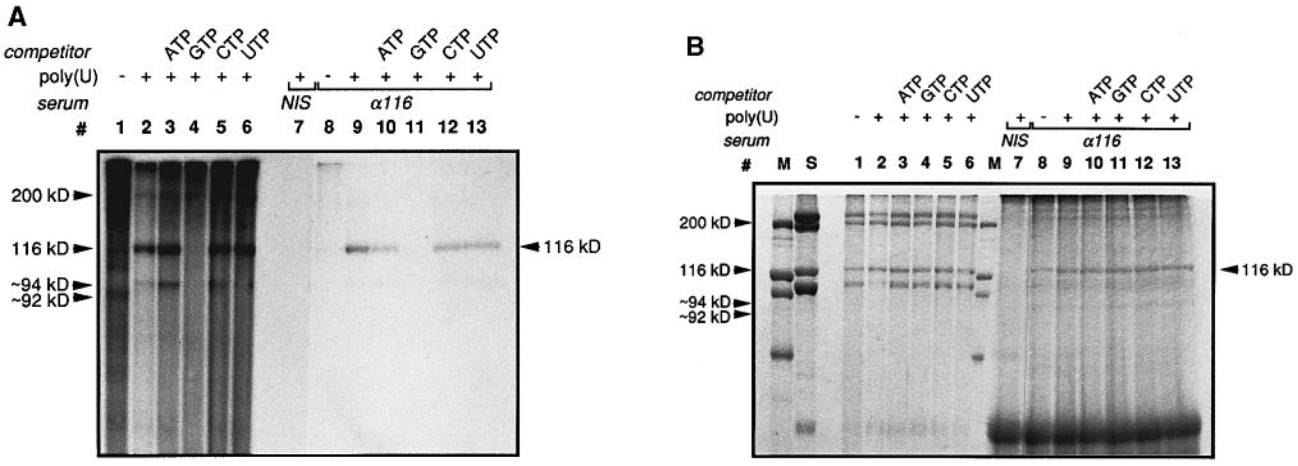


Fig. 5. UV cross-linking of [α-^{32}P]GTP to purified U5 snRNPs. (**A**) Autoradiography of proteins cross-linked to [α-^{32}P]GTP in the absence (lane 1) or presence (lanes 2–6) of poly(U). The NTP specificity of the cross-link signals was determined by competition with non-labeled nucleotide, as indicated at the top (lanes 3–6); only cold GTP abolishes the radioactive labeling of the 116 kDa protein. The identity of the U5-116kD protein as a GTP cross-link signal was confirmed by immunoprecipitation of cross-linked proteins that had been dissociated from U5 snRNPs before incubation with anti-U5-116kD antibody (lanes 8–13). The immunoprecipitation reactions shown in lanes 8–13 correspond to the cross-link reactions in lanes 1–6. Lane 7 shows a control reaction of cross-linked U5 snRNP proteins using non-immune serum. (**B**) Coomassie staining of proteins from the cross-link reactions. The lane numbers correspond to the lanes displayed in (A). Lanes M, molecular weight standards of 200, 116, 96, 66, 45, 31 and 21 kDa. Lane S, protein composition of 10 μg of purified U5 snRNP. An arrow to the right indicates the position of the U5-116kD protein.

yeast U5 snRNP is essential for cell growth. To this end, the *SNU114* gene was cloned by PCR amplification using specific primers and yeast genomic DNA as template. Subsequently, the *SNU114* gene was disrupted in a diploid strain by homologous recombination (Scherer and Davis, 1979). A 1.47 kb fragment of the *SNU114* gene was replaced by the selective marker *HIS3*. The deleted *snu114Δ::HIS3* allele was inserted at the *SNU114* locus in a diploid strain by homologous recombination. Correct integration at the *SNU114* locus was verified by Southern analysis (data not shown). Diploid cells were viable, indicating that the mutation was recessive. The heterozygous diploid was sporulated and tetrads were dissected. All tetrads gave rise to two viable and two non-viable spores, and the two viable spores exhibited a His$^-$ phenotype, confirming that cells containing only the disrupted *SNU114* gene were non-viable (data not shown). The non-viable spores germinated and divided two or three times before final cell death, indicating that the *SNU114* gene is essential for yeast cell growth and not for spore germination. The viability of cells containing the disrupted *SNU114* gene was restored by transformation with a single-copy plasmid containing the wild-type *SNU114* gene under control of its natural promoter (data not shown).

Since *SNU114* is essential for viability, we studied the effect of its genetic depletion on the splicing of pre-mRNA. We constructed a conditional lethal allele of *SNU114* cloned under the control of the *GAL1* promoter (Johnston and Davis, 1984). This promoter is active when cells are grown in galactose, but repressed by growth in glucose. Growth of the conditional strain is galactose dependent, i.e. the cells grow on galactose as the sole carbon source but not on glucose (see Figure 6). When the growth rates in galactose media were compared with those in glucose, the doubling times were almost indistinguishable for the first 12 h. However, after 24 h, cell growth stopped completely, indicating that depletion of *SNU114* arrests cell growth (Figure 6A). To investigate the effect of *SNU114* depletion on pre-mRNA splicing, we examined splicing of pre-U3 RNA by primer extension. Both yeast U3 genes *SNR17A* and *SNR17B* (Hughes *et al.*, 1987; Myslinski *et al.*, 1990) contain introns. An oligonucleotide complementary to the identical exon 2 of U3A and U3B was labeled, annealed to total RNA which was extracted from the conditional strain grown in galactose or shifted to glucose medium, and extended with reverse transcriptase. Since the two U3 genes have introns of different sizes, accumulation of both pre-U3A and pre-U3B can be detected in the same reaction (Figure 6B). Since U3 is a relatively stable and abundant RNA, we did not expect to observe a decrease in the amount of spliced U3 during depletion of Snu114p. Interestingly, a significant increase in both pre-U3 RNA species could be detected already after 4 h following the shift to glucose, with no further increase of pre-mRNA levels after 8 and 12 h (Figure 6B). Growth of this strain in galactose shows only background levels of pre-U3A at all time points, while the parental strain, which contains two chromosomal copies of *SNU114*, does not accumulate any detectable pre-U3 RNAs. These data thus demonstrate that Snu114p is an essential splicing factor *in vivo*.

Single amino acid substitution in the P-loop motif of Snu114p leads to a lethal phenotype in yeast

Since Snu114p is essential for cell viability and pre-mRNA splicing *in vivo*, it was possible to test, by site-specific mutagenesis of the G domain, whether GTP binding might contribute to its function *in vivo*. We thus introduced an amino acid substitution known to affect the extent of NTP binding in other G domain-containing proteins. In this mutant, the glycine at position 145 of the P-loop was replaced with an arginine (GKT→RKT) (Figure 7A). To study the effect of this mutation on growth, we used a yeast strain in which the chromosomal *SNU114* locus was disrupted and a wild-type copy of the gene was carried on a plasmid with the *URA3* marker (i.e. YPF8 cells, see Materials and methods). The *snu114*G145R ORF was cloned into a *TRP1* centromeric plasmid down-

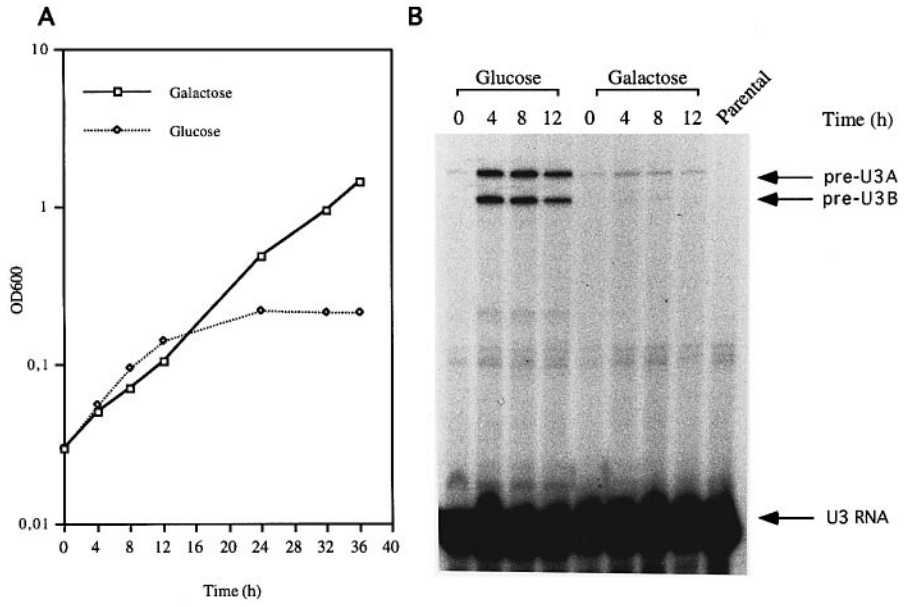


Fig. 6. (**A**) Arrest of cell growth by depletion of Snu114p. Strain YPF14 was grown to mid-logarithmic phase in 2% galactose medium, harvested, washed and resuspended in sterile water. Half of this suspension was used to inoculate either pre-warmed galactose or glucose media to an initial OD_{600} of 0.05. Cultures were diluted to keep all OD readings below 0.6, maintaining the cells in logarithmic growth. The OD_{600} of the cultures was monitored and plotted as a function of time. (**B**) Requirement for Snu114p for pre-mRNA splicing *in vivo*. Total RNA was extracted from cells grown in galactose or at various times following a shift to glucose medium, and primer-extended with an oligonucleotide probe complementary to a region common to both U3A and U3B. As a control, pre-U3 levels in the parental strain YPF1 were also monitored (Parental). The positions of pre-U3A, pre-U3B and mature U3 RNA are indicated on the right.

stream of the *SNU114* wild-type promoter sequence. YPF8 cells were transformed with the mutant pRS314/*snu114*G145R and transformants were cured of the wild-type *SNU114* gene by plating on 5-fluoroorotic acid (5-FOA)-containing plates (Boeke *et al.*, 1987) (Figure 7B). As shown in Figure 7C, the loss of the wild-type plasmid clearly leads to a lethal phenotype, as evidenced by the fact that yeast cells containing *snu114*G145R are unable to grow on 5-FOA plates. This result indicates that the wild-type sequence of the P-loop is required to support cell growth, and strongly suggests that GTP binding and perhaps hydrolysis are necessary for Snu114p function *in vivo*.

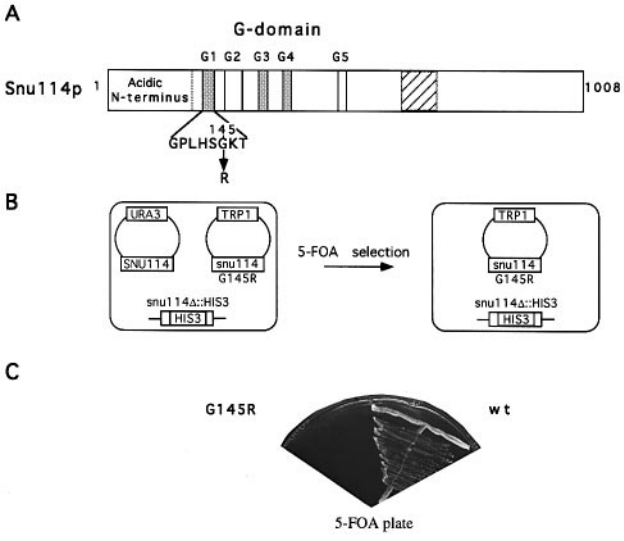


Fig. 7. A single amino acid change in the P-loop of Snu114p leads to a lethal phenotype. (**A**) Diagram of the primary structure of the U5-specific protein Snu114p as described in the legend of Figure 1. (**B**) Plasmid-shuffling strategy. Yeast cells which have an interrupted *SNU114* locus and contain a *URA3* plasmid harboring the wild-type *SNU114* gene were transformed with a *TRP1* plasmid containing the *SNU114* gene with a point mutation in the P-loop (*snu114*G145R). Transformants were cured of the wild-type *SNU114* gene by plating on 5-fluoroorotic acid-containing plates which specifically remove the *URA3* plasmid (5-FOA). (**C**) Growth comparison on a 5-FOA plate of yeast cells transformed with *TRP1* plasmids carrying the wild-type (wt) or the mutant (G145R) *SNU114* genes.

Discussion

An evolutionarily conserved spliceosomal snRNP protein is a close homolog of the ribosomal elongation factor EF-2

Here we have identified the human U5 snRNP-specific 116 kDa protein (U5-116kD) as a close homolog of the ribosomal elongation factor EF-2. Aside from an amino-terminal acidic domain which is not present in EF-2 (Figure 1), the human U5 protein exhibits the same domain structure as EF-2, including the G domain. The overall sequence identity between U5-116kD and EF-2 ranges from 27 to 50%, depending on the region compared (Figure 1). U5-116kD resembles EF-2 more closely than it does other eukaryotic or prokaryotic elongation factors (data not shown). For example, the large insert of the G domain of U5-116kD (residues 316–424) exhibits a strong homology with the G″ subdomain of EF-2 (Figure 1A). Since the G″ subdomain is considered to be specific for the EF-2 family of eukaryotic elongation factors (see Ævarsson, 1995 and references therein for a review), this suggests that the two proteins may also share, at least in part, functional properties (see below).

As would be expected for an essential snRNP protein, the yeast *S.cerevisiae* (denoted Snu114p) and the nematode *C.elegans*, possess evolutionarily conserved homologs of

the human U5-116kD (Figure 1A). Interestingly, the yeast and *C.elegans* homologs also contain the acidic amino-terminal stretch of >100 amino acids, which is not found in the ribosomal elongation factors (Figure 1). Thus, this supports the notion that the *S.cerevisiae* and *C.elegans* proteins are functional counterparts of the U5-116kD and not of elongation factor EF-2. For yeast, we could indeed demonstrate by biochemical fractionation that Snu114p is associated with the yeast U5 snRNP (Neubauer *et al.*, 1997, and data not shown).

U5-116kD/Snu114p is essential for pre-mRNA splicing

Several pieces of experimental evidence support our notion that U5-116kD/Snu114p plays an essential role in pre-mRNA splicing. First, a functional *SNU114* gene is essential for yeast cell viability. Secondly, and most importantly, genetic depletion of Snu114p results in accumulation of unspliced pre-U3 RNAs, demonstrating that Snu114p is an essential splicing factor *in vivo* (Figure 6). Thirdly, antibodies specific for U5-116kD inhibited pre-mRNA splicing in a HeLa nuclear extract (Figure 4). The low level of splicing inhibition observed may be due to the fact that the antibody used was raised exclusively against the amino-terminal acidic domain of U5-116kD and not against potentially more functionally significant regions of the protein. We are currently raising monoclonal antibodies against other regions of the U5-116kD protein, in particular the G domain and domains IV and V (see below), in order to investigate in more detail the contribution of U5-116kD to pre-mRNA splicing *in vitro*. We have also attempted to carry out immunodepletion/complementation studies with U5-116kD but thus far have been unsuccessful. This appears to be due partly to the fact that U5-116kD is associated with several other U5-specific proteins, making it difficult selectively to deplete U5-116kD from nuclear extracts (data not shown).

U5-116kD/Snu114p is a GTP-binding protein and an intact G domain of Snu114p is essential for yeast cell viability

Apart from the identification of U5-116kD/Snu114p as a G domain-containing protein (Figure 1A), the data presented here raise the interesting possibility that GTP binding may be related to its function as a splicing factor. The presence of GTPase consensus elements in U5-116kD provides a powerful argument that the function of this protein involves both the binding and hydrolysis of GTP. All of the conserved consensus motifs of the G domain, including G1, G3 and G4, as well as two less obvious elements, G2 and G5, are well conserved in terms of sequence and relative spacing within the G domain of U5-116kD, when compared with the sequence of ribosomal translocases (Figure 1). Most significantly, all of the residues known from the crystal structure of ribosomal elongation factors to play an important role in GTP binding specificity (Berchtold *et al.*, 1993) are also found in the appropriate G domain consensus elements of U5-116kD. These include asparagine, lysine and aspartic acid in the G4 NKID motif, as well as serine together with the main chain NH group of the downstream residue in the G5 SSSQ motif (Figure 1A). The putative GTPase activity of U5-116kD is supported further by the observation that U5-116kD and the ribosomal translocases also share functionally important, conserved sequence motifs in regions other than the G domain. For example, domain II of U5-116kD contains a sequence at positions 488–507 that perfectly matches the conserved sequence motif located in domain II of all ribosomal translation factors (Figure 1A) (Ævarsson, 1995). In the crystal structure of EF-G and EF-Tu·GTP, this region of domain II is located at the interface of the G domain. Specifically, the conserved arginine residue at position 8 of the domain II consensus element forms a salt bridge with a conserved aspartate residue in the G domain. Strikingly, both residues are conserved in the U5-116kD protein (R495 and D223, Figure 1A), indicating that not only are the individual domains I and II of the U5-116kD structurally highly homologous to those of the ribosomal translocases, but also the domain contacts (i.e. the quaternary structure of this functional unit could be at least partially conserved).

We have at least two direct lines of evidence that the G domain of U5-116kD/Snu114p could be important for the function of this protein. First, by UV cross-linking we have shown that U5-116kD as part of isolated U5 snRNPs binds GTP specifically (Figure 5). Interestingly, the GTP cross-link to U5-116kD could be enhanced significantly upon addition of poly(U) to U5 snRNPs (Figure 5). Whether this effect is due to direct binding of poly(U) to U5-116kD or, indirectly, via other U5 proteins, is not yet clear. Second, a point mutation in the P-loop of the G domain of Snu114p (G145R), expected to inhibit GTP-binding activity, was lethal (Figure 7). Thus, this provides strong support for the idea that GTP binding (and most likely also hydrolysis) is essential for the function of Snu114p/U5-116kD. While we presently cannot rigorously exclude the possibility that the lethal phenotype of this mutant is due to the inhibition of a cellular process other than, or in addition to, splicing, we do not think that it is very likely for the following reasons. We have shown that U5-116kD is tightly associated with U5 snRNP and is a major protein component of purified 20S U5 and [U4/U6·U5] tri-snRNPs (Behrens and Lührmann, 1991; this work). Moreover, U5-116kD has also been identified as an intrinsic component of highly purified mammalian spliceosomal complexes B and C (Bennett *et al.*, 1992; Gozani *et al.*, 1994; U5-116kD is identical to SAP116), demonstrating that U5-116kD does not interact with spliceosomes in a transient manner. Most importantly, and consistent with the biochemical association of U5-116kD with U5 RNA, U5-116kD was localized predominantly in the nuclear compartment of HeLa cells, as shown by immunofluorescence microscopy (Figure 3). In the nucleus, U5-116kD co-localizes with nuclear speckles which have been identified as typical snRNP-containing structures. Thus we have, to date, no evidence for a free non-U5 snRNP-bound pool of U5-116kD in the cell. Taken together, our results suggest that U5-116kD (or, in yeast, Snu114p) functions in close association with U5 snRNP and has a function related to splicing that involves the binding and, perhaps, hydrolysis of GTP. We note, however, that we presently cannot distinguish between a direct role for the U5-116kD GTPase in the spliceosome or in the post-spliceosomal recycling of snRNPs (see below).

In an effort to test the hypothesis that GTP may be involved in splicing, we have investigated whether non-

hydrolyzable GTP analogs such as γS-GTP or GMPPNP affect pre-mRNA splicing in HeLa nuclear extracts. While these GTP analogs reproducibly and specifically inhibited splicing, significant inhibition was only observed at concentrations of γS-GTP or GMPPNP >4 mM (data not shown). Currently, we cannot explain why the concentration of GTP analogs required to compete for the binding of GTP should be so high, since inhibition by such analogs of, for example, protein synthesis could be observed at substantially lower concentrations (Kaziro, 1978). Moreover, we cannot exclude that the affected protein is not exclusively U5-116kD, but rather one or more additional factors required for pre-mRNA splicing *in vitro*.

Possible roles of U5-116kD in light of the structure and function of the ribosomal translocase

The U5-116kD protein shares a strong overall homology with the ribosomal translocase EF-2 (Figure 1), and it is expected that U5-116kD will have a domain structure similar to that of the bacterial ribosomal translocase EF-G (see discussion above). While it is at present unknown to what extent the functions of these proteins will prove to be similar (see discussion above), it is tempting to speculate that there is a common mechanistic principle at work in the ribosome and the spliceosome. Both the ribosome and spliceosome are multifactorial RNP complexes which contain both stably and transiently associated proteins. EF-2 (EF-G) is a GTP-dependent translocase. Following association of GTP-bound EF-G with a ribosome containing a peptidyl-tRNA bound at the A site and a deacylated tRNA at the P site (for simplicity only the A and P sites of tRNA binding are considered), hydrolysis of GTP facilitates an EF-G-mediated translocation of peptidyl-tRNA and mRNA from the A to the P site (for review, see Abel and Jurnack, 1996). Recently, experimental evidence has been provided that EF-G may possibly act as a motor protein in the ribosome (Rodnina *et al.*, 1997). Like the ribosome, the spliceosome undergoes many conformational rearrangements at the RNA and protein level (see Introduction). It is thus conceivable that the U5-116kD protein may mediate one or more of these RNA or protein conformational transitions, and act like a 'spliceosomal translocase'.

Aside from a role for U5-116kD and its yeast counterpart in the spliceosome, which is similar to that of the ribosomal translocases, additional functions could also be envisaged. It is not known whether U5-116kD binds specifically to proteins, RNA or both. In this context, it is interesting that the polyribonucleotide poly(U) strongly stimulated GTP binding by U5-116kD. As observed for domains III, IV and V in EF-G, the corresponding domains in U5-116kD probably also share structural homologies with RNA-binding proteins (Ævarsson *et al.*, 1994; Czworkowski *et al.*, 1994), and may therefore interact directly with RNA. U5-116kD could be involved in splice site selection, for example by binding to the polypyrimidine tract and scanning until the 3′ splice site is recognized. As discussed in the Introduction, the 25S [U4/U6·U5] tri-snRNP complex most likely dissociates and reassociates during splicing. At present, little is known about how the dissociated U4, U6 and U5 snRNPs reassemble, and a GTPase, such as the U5-116kD protein, could also be envisaged to play a role in this process.

A comparison of the three-dimensional structure of the elongation factor EF-G with the recently determined structure of the ternary complex EF-Tu·GDPNP·Phe-tRNA revealed that domain IV of EF-G is structurally similar to the anticodon stem–loop of the tRNA, and parts of domains III and V with the rest of the tRNA molecule (Nissen *et al.*, 1995). This molecular mimicry lends support to the idea that domain IV may have a role in the catalysis of translocation (Czworkowski *et al.*, 1994), and it is thought that domain IV may compete with the ternary complex at the ribosomal A site, thereby forcing the system toward peptidyl-tRNA translocation (Nissen *et al.*, 1995; Abel and Jurnak, 1996). Since the same domains appear to be present in U5-116kD and are structurally highly homologous to those of EF-2 (~50% identity), it is conceivable that this spliceosomal protein also mimics an RNA stucture. As mentioned earlier, several RNA tertiary structures participate in splicing and undergo conformational transitions. If U5-116kD could compete for the binding site of such an RNA structure, it might also be able to modify the structure of that site. Future mutagenesis studies of Snu114p/U5-116kD, in particular of domains III–V, should help to answer the questions raised above.

Materials and methods

Isolation and microsequencing of snRNP proteins

HeLa cells were grown in suspension culture, as described by Lauber *et al.* (1996), or obtained from Computer Cell Culture Company (Mons, Belgium). Nuclear extracts were prepared by the method of Dignam *et al.* (1983). U1, U2, U5 and U4/U6 snRNPs were purified from nuclear extract in buffer C-5 [20 mM HEPES/KOH, pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA, pH 8; 0.5 mM dithioerythritol (DTE); 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 4 μg/ml leupeptin; 5% v/v glycerol] by affinity chromatography using the monoclonal antibody H20, covalently attached to CNBr-activated Sepharose 4B (Pharmacia) (Bochnig *et al.*, 1987). snRNPs were eluted with the nucleoside m^7G in buffer C-5 (Bach *et al.*, 1990).

snRNP proteins were extracted from 20 mg of purified snRNPs and fractionated as described by Lauber *et al.* (1996). Partial amino acid sequences of tryptic peptides of the snRNP proteins were determined by microsequencing on an ABI 477A protein sequencer.

The following peptide sequences were obtained from U5-116kD: 116 a, IYADTFGDINYQEFA; 116 b, YYPTAEEVYGPEVETIVQEEDTQPLTE; 116 c, YDWDLLAARSIWAFGPDATGPNILVDDTLPSEV.

Database search

A database search was carried out on the NIH mail server using the TBLASTN and BLASTP programs (Altschul *et al.*, 1990). A 3.8 kb sequence, with an ORF matching the peptide sequences of U5-116kD, is the human cDNA clone with accession no. D21163 (Nomura *et al.*, 1994). A gene derived from chromosome XI of *S.cerevisiae* (*YKL173w*) codes for Snu114p (accession nos Z28173, P36048), which is homologous to U5-116kD (Dujon *et al.*, 1994). A gene derived from chromosome III of *C.elegans* (locus *CELZK328*), codes for Caell-116H (accession no. U5-193) (Wilson *et al.*, 1994).

Oligonucleotides used in this work

1, 5′-GGGGGGCTCGAGGTAGCGTATACTCTGGCA-3′; 2, 5′-GGGGGGGAGCTCGCCATTCTCTATATACCG-3′; 3, 5′-GCTCGGGTCGACAGTACGGATTAGAAGCCG-3′; 4, 5′-CTGGGGAGATCTGGCCGCAAATTAAAGCCT-3′; 5, 5′-GCTCGGGGATCCATGGAAGGTGACGATTTA-3′; 6, 5′-CTGGGGCTCGAGTCACGGTACTAAGCCATT-3′; 7, G145R-1 5′-CCATCAAAGAGGTCTTACgTGAGTGAAGAGGTCC-3′; 8, G145R-2 5′-GGACCTCTTCACTCAcGTAAGACCTCTTTGATGG-3′; 9, 5′-TGAAAACACGTTTCCCTTCG-3′; 10, 5′-GACTCCACTGGTAGAGCCGG-3′; 11, U3 exon 2, 5′-CCAAGTTGGATTCAGTGGCTC-3′. Underlined sequences indicate the following

restriction sites: 1 and 6, *Xho*I; 2, *Sac*I; 3, *Sal*I; 4, *Bgl*II; 5, *Bam*HI; and the following substitutions: 7 and 8, G145R, GGT→CGT.

cDNA cloning and sequencing

The cDNA clone with accession number D21163 encoding a protein (U5-116kD) of human myeloblast was kindly provided by S.Tabata (Nomura *et al*., 1994). Characterization of the cDNA clone confirmed the published sequence. The myeloblast cDNA was also used for the isolation of a mouse cDNA encoding the full-length mouse U5-116kD protein. Sequence analysis was carried out on exonuclease III/nuclease S1 deletion subclones using a nested deletion kit (Pharmacia) or by using primers designed from the new sequence. Sequences were determined manually by the dideoxy termination method and with an automated DNA sequencer (Applied Biosystems) using *Taq* polymerase and double-stranded templates (PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit, Applied Biosystems).

Antibody production

Antibodies were raised against the amino-terminal acidic domain of U5-116kD, expressed as a GST fusion protein, according to Lauber *et al.* (1996). Anti-U5-116kD antibodies were affinity purified on columns containing gel-purified fusion protein coupled to CNBr-activated Sepharose according to the manufacturer's protocol (Pharmacia). All steps were carried out at 4°C, as described by Lauber *et al*. (1996).

Immunofluorescence microscopy

HeLa cells, grown on glass coverslips to ~70% confluency, were processed for immunofluorescence studies at room temperature. Coverslips were rinsed three times for 5 min with cold phosphate-buffered saline (PBS), fixed with 5% (v/v) paraformaldehyde (Merck) for 10 min, rinsed three times with PBS and permeabilized with PBS/0.5% (v/v) Triton X-100 (Sigma) for 5 min. After washing three times for 5 min with cold PBS, the cells were incubated for 1 h with the primary antibodies diluted (1:1000) in PBS/1% bovine serum albumin (BSA). After washing three times for 5 min with PBS, the cells were incubated for an additional hour with either Cy2-conjugated GAR (goat anti-rabbit) or Cy3-conjugated DAM (donkey anti-mouse) secondary antibodies (Amersham) diluted (1:1000) in PBS/1% BSA. Finally, coverslips were washed three times, air dried, and mounted with Fluoprep. Fluorescence microscopy was carried out using a confocal laser scanning microscope (Zeiss) with excitation wavelengths of 488 nm (Cy2) and 543 nm (Cy3). Each channel was recorded independently, and photographs were taken from an Imagecorder plus (Focus).

In vitro splicing assays

Splicing was performed with ^{32}P-labeled pre-mRNA derived from the adenovirus 2 major late transcription unit. SP6 run-off transcripts, synthesized *in vitro* from pSP62Δi (Frendewey and Keller, 1985), were capped with G(5′)ppp(5′)G (Pharmacia) and had a specific activity of 4×10^6 c.p.m./pmol. Splicing reactions were carried out *in vitro* according to Krainer *et al*. (1984). Standard assays contained 2.5 µl of nuclear extract (20% final concentration), 3 mM $MgCl_2$, 60 mM KCl, 10 mM creatine phosphate, 2 mM ATP, 1.5 µl ^{32}P-labeled pre-mRNA (3×10^4 c.p.m.) and Tris–HCl (40 mM, pH 7.4) or antibody solution in a final volume of 12.5 µl. Creatine phosphate was not included in inhibition assays which utilized GTP or its analogs. For antibody inhibition of splicing activity *in vitro*, standard splicing reaction mixtures were pre-incubated with increasing concentrations of affinity-purified anti-U5-116kD antibodies for 30 min on ice, followed by incubation at 30°C for 60 min The reactions were stopped as described by Lauber *et al.* (1996).

GTP cross-linking assay

UV-induced cross-linking of GTP was performed essentially as described by Laggerbauer *et al.* (1996). 20S U5 snRNPs were obtained by anti-m_3G immunoaffinity purification and purified further by anion-exchange FPLC and glycerol gradient centrifugation. Then 2–3 pmol of 20S U5 snRNPs were pre-incubated for 15 min at 37°C with 40 µCi of [α-^{32}P]GTP (13.3 pmol) in the presence or absence of poly(U) (0.6 µg/µl) and 75-fold excess of non-labeled NTP, respectively. After pre-incubation, the samples were transferred to ice and cross-linked for 7.5 min. The cross-linked proteins were monitored by electrophoresis on SDS–12% polyacrylamde gels, followed by Coomassie staining and autoradiography.

Immunoprecipitation of snRNP particles and cross-linked proteins

For immunological detection of proteins, HeLa nuclear extract or affinity-purified snRNPs were separated on SDS–polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schüll). The blots were probed with anti-U5-116kD antibodies as described by Lauber *et al.* (1996). Immunoprecipitation of snRNPs and detection of [^{32}P]pCp-labeled snRNAs was carried out essentially as described by Lauber *et al.* (1996). In order to establish that the U5-116kD protein was cross-linked to GTP, after cross-linking the reactions were incubated with either anti-U5-116kD or non-immune serum that had previously been bound to protein A–Sepharose (Pharmacia) and washed with PBS (pH 8.0). BSA (10 µg/µl) was added to the coupling reactions. After washing with PBS (pH 8.0), the cross-linked samples were added to the coupled antibody, the volume was adjusted with PBS (pH 8.0) to 350 µl and the samples were incubated at 4°C for 1 h. The U5 snRNP was dissociated prior to incubation with antibody by heating cross-linked samples at 70°C for 5 min in the presence of 1% SDS (w/v) and subsequently adding Triton X-100 to 5% (v/v). After washing with PBS (pH 8.0), the immunoprecipitated proteins were separated from protein A–Sepharose by heating at 90°C for 3 min in a volume of 30 µl. This step was repeated twice. The pooled supernatants were precipitated with acetone and the proteins analyzed as described for the cross-linking assay.

Yeast manipulation and disruption of SNU114

Standard methods were used for growth and maintenance of yeast (Guthrie and Fink, 1991). Transformation of yeast cells with DNA was carried out by the lithium acetate method (Ito *et al.*, 1983). The *SNU114* gene was cloned by PCR amplification of yeast genomic DNA using two primers complementary to the 5′- and 3′-untranslated regions of the gene (oligonucleotides 1 and 2). The PCR reaction was carried out as follows: one cycle (5 min at 95°C), 30 cycles (30 s at 95°C, 30 s at 55°C, 3 min at 72°C), one cycle (5 min at 72°C). *Taq* Plus or *Pfu* polymerases (Stratagene) were used to ensure high-fidelity polymerization. PCR amplification resulted in a unique product with the expected size of 3.5 kb, which was cloned into pRS316 (Sikorski and Hieter, 1989) at the *Xho*I–*Sac*I sites to generate pRS316/*SNU114*. The identity of the insert was verified by sequencing the complete gene in both directions. A null *snu114* allele was created from pRS316/*SNU114* by deletion of a 1.47 kb *Xba*I–*Eco*NI fragment from the *SNU114* ORF (amino acids 207–699). pRS316/*SNU114* was first digested with *Eco*NI. Blunt ends were obtained by incubation with Klenow polymerase at room temperature for 15 min and, finally, digested with *Xba*I. A 1.1 kb *Sna*BI–*Xba*I fragment containing the full-length *HIS3* gene was obtained from the plasmid pRS313 (Sikorski and Hieter, 1989) by PCR and cloned into *SNU114*. The resulting plasmid is pRS316/*snu114*Δ*::HIS3*. A 2.1 kb *Bsp*EI–*Pvu*II fragment from pRS316/*snu114*Δ*::HIS3* containing *snu114*Δ*::HIS3* was used to replace one copy of *SNU114* in the diploid yeast strain YPF1 [MAT**a**/α, *trp1-*Δ*1/trp1-*Δ*1; his3-*Δ*/his3-*Δ*; ura3-52/ura3-52; lys2-801/lys2-801; ade2-101/ade2-101* (Sikorski and Hieter, 1989)] by homologous recombination (Scherer and Davis, 1979). Viable His$^+$ transformants were obtained, and correct integration of the *snu114*Δ*::HIS3* gene at the homologous locus was confirmed by Southern analysis (Sambrook *et al.*, 1989). After digestion with *Xmn*I and *Nde*I, DNA fragments were subjected to electrophoresis, stained, transferred to nylon membranes (Qiagen) and irradiated with UV light for 2.5 min. A 3.028 kb *Nhe*I–*Eco*47III DNA fragment of *SNU114* was used as a probe for Southern hybridization. Pre-hybridization, hybridization and washing of the membrane were carried out as described previously (Fabrizio *et al*., 1989). One correct integrant, YPF5 (MAT**a**/α, *trp1-*Δ*1/trp1-*Δ*1; his3-*Δ*/his3-*Δ*; ura3-52/ura3-52; lys2-801/lys2-801; ade2-101/ade2-101; SNU114/snu114*Δ*::HIS3*), was sporulated on YPA plates and tetrads were dissected on YPD plates (Guthrie and Fink, 1991). YPF5 subsequently was transformed with pRS316/*SNU114* (see above), sporulation and asci dissection gave YPF8 [MATα, *trp1-*Δ*1; his3-*Δ*; ura3-52; lys2-801; ade2-101; snu114*Δ*::HIS3* (*URA3* pRS316/*SNU114, ARS, CEN6*)].

Growth curves and primer extension

To obtain *GAL1::SNU114*, the *SNU114* coding sequence was amplified from yeast genomic DNA by PCR using specific primers 5 and 6. The 3.0 kb PCR product harboring the ORF of *SNU114* was cloned into pRS314-*GAL1* and, for sequencing, into pBSIIKS(–). To obtain pRS314-*GAL1*, a 0.87 kb PCR product, obtained with primers 3 and 4, from plasmid pYES (Stratagene), containing the *GAL1* promoter, the polylinker and transcription termination signals, was cloned into pRS314 (Sikorski and Hieter, 1989). The plasmid containing the *GAL1::SNU114* allele was transformed into the heterozygous diploid strain YPF5 (see above). Viable His$^+$/Trp1$^+$ transformants were selected in synthetic media lacking histidine and tryptophan, sporulated on YPA plates and dissected

on galactose-based YP medium. The haploid progeny was analyzed for the presence of the chromosomal *snu114Δ::HIS3* disruption and for the presence of pRS314-*GAL1/SNU114* on synthetic media lacking histidine and tryptophan.

Growth of the strain was monitored on galactose- and glucose-based plates. Strain YPF14 [MATα, *trp1-Δ1; his3-Δ; ura3-52; lys2-801; ade2-101; snu114Δ::HIS3* (*TRP1* pRS314-*GAL1/SNU114, ARS,CEN6*)] was identified among the meiotic progeny which possessed the desired phenotype. To monitor growth, YPF14 cells were grown in liquid medium containing 2% galactose, pelleted, washed and resuspended in YP medium. The suspension was used to inoculate fresh pre-warmed YP-medium containing 2% galactose or 2% glucose to an initial OD_{600} of 0.05 and aliquots were removed at various times thereafter. In order to maintain logarithmic growth, cultures were diluted as necessary to keep all OD_{600} readings below 0.6. For primer extension, total RNA was extracted from strain YPF14 at each time point, by the method of Vijayraghavan *et al.* (1989). Five micrograms of RNA from each sample were used together with 5×10^4 c.p.m. of radiolabeled oligonucleotide 11. Primer extension was carried out as described by Vijayraghavan *et al.* (1989).

Site-directed mutagenesis and plasmid shuffling

The QuikChange site-directed mutagenesis method (Stratagene) was used to introduce the point mutation G145R (GGT→CGT) into *SNU114*. Two oligonucleotides primers, each complementary to opposite strands of *SNU114* and containing the desired substitution, were designed (primers 7 and 8). The PCR cycling parameter suggested by the QuickChange method was the following: one cycle (30 s at 95°C), 12 cycles (30 s at 95°C, 1 min at 55°C and 17 min at 68°C), using native *Pfu* DNA polymerase. The mutant allele was screened for the desired mutation by sequencing with primers 9 and 10. Plasmids pRS314/*SNU114* and pRS314/*snu114*G145R were obtained and transformed separately into strain YPF8. The plasmid-shuffling strategy was applied (Boeke *et al.*, 1987). 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-FOA plates to select for cells lacking the *URA3* plasmid.

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