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Reassembly and protection of small nuclear ribonucleoprotein particles by heat shock proteins in yeast cells

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ABSTRACT

The process of mRNA splicing is sensitive to in vivo thermal inactivation, but can be protected by pretreatment of cells under conditions that induce heat-shock proteins (Hsps). This latter phenomenon is known as "splicing thermotolerance". In this article we demonstrate that the small nuclear ribonucleoprotein particles (snRNPs) are in vivo targets of thermal damage within the splicing apparatus in heat-shocked yeast cells. Following a heat shock, levels of the tri-snRNP (U4/U6.U5), free U6 snRNP, and a pre-U6 snRNP complex are dramatically reduced. In addition, we observe multiple alterations in U1, U2, U5, and U4/U6 snRNP profiles and the accumulation of precursor forms of U4- and U6-containing snRNPs. Reassembly of snRNPs following a heat shock is correlated with the recovery of mRNA splicing and requires both Hsp104 and the Ssa Hsp70 family of proteins. Furthermore, we correlate splicing thermotolerance with the protection of a subset of snRNPs by Ssa proteins but not Hsp104, and show that Hsp70 directly associates with U4- and U6-containing snRNPs in splicing thermotolerant cells. In addition, our results show that Hsp70 plays a role in snRNP assembly under normal physiological conditions.

Keywords: Hsp70; Hsp104; mRNA splicing; *Saccharomyces cerevisiae*; snRNPs; splicing thermotolerance

INTRODUCTION

The mRNA splicing apparatus, or the spliceosome, is a large, highly conserved, multi-component complex (Staley & Guthrie, 1998). It is constituted of several small nuclear ribonucleoprotein particles (snRNPs) and other associated protein factors (Will & Lührmann, 1997). Each snRNP is generally composed of a single small nuclear RNA (U1, U2, U4, U5, and U6 snRNAs), a common set of proteins referred to as Sm proteins, and a group of proteins unique to each snRNP (Will & Lührmann, 1997). The exception to this composition is the U4/U6 snRNP, which contains two snRNAs, U4 and U6. The formation of the spliceosome is an ordered process that involves the stepwise interaction of snRNPs with the pre-mRNA (Krämer, 1996; Staley & Guthrie, 1998). The U1 and U2 snRNPs interact with the pre-mRNA as single particles, whereas the tri-snRNP (U4/U6.U5) is first assembled from the U4/U6 and the U5 snRNPs before entry into the spliceosome. The primary function of snRNPs within the spliceosome is to facilitate mRNA splicing by juxtaposing the spatially separated 5′ and 3′ splice sites through specific base-pairing interactions between snRNAs and the pre-mRNA and through protein–protein interactions involving snRNP proteins and other splicing factors.

The process of mRNA splicing is extremely sensitive in vivo thermal inactivation in many different organisms (Bond & Schlesinger, 1986; Yost & Lindquist, 1986; Bond, 1988; Vogel et al., 1995). Treatment of cells at sublethal heat shock temperatures for an hour or more results in a complete but reversible inactivation of mRNA splicing. In heat-shocked HeLa cells, alterations in snRNPs have been correlated with the inactivation of mRNA splicing (Bond, 1988; Shukla et al., 1990; Utans et al., 1992). Alterations of hnRNP particles have also been observed in heat-shocked HeLa and *Drosophila* cells, suggesting that RNP particles, in general, are particularly sensitive to heat shock (Mayrand & Pederson, 1983; Gattoni et al., 1996; Mahé et al., 1997). Despite the numerous effects of heat on RNPs, studies have shown that in vitro splicing activity in extracts prepared from heat-shocked cells can be restored by the addition of a protein fraction consisting minimally of five proteins (Utans et al., 1992). This fraction is capable of reassembling the U4/U6 and the U5 snRNPs into the U4/U6.U5 tri-snRNP, suggesting that the primary

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splicing defect in heat-shocked HeLa cells may be the lack of a functional tri-snRNP.

The phenomenon of "splicing thermotolerance," in which mRNA splicing can be protected from thermal inactivation if cells are first subjected to a mild heat pretreatment before a severe heat shock, has been observed in both HeLa and *Saccharomyces cerevisiae* cells (Bond, 1988; Yost & Lindquist, 1991). Heat-shock proteins (Hsps) are known to be induced during this pretreatment in both organisms (Morimoto et al., 1994, 1997). Interestingly, however, *de novo* protein synthesis is not required during the pretreatment to confer splicing thermotolerance in *S. cerevisiae* (Yost & Lindquist, 1991). This latter result suggests that preexisting proteins such as those of the constitutively expressed members of the Ssa subfamily of Hsp70s, Ssa1 and Ssa2, and/or other factors may become activated or may relocalize within the cell during the pretreatment to subsequently confer splicing thermotolerance. Evidence of the interaction of Hsps with the splicing machinery comes from the fact that Hsp104 is vital for the recovery of mRNA splicing when cells are returned to normal growing temperatures (Yost & Lindquist, 1991). Ssa proteins also assist in this recovery (Yost & Lindquist, 1991; Vogel et al., 1995). Furthermore, splicing can be restored to in vitro heat-inactivated extracts by the addition of Hsp104 and to a lesser degree by the addition of Ssa protein (Vogel et al., 1995).

Our aim in this article was to identify the specific in vivo targets of thermal damage within the splicing apparatus of *S. cerevisiae* and to elucidate any role played by Hsps in the protection and restoration of these targets. In this article, we show that the inactivation of mRNA splicing in heat-shocked yeast cells is correlated with both qualitative and quantitative alterations in snRNPs and the accumulation of snRNP intermediates. Both Hsp104 and Ssa Hsp70 proteins, acting in synergy, are required for the reassembly of snRNPs following heat shock. In addition we find that splicing thermotolerance is correlated with protection of a subset of snRNPs and requires the presence of at least one member of the Ssa Hsp70 protein subfamily. Finally, we provide evidence that Hsp70 directly associates with U4- and U6-containing snRNPs in splicing thermotolerant cells in addition to playing a role in snRNP assembly, even under normal physiological conditions.

RESULTS

The inactivation of mRNA splicing by heat shock is correlated with qualitative and quantitative alterations in snRNP profiles in yeast

To establish experimental conditions that result in an inactivation of mRNA splicing, exponentially growing yeast cells were subjected to a heat shock at various temperatures for 1 h. Inactivation of mRNA splicing was monitored by the appearance of intron-containing pre-mRNA species on Northern blots hybridized with an actin-specific DNA probe. Complete inactivation was observed when cells were incubated at temperatures of 42 °C or greater (Fig. 1). Incubation at temperatures below 42 °C resulted in a partial inactivation of splicing (data not shown).

Having established conditions for the inactivation of mRNA splicing, we next examined the state of the major snRNPs to determine if these are altered as a result of the heat shock. We initially concentrated on an analysis of the U4, U5, and U6 snRNA-containing snRNPs, as previous results had indicated that the U4/U6.U5 tri-snRNP was drastically altered in heat-shocked HeLa cells (Bond, 1988). Yeast cell extracts were electrophoresed on nondenaturing polyacrylamide gels that allow the separation of RNA–protein complexes (Konarska & Sharp, 1986; Raghunathan & Guthrie, 1998). Following transfer to nylon membranes, blots were hybridized with U4-, U5-, and U6-specific DNA probes (Fig. 2A). The tri-snRNP migrates as a single complex as judged by hybridization to all three probes (Fig. 2A, lanes 2, 5, and 8). A significant decrease in the level of this complex is observed in the heat-shocked samples, but no alteration in its mobility is observed (Fig. 2A, lanes 3, 6, and 9). This result was confirmed by separation of these samples on glycerol gradients (data not shown). Quantitation of levels of the tri-snRNP from a number of separate experiments indicates that on average, levels decreased to 5–15% of the normal level after a heat shock at 42 °C. Figure 2A also shows that the U4/U6 snRNP appears to be altered. Two complexes (designated as U4/U6) hybridize to both U4 and U6 specific probes in untreated cells (Fig. 2A, lane 2 and 8). Following a heat shock at 42 °C, both species are reduced, although the slower-migrating species appears to be

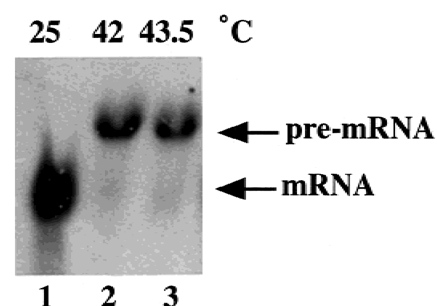

FIGURE 1. mRNA splicing is inhibited in heat-shocked yeast cells. Yeast cells were heat shocked for 1 h at the indicated temperatures as described in Materials and Methods. RNA extracted following the treatment was separated on 0.9% formaldehyde-agarose gels and transferred to nylon membranes. The membranes were hybridized with a Digoxygenin-UTP-labeled DNA probe specific for the actin gene (ACT1). The position of the mature mRNA and the pre-mRNA are indicated by arrows.

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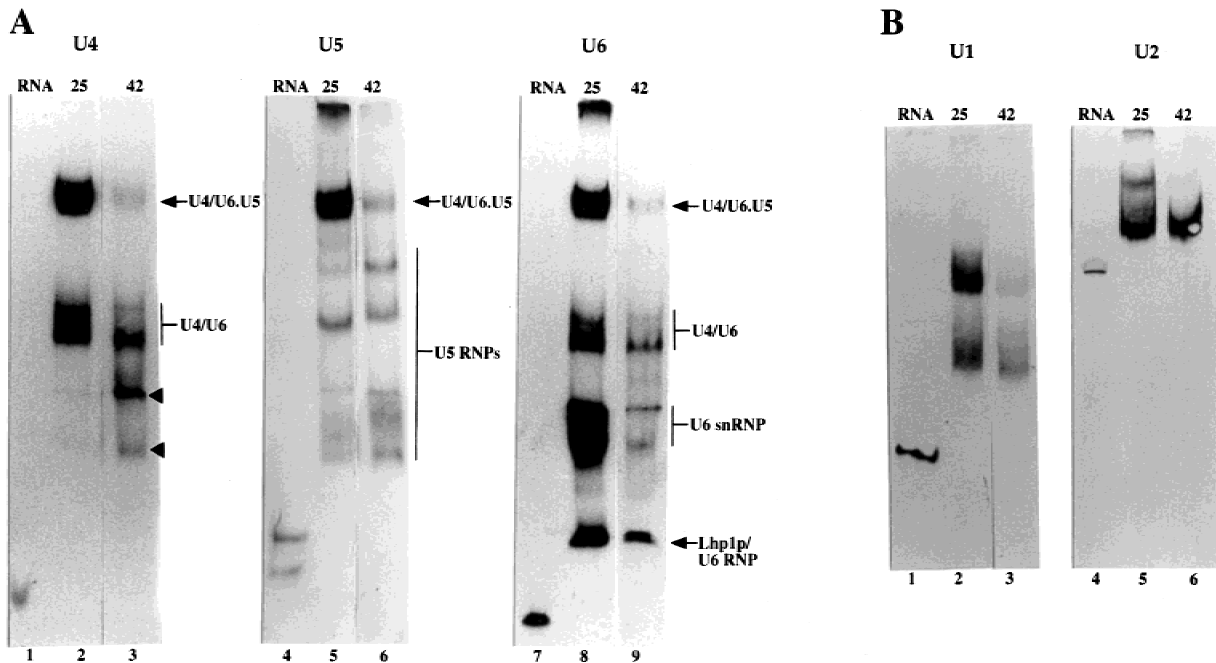

FIGURE 2. snRNPs are altered in heat-shocked yeast cells. Cell extracts were separated on nondenaturing polyacrylamide gels as described in Materials and Methods and transferred to nylon membranes. The membranes were probed with Digoxygenin-UTP-labeled U1, U2, U4, U5, and U6 specific DNA probes as indicated. **A**: Effects of heat shock on U4, U5, and U6 containing snRNPs. The position of the U4/U6.U5 snRNP and the Lh1p1/U6 RNP are indicated by arrows. The U4/U6 and the U5 snRNPs are indicated by lines. Lanes 1, 4, 7: deproteinized RNA. Lanes 2, 5, 8: extracts from cells grown at 25 °C. Lanes 3, 6, 9: extracts from cells heat shocked at 42 °C for 1 h. **B**: Effects of heat shock on the U1 and U2 snRNPs. Lanes 1 and 4, deproteinized RNA. Lanes 2 and 5: extracts from cells grown at 25 °C. Lanes 3 and 6: extracts from cells heat shocked at 42 °C for 1 h.

most affected. Two faster-migrating species, which hybridize to the U4 probe, are also evident in the heat-shocked samples (Fig. 2A, lane 3; arrowheads). Lower levels of these two species are also evident in the untreated samples (Fig. 2A, lane 2), suggesting that these species are precursors of U4/U6 and/or the tri-snRNP.

The U6 probe detects free U6 snRNP in addition to the U4/U6 and the tri-snRNP complexes (Fig. 2A, lane 8). The level of this snRNP is significantly reduced following heat shock (Fig. 2A, lane 9). It has been shown that the U6 snRNA first associates with the yeast La protein before assembly into the U6 snRNP (Pannone et al., 1998). This complex, designated Lhp1p/U6 RNP, is also detected on this gel system as judged by its hybridization to a U6 probe only. This pre-U6 RNP is reduced following a heat shock (Fig. 2A, lane 9).

A number of U5 snRNA-containing RNP complexes are also resolved in this gel system (Fig. 2A, lane 5). The predominant band corresponds to the tri-snRNP as judged by its hybridization to U4 and U6 probes (arrow). At least six additional bands are detected with the U5 probe (bar). The exact composition of the multiple forms of U5-hybridizing complexes is not known at present, but may reflect the fact that at least two forms of U5 snRNA, U5L and U5S, are found in yeast (Patterson & Guthrie, 1987; Chanfreau et al., 1997; Fig. 2A, lane 4). As observed with the other snRNPs, the U5-snRNP profile is altered following heat shock (Fig. 2A, lane 6).

We also examined the effects of heat shock on the other major snRNPs, U1 and U2. At least two distinct U1-containing species are observed in cells under normal physiological conditions (Fig. 2B, lane 2). Following a heat shock at 42 °C, the levels of both U1-containing snRNPs are reduced, although the slower-migrating species appears to be most affected (Fig. 2B, lane 3). We also observe at least two U2-containing species in cells grown at 25 °C (Fig. 2B, lane 5), and again there is a complete reduction in the level of the slower-migrating species following a heat shock whereas the faster-migrating species appears to be less affected (Fig. 2B, lane 6).

Concomitant with the qualitative and quantitative changes to the spliceosomal snRNPs following a heat shock, we observed an approximately 50% reduction in U4 and U6 snRNA levels in cell extracts, whereas U5, U1, and U2 snRNAs are less affected and are decreased by approximately 25–30% (data not shown).

Hsps are essential for the reassembly of snRNPs after a heat shock

Previous data have shown that Hsp104 and the Ssa proteins (Hsp70) play a role in the recovery of mRNA

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splicing following a heat shock (Yost & Lindquist, 1991; Vogel et al., 1995). To determine if there is a correlation between the recovery of mRNA splicing and the restoration of normal snRNP profiles and to determine if this restoration was dependent on the presence of Hsps, we monitored the recovery of mRNA splicing, in parallel with an analysis snRNP species, after a heat shock in a variety of yeast *HSP* mutant strains (Fig. 3). In *S. cerevisiae*, a large family of Hsp70 proteins exists. The Ssa subfamily consists of four members, Ssa1–4, of which three are heat inducible. The strain SL324-1B lacks the genes coding for these three heat inducible Ssa proteins; *SSA1, SSA3,* and *SSA4* (Table 1). To maintain viability in this strain, the *SSA2* gene is required. Strain SL304A lacks the single copy *HSP104* gene, whereas SL325-1B lacks *SSA1*, *SSA3*, *SSA4*, and *HSP104* (Table 1).

The mRNA splicing pattern (Fig. 3A) and snRNP profiles (Fig. 3B,C) were analyzed for all four strains following a heat shock at 42 °C. In the wild-type strain (SL303-1A), mRNA splicing is completely inactivated at 42 °C but recovers rapidly after heat shock with levels of mRNA returning to normal within 60 min of recovery at 25 °C (Fig. 3A, lanes 1–5). Strain SL324-1B (*ssa1, ssa3, ssa4*) shows a slower rate of recovery of splicing, with mRNA levels not returning to normal until 90 min (Fig. 3A, lanes 6–10). In the *hsp104* mutant SL304A, recovery of mRNA splicing is considerably slower (Fig. 3A, lanes 11–15), as has previously been reported (Yost & Lindquist, 1991; Vogel et al., 1995), whereas strain SL325-1B, (*hsp104, ssa1, ssa3, ssa4*) is the most affected of the strains examined in this study with minimal recovery of splicing activity (Fig. 3A, lanes 16–20). The levels of pre-mRNA accumulating following the heat shock (Fig. 3A, lanes 2, 7, 12, and 17) are lower than previously observed in Figure 1 (lanes 2 and 3). This reflects a difference in heat shock conditions used in the two experiments (see Materials and Methods). The heat shock regime used in Figure 3 appears to affect both transcription and splicing, resulting in a lower accumulation of pre-mRNA. Interestingly, pre-mRNA levels accumulate in strains SL304A (Fig. 3, lanes 13–15) and strain SL3251B (Fig. 3, lanes 18–20) during the recovery period, suggesting

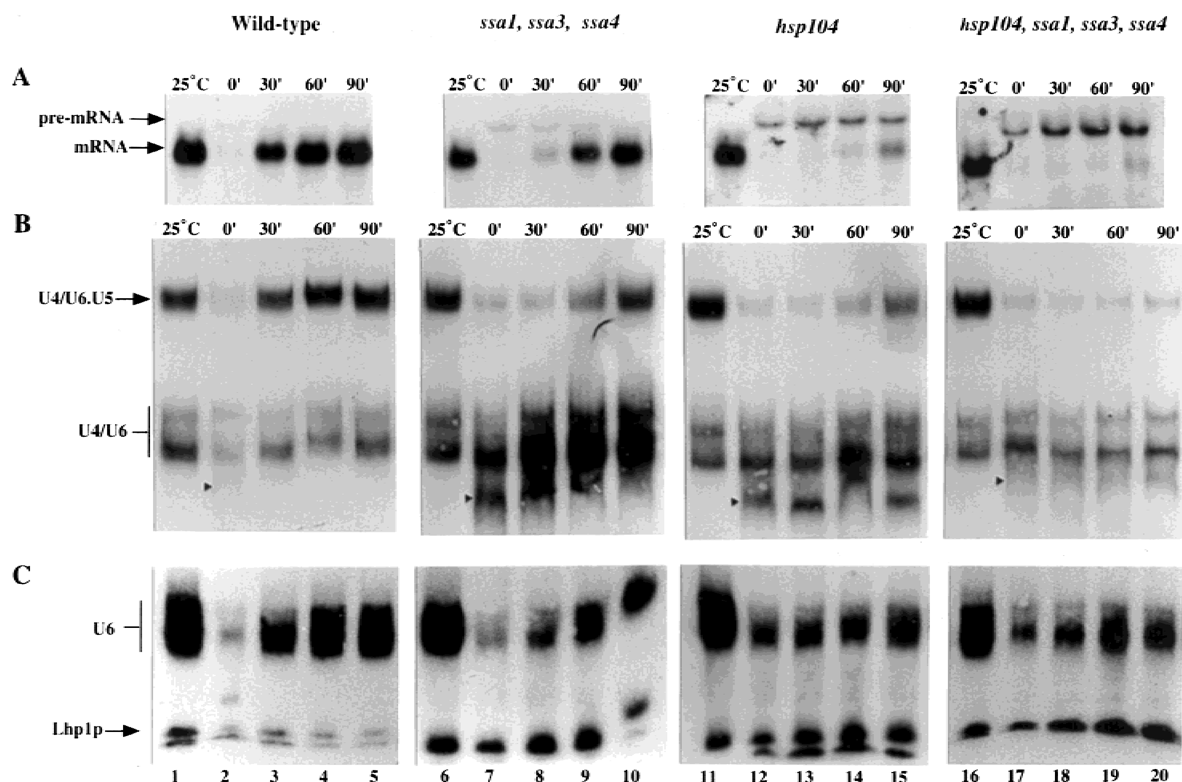

FIGURE 3. Splicing recovery after heat shock correlates with snRNP reassembly by Hsps. Wild-type (SL303-1A) and mutant strains (SL304A, SL324-1B, and SL325-1B) were heat shocked for 1 h at 42 °C in prewarmed media (see Materials and Methods) and then allowed to recover at 25 °C. Cell extracts and RNA were prepared before heat shock (25 °C), immediately following the heat shock (0′) and during recovery at 30-min intervals as indicated above the lanes. **A**: Northern blots probed for actin RNA species. The positions of the pre-mRNA and mRNA are indicated by arrows. **B**: Cell extracts were electrophoresed on nondenaturing gels and transferred to nylon membranes. The membranes were probed with a U4 specific DNA probe. The position of the U4/U6.U5 and the U4/U6 snRNPs are indicated. Arrowheads denote the faster-migrating snRNP species that persists during recovery in the mutant strains. **C**: As in **B** except the membranes were probed with a U6-specific DNA probe. The section of the blot showing the free U6 snRNP and the Lh1p1/U6 RNP complex (arrow) is shown.

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TABLE 1. *S. cerevisiae* strains used.

Strain	Genotype	Source
SL303-1A	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2*	Vogel et al., 1995
SL304A	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (hsp104::LEU2)*	Vogel et al., 1995
SL324-1B	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa1::HIS3) (ssa3::TRP1) (ssa4::LYS1)*	Vogel et al., 1995
SL325-1B	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (hsp104::URA3) (ssa1::HIS3) (ssa3::TRP1) (ssa4::LYS1)*	Vogel et al., 1995
JN55	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2*	Becker et al., 1996
JN516	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa2::LEU2) (ssa3::TRP1) (ssa4::LYS2)*	Becker et al., 1996
JN519	MAT**a** *leu2 trp1 ura3 ade2 his3 lys2 (ssa1-45BKD) (ssa2::LEU2) (ssa3::TRP1) (ssa4::LYS2)*	Elizabeth Craig (University of Wisconsin)

that transcription but not mRNA splicing has been restored.

The quantitative and qualitative restoration of snRNPs correlates very well with the rate of recovery of mRNA splicing (Fig. 3B). In the wild-type strain (SL303-1A), tri-snRNP levels, as judged by its hybridization to a U4-specific probe, return to normal within 30 min of recovery at 25 °C (Fig. 3B, lanes 1–5). Again recovery of tri-snRNP levels is slower in the mutant strains. Little or no tri-snRNP restoration is observed in strain SL325-1B (*hsp104, ssa1, ssa3, ssa4*), reflecting the pattern of mRNA splicing (Fig. 3B, lanes 16–20), whereas levels are restored slightly faster in strain SL324-1B (*ssa1, ssa3, ssa4*) (Fig. 3B, lanes 6–10) than in strain SL304A (*hsp104*) (Fig. 3B, lanes 11–15).

Qualitative restoration of the U4/U6 snRNP to its normal profile is evident during the recovery period, as judged by its hybridization with a U4-specific probe (Fig. 3B). In the wild-type strains, the U4/U6 profile is returned to normal within 60 min of recovery (Fig. 3B, lane 4). In strain SL324-1B (*ssa1, ssa3, ssa4*) (Fig. 3B, lanes 6–10), the snRNP species previously noted in Figure 2A, lane 3, denoted by an arrowhead, is present in significantly greater amounts immediately after the heat shock and is progressively lost as recovery proceeds. Significantly, this species persists for up to 90 min into the recovery period in both strains SL304A (*hsp104*) (Fig. 3B, lanes 11–15), and SL325-1B (*hsp104, ssa1, ssa3, ssa4*) (Fig. 3B, lanes 16–20).

Restoration of free U6 snRNP levels in the wild-type strain is also observed during the recovery after heat shock, as judged by a hybridization with a U6-specific probe (Fig. 3C, lanes 1–5). Recovery is impeded in all of the mutant strains (Fig. 3C, lanes 6–20). The U6 snRNA probe also detects a U6 snRNP precursor that has mobility properties on these native gels similar to the Lhp1p/U6 RNP complex (Fig. 3C; Pannone et al., 1998). This complex is reduced following a heat shock in all strains (Fig. 3C, lanes 2, 7, 12, and 17); however it accumulates during the recovery period in all mutant strains (Fig. 3C, lanes 8–10, 13–15, and 18–20) but not in the wild-type strain (Fig. 3C, lanes 3–5).

The U1 and U2 snRNP profiles are also restored during the recovery period (data not shown). Again, restorations were fastest in the wild-type strain and slowest in strains lacking Hsp104. Thus, both Hsp104 and the Ssa Hsp70 proteins are essential after heat shock for both the quantitative and qualitative restoration of normal snRNP profiles. In addition, both proteins appear to be acting in synergy.

Splicing thermotolerance is independent of the Hsp70 Ssa subfamily

Previous studies have shown that mRNA splicing is protected in cells rendered splicing thermotolerant by a preincubation at mild temperatures before exposure to temperatures that would otherwise inactivate the process (Bond, 1988; Yost & Lindquist, 1991). *De novo* protein synthesis is not required for this phenomenon in *S. cerevisiae* (Yost & Lindquist, 1991). It has been hypothesized that preexisting factors, such as the constitutive members of the Hsp70, Ssa subfamily, Ssa1 and Ssa2, become activated or relocalized to the nucleus by the pretreatment and subsequently confer splicing thermotolerance (Yost & Lindquist, 1991). To test this hypothesis, we investigated whether the mutant strain JN519, which lacks functional genes coding for *SSA2*, *SSA3*, and *SSA4* and which contains a temperature-sensitive mutation in the *SSA1* gene, was capable of acquiring splicing thermotolerance. The temperature-sensitive *SSA1-45BKD* allele contains a single point mutation changing P-417 to L (Becker et al., 1996). The *URA* gene that was used for replacing the chromosomal copy of *SSA1* with the *SSA1-45BKD* gene was subsequently deleted, resulting in strain JN519 (E. Craig, University of Wisconsin, pers. comm.). This strain is viable, but slow growing, at the permissive temperature of 25 °C, whereas all four *SSA* gene products are absent when incubated at the nonpermissive temperature of 37 °C, the temperature normally used to induce thermotolerance. In addition, we examined the protection of mRNA splicing in strain SL304A (*hsp104*) and in strain JN516 (*ssa2, ssa3, ssa4*).

The wild-type isogenic strain (JN55) and mutant strains were preincubated at 37 °C for 45 min and then immediately challenged with a heat shock at 42 °C for 1 h. Surprisingly, we find that mRNA splicing is pro-

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tected from heat inactivation in pretreated cells regardless of the mutant background, as judged by the lack of intron-containing pre-mRNAs (Fig. 4, lanes 2, 5, 8, and 11; TT). Splicing was also protected in the mutant strains SL324-1B (*ssa1, ssa3, ssa4*) and SL325-1B (*ssa1, ssa3, ssa4, hsp104*) (data not shown). Significantly however, in strain JN519 we observe an approximately 80% reduction in spliced actin mRNA levels in pretreated cells after the 42 °C heat shock (Fig. 4, compare lanes 4 and 5) as compared to a 50% reduction in wild-type and other mutant strains (Fig. 4, compare lanes 1 and 2, 7 and 8, and 11 and 12).

Protection of snRNPs in thermotolerant cells requires at least one member of the Ssa protein subfamily

To determine if the protection of mRNA splicing observed in heat-shocked pretreated cells is a direct consequence of the protection of the snRNP profiles, we analyzed the tri-snRNP, U4/U6, and free U6 snRNPs in these cells. Tri-snRNP levels are consistently higher in cells heat shocked after a pretreatment as compared to cells heat shocked without a prior pretreatment (Fig. 5, compare lanes marked TT and 42). Protection of tri-snRNP levels was also observed in the strain SL325-1B (*ssa1, ssa3, ssa4, hsp104*) and in strain SL324-1B (*ssa1, ssa3, ssa4*) (data not shown). However, in the strain JN519 (*ssa1-45BKD, ssa2, ssa3, ssa4*), the degree of protection of the tri-snRNP levels in heat-shocked pretreated cells is significantly less than that observed for the wild-type or any of the other strains (Fig. 5, lane 5). Furthermore, the amount of tri-snRNP at 25 °C was markedly lower in this strain than that observed in the other strains (Fig. 5, compare lanes 4 with lanes 1, 7, and 10).

As noted previously in Figure 2, only minor reductions in the levels of U4/U6 snRNP are observed in cells heat shocked at 42 °C (Fig. 5, lanes 3, 6, 9, and 12). These levels are maintained in pretreated cells (Fig. 5, lanes 2, 5, 8, and 11). In this gel, two U6 hybridizing snRNPs are resolved. Interestingly, the levels of the free U6 snRNP are not protected in the wild-type or the mutant strains (Fig. 5, compare lanes marked TT and 42).

We also observed that both the U4/U6 and the tri-snRNP exhibit upward shifts in mobility in pretreated cells after a heat shock. This is most pronounced in the strain SL-304A (*hsp104*) (Fig. 5, lane 11). To emphasize the shift in this strain, cell extracts were electrophoresed for a longer time period (see Materials and Methods; Fig. 5B). Under these electrophoretic conditions, the upward shifts in both U4/U6 snRNP and the tri-snRNP are more noticeable (Fig. 5B, lanes 2 and 3). Furthermore, upward shifts were also observed for the U1, U2, and U5 snRNPs (data not shown). These shifts do not occur if cells are immediately placed at the higher temperature of 42 °C without the pretreatment (Fig. 5B, lane 4).

To determine if the upshifts observed in thermotolerant samples are a result of direct association between Hsp70 and snRNPs, cell extracts from untreated cells or from cells heat shocked with or without a prior pretreatment to induce thermotolerance were immunoprecipitated with either an anti-Hsp70 or an anti-Prp4 antibody. The latter recognizes the Prp4 protein that is a constituent of the U4/U6 and the U4/U6.U5 snRNPs. Pellet and supernatant fractions were probed for the presence of U4 and U6 snRNAs to confirm immunoprecipitation with the anti-Prp4 antibody (data not shown). Proteins present in the pellet fraction of the immunoprecipitation were Western blotted onto nitrocellulose membranes and the presence of Hsp70 was detected using the anti-Hsp70 antibody. Figure 6 shows the results of the immunoprecipitation. The immunoglobulin heavy chains are apparent in each lane (star). Figure 6, lanes 1–3, show samples immunoprecipitated with anti-Hsp70 antibody. Because this antibody recognizes both constitutive and induced members of the Ssa Hsp70 family and constitutively expressed Hsp70 predominates over inducible Hsp70, the levels of Hsp70 (arrowhead) are similar in the untreated sample (Fig. 6, lane 1, 25), the thermotolerant sample (Fig. 6, lane 2, TT), and in the heat-shocked sample (Fig. 6, lane 3, 42). The samples immunoprecipitated with anti-Prp4 antibody are shown in Figure 6, lanes 4–7. We observe that Hsp70 is immunoprecipitated by anti-Prp4 in thermotolerant cells (Fig. 6, lane 5) and to a much lesser extent in cells heat shocked without a prior induction of thermotolerance (Fig. 6, lane 6). Hsp70 is not observed in the anti-Prp4 immunoprecipitate from untreated cells (Fig. 6, lane 4) nor when extract is omitted from the immunoprecipitate (Fig. 6, lane 7). In conclusion, we infer from these results that Hsp70 physically associates with U4- and U6-containing snRNPs in thermotolerant cells.

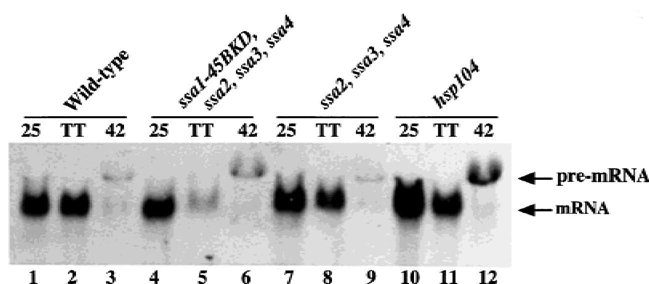

FIGURE 4. Splicing thermotolerance is independent of all four Ssa proteins and Hsp104. The wild-type and mutant strains, indicated above the lanes, were subjected to a heat shock at 42 °C for 1 h without a prior pretreatment at 37 °C (42: lanes 3, 6, 9, 12) or with a prior pretreatment at 37 °C (TT: lanes 2, 5, 8, 11). Samples from untreated cells (25 °C) are shown in lanes 1, 4, 7, and 10. RNA from these cells was separated on a denaturing agarose gel and probed with a Digoxygenin-UTP-labeled actin probe. The position of the actin pre-mRNA and the mRNA are indicated by arrows.

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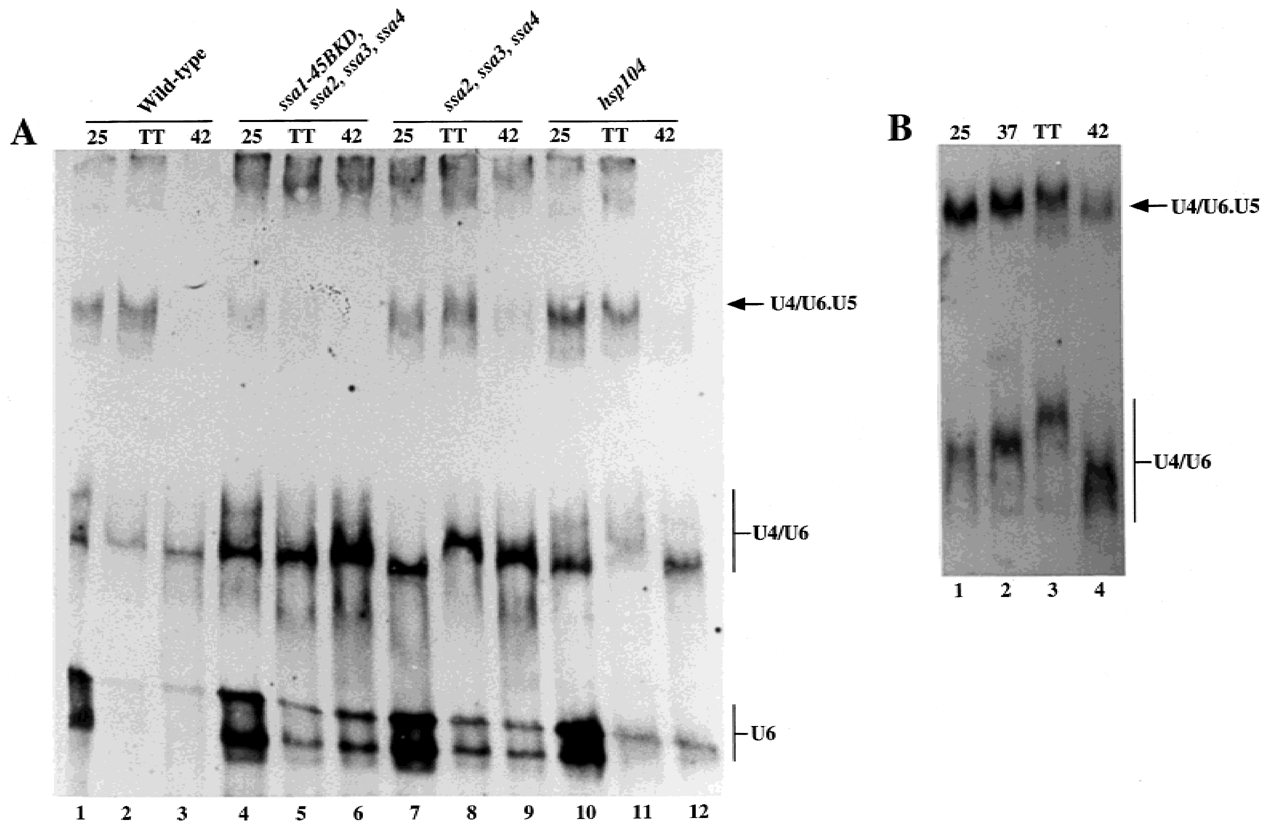

FIGURE 5. Protection of tri-snRNP levels in pretreated heat-shocked cells. Cell extracts from wild-type (JN55) and mutant strains, as indicated above the lanes, were electrophoresed on nondenaturing polyacrylamide gels and transferred to nylon membranes. The membranes were probed with a U6 specific DNA probe. The positions of the U4/U6.U5 tri-snRNP, the U4/U6 and U6 snRNPs are indicated. Lanes 1, 4, 7, 10: extracts from untreated cells (25 °C). Lanes 2, 5, 8, 11: extracts from cells pretreated at 37 °C for 45 min and subsequently heat shocked at 42 °C for 1 h (TT). Lanes 3, 6, 9, 12: extracts from cells heat shocked at 42 °C without a prior pretreatment at 37 °C. **B:** Cell extracts from strain SL304A (*HSP104*) were electrophoresed for 7 h (see Materials and Methods) to illustrate the upward shift in the U4/U6.U5 and the U4/U6 snRNPs. Lane 1: untreated cells (25 °C). Lane 2: cells treated at 37 °C for 45 min. Lane 3: cells treated at 37 °C for 45 min and then heat shocked at 42 °C for 1 h. Lane 4: cells heat shocked at 42 °C for 1 h.

DISCUSSION

Inactivation of mRNA splicing in heat-shocked cells is correlated with both qualitative and quantitative alterations in snRNPs

Previous data have shown that the process of mRNA splicing is extremely sensitive to in vivo heat inactivation in a variety of eukaryotic cells (Bond & Schlesinger, 1986; Yost & Lindquist, 1986; Bond, 1988). In addition, the recovery of mRNA splicing after a heat shock is slower in *S. cerevisiae* mutants lacking the *HSP104* gene and the heat inducible *SSA* genes (*SSA1*, *SSA3*, and *SSA4*) (Vogel et al., 1995), suggesting that these Hsps may interact with components of the spliceosome. To examine more closely the individual components within the splicing process that are sensitive to heat shock, and to determine if Hsps protect and/or restore them during and after heat shock, we have analyzed alterations in snRNP profiles in heat-shocked cells from wild-type and mutant strains lacking specific *HSP* genes. The data presented in this paper confirm that splicing is completely inactivated when yeast cells are exposed to high temperatures and demonstrate that this inactivation correlates with both qualitative and quantitative alterations in the major snRNP species involved in mRNA splicing.

We find that all snRNPs are sensitive to in vivo thermal treatments in *S. cerevisiae*. The levels of the tri-snRNP, the free U6 snRNP, and the U6 pre-snRNP are drastically reduced following a heat shock at 42 °C. We also observed alterations in the U1, U2, U4/U6, and U5 snRNPs. Interestingly, multiple forms of these snRNPs are observed under normal physiological conditions. In the case of U1, U2, and U4/U6 snRNPs, two distinct hybridizing bands are observed (Fig. 2A,B). The slower-migrating species, in each case, is more sensitive to heat stress. The differential sensitivities of these snRNP species may suggest that in each case the faster-migrating species is a precursor of the slower-migrating species.

Additional snRNP species are also uncovered following the heat shock treatment. Two additional faster-migrating U4-hybridizing snRNP species accumulate

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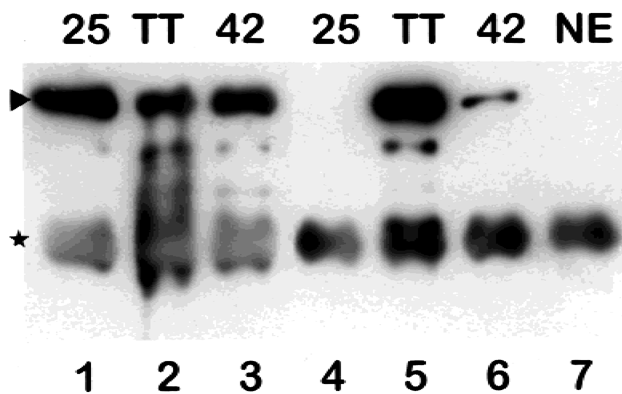

FIGURE 6. Hsp70 associates with U4- and U6-containing snRNPs in thermotolerant cells. Cell extracts from the wild-type strain (SL-304A) were immunoprecipitated with anti-Hsp70 antibody (lanes 1–3) or with anti Prp4 antibody (lanes 4–7). Protein samples from the pellet fraction were Western blotted onto nitrocellulose membranes and the presence of Hsp70 in the samples detected by incubation with anti-Hsp70 antibody. Lanes 1 and 4: extracts from untreated cells grown at 25 °C. Lanes 2 and 5: extracts from cells pretreated at 37 °C for 45 min to induce thermotolerance (TT) prior to a 42 °C heat shock. Lanes 3 and 6: extracts from cells heat shocked at 42 °C without a prior pretreatment. Lane 7: anti-Prp4 immunoprecipitation without added cell extract. Arrowhead shows Hsp70 and the star shows the immunoglobulin heavy chains from the antibodies. Lanes 4–7 were exposed to X-ray film for 10 times longer than lanes 1–3.

in heat-shocked cells (Fig. 2A, lane 3, see arrowheads). These species are also observed in untreated cells but in much lower amounts, which suggests that they are precursors of the U4/U6 and/or the tri-snRNP. Thus, heat shock treatments may block the assembly of snRNPs or alternatively, may disassemble snRNPs into *bona fide* precursor species. Pulse chase experiments will be required to differentiate between these two possibilities. In general, the levels of snRNAs remain relatively constant following a heat shock: we observed no more than a twofold reduction in U4 and U6 snRNAs, whereas U5, U1, and U2 levels were less affected (data not shown).

snRNPS do not appear to be the only RNPs affected by heat shock. A number of studies have also reported alterations in hnRNP particles in heat-shocked cells (Mayrand & Pederson, 1983; Lutz et al., 1988; Mähl et al., 1989; de Graaf et al., 1992; Buchenau et al., 1997). Many of these alterations are associated with mRNA splicing inactivation (Gattoni et al., 1996; Mahé et al., 1997). Thus, the inactivation of mRNA splicing in heat-shocked cells appears to be multi-faceted, but specifically involves alterations in RNP complexes.

Hsps are essential for the reassembly of snRNPs following heat shock

Our data also demonstrate that snRNP precursor forms that accumulate upon heat shock can be chased into normal forms of snRNPs during the recovery period following a heat shock. Hsp104 and the Ssa (Hsp70) proteins, acting in synergy, are essential for this process. Hsp104 appears to play the more prominent role in snRNP reassembly as restoration of normal snRNP species is slower in a *HSP104* mutant than it is in the SL324-1B mutant (*ssa1*, *ssa3*, *ssa4*). In the latter mutant, the Ssa2 protein, although generally considered to be cytosolic, may redistribute to the nucleus to aid in the restoration. The role of Hsps in the reassembly of snRNP species is most noticeable with the U6 snRNP precursor (Fig. 3C) and the putative U4/U6 or U4/U6.U5 precursor forms described above (Fig. 3B; arrowheads). The former complex accumulates in all *HSP* mutant strains, suggesting that the Ssa proteins, in association with Hsp104, are crucial for the chaperoning of the U6 snRNA into the mature U6 snRNP, and points to the assembly of this snRNP as being a critical step in snRNP assembly affected by heat stress. The lack of U6 snRNP assembly in *HSP* mutants may affect the subsequent assembly of the U4/U6 snRNP and the tri-snRNP.

mRNA splicing thermotolerance is independent of the Hsp70s encoded by the *SSA* gene family

We have also examined mRNA splicing following a heat shock of splicing thermotolerant cells (Fig. 4). The observation that splicing thermotolerance is maintained in strain JN519, which lacks all four of the SSA gene products, suggests that none of the *SSA* genes are required for splicing thermotolerance at least under conditions when other Hsps are present. One explanation for this result is that, in the absence of Hsp70, Hsp104 may substitute to confer splicing thermotolerance and *vice versa*. A similar functional relationship between these two proteins has previously been observed with regard to their role in cellular thermotolerance (Sanchez et al., 1993).

Although mRNA splicing appears to proceed normally in heat-shocked thermotolerant cells lacking all the *SSA* genes, the level of spliced mRNA was approximately 20% of the level observed in cells grown at the permissive temperature of 25 °C. In the other mutant and wild-type strains, spliced mRNA levels were approximately 50% reduced compared to the levels found at 25 °C. This greater reduction in mature mRNA levels in JN519 cells points to a role for at least one member of the Ssa subfamily, possibly Ssa1 or Ssa2, in maintaining spliced mRNA levels in heat-shocked thermotolerant cells by allowing continued transcription and splicing and/or stabilizing preexisting mRNA levels.

Protection of the structural integrity of a subset of snRNPs in splicing thermotolerant cells

The major snRNPs required for spliceosome assembly, the tri-snRNP (Fig. 5) and the U1 and U2 snRNPs (data

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not shown), can be protected from thermal damage if cells are pretreated under conditions that induce thermotolerance. This protection requires at least one member of the Ssa, Hsp70 proteins. The free U6 snRNP and the U6 RNP precursor are not protected in thermotolerant cells. The lack of protection of the U6 snRNA-containing complexes may be a consequence of U6 snRNP's transcription, as it is the only splicing snRNA transcribed by RNA polymerase III. Alternatively, the fact that the U6 snRNP is assembled in a different manner than the other snRNPs may account for the lack of protection.

Interestingly, we note that in strain JN519 (*ssa1-45BKD, ssa2, ssa3, ssa4*), the level of the tri-snRNP was significantly lower even at the permissive temperature (25 °C) compared to levels in the wild-type or any other strain. Furthermore, the levels of free U6 and U4/U6 snRNPs, precursors of the tri-snRNP, accumulate in this strain as compared to the levels in other strains (Fig. 5A). Thus, the *SSA* gene products appear to play a role in tri-snRNP assembly under normal growing conditions and in maintaining snRNP integrity in heat-shocked cells.

We observed a noticeable upshift in the mobility of a subset of snRNPs in splicing thermotolerant cells, suggesting the association of additional proteins with snRNPs under these conditions. Our immunoprecipitation results indicate that, at a minimum, Hsp70 physically interacts with U4- and U6-containing snRNPs. Because upshifts in other snRNPs are also observed (data not shown), we can speculate that Hsp70 physically associates with all snRNPs, with the exception of the U6 snRNP, perhaps in the context of the spliceosome to protect these large RNP-complexes from thermal damage. We did not detect Hsp70 in anti-Prp4 immunoprecipitations from untreated cells. This most likely reflects the low levels of Hsp70 associating with snRNPs under these conditions. Surprisingly the upshifts in snRNPs on native gels are more pronounced in the *HSP104* strain. Based on our data, we present a model that may explain this phenomenon and is consistent with the known interrelationship between Hsp70 and Hsp104 (Sanchez et al., 1993; Vogel et al., 1995; Glover & Lindquist, 1998; Newnam et al., 1999). Activated or relocalized constitutive Hsp70 may bind to exposed hydrophobic surfaces of partially unfolded snRNPs upon heat shock in thermotolerant cells, accounting for the upshift of snRNPs observed on nondenaturing gels. Hsp104 may be required to complete the repair process by removing bound Hsp70. In the absence of Hsp104, Hsp70 remains bound to the snRNPs, thereby resulting in a greater upshift observed in *HSP104* mutants. Future studies to investigate the nature of the association between snRNPs and Hsps will help to elucidate the role of Hsps in the assembly and maintenance of snRNPs during normal growth and in heat-shocked cells.

MATERIALS AND METHODS

Yeast strains, culture, and heat shock conditions

The yeast strains used in this study are listed in Table 1. Cells were cultured at 25 °C in YEPD (1.0% yeast extract, 2.0% Bacto Peptone, 2.0% glucose) to between 4.0×10^6 and 1.0×10^7 cells/mL. For experiments depicted in Figures 2–6, before all heat treatments, cells were centrifuged and resuspended in the same prewarmed media and heat shocked at the temperatures (±0.5 °C) and times indicated in the text. For the experiment depicted in Figure 1, cells were heated gradually to the temperatures indicated in media that was not prewarmed. In general a period of 12–15 min is required to bring the temperature to 42 °C. Recovery experiments involved immediate transfer of heat-shocked cells to water baths at 25 °C following the heat shock treatment. Mild heat pretreatments were performed by incubating cells at 37 °C for 45 min before immediately transferring to 42 °C for 1 h. After all heat treatments, cultures were chilled on ice, centrifuged at 5,000 rpm for 5 min at 4 °C, quick frozen on dry ice, and stored at −70 °C for subsequent splicing extract and RNA preparations. In general 450- and 50-mL aliquots of cultures were used for the preparation of splicing extracts and RNA respectively.

RNA preparation and Northern hybridization analysis

Total RNA was isolated by the hot-phenol method (Krieg, 1996). RNA (10–15 μg per lane) was separated on 0.9% formaldehyde-agarose gels (Maniatis et al., 1989) and subsequently blotted to Nytran membranes (Schleicher & Schuell). Digoxygenin-UTP (Roche) labeled DNA probes specific for the actin gene (ACT1) were generated by PCR amplification using the primers 5′-TAATACGACTCACTATAGGG-3′ and 5′-GGCTGCAGGTCGACTCTAGA-3′ according to manufacturer's instructions. Blots were prehybridized for 1–3 h in EasyHyb buffer (Roche) at 50 °C, and hybridized for 15–20 h at 50 °C with approximately 25 ng labeled probe per milliliter of EasyHyb solution. Blots were detected with the chemiluminescent substrates CSPD or CDP-Star (Roche) as described by manufacturer's instructions.

Preparation of yeast splicing extracts

Cell pellets were thawed and washed with 10 mL of ice-cold, sterile distilled water and then suspended in 3.5 mL of extraction buffer A (10 mM HEPES-KOH, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT). Cells were subsequently broken by vortexing with 3 mL of acid-washed glass beads in Corex tubes as previously described (Séraphin & Rosbash, 1989).

Native gel analysis of snRNP complexes

Splicing extracts (40–60 μg total protein/lane) were separated on 4% acrylamide:bisacrylamide (80:1) nondenaturing gels in TG buffer (50 mM Tris base, 50 mM glycine, pH 8.9) (Raghunathan & Guthrie, 1998). Samples were electropho-

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resed for 3 to 4 h at 230 V at 4 °C. To observe upward shifts in snRNPs, the gels were electrophoresed for 7 h. Gels were electroblotted to Nytran membranes and hybridized with PCR-generated Digoxygenin-labeled DNA probes specific for U1, U2, U4, U5, and U6 snRNAs. Hybridizations were performed as described above. Plasmids containing snRNA sequences (Ares, 1986; Kretzner et al., 1987; Patterson & Guthrie, 1987; Siliciano et al., 1987) were kindly provided by Bertrand Séraphin (European Molecular Biology Laboratory (EMBL), Heidelberg).

Immunoprecipitation analysis

Immunoprecipitation of snRNPs with anti-Prp4 antibody was performed as previously described (Banroques & Abelson, 1989). Briefly, 6 μL of anti-Prp4 antibody was coupled to Protein A-Sepharose (Pharmacia, Inc.) at 4 °C for 1.5 h in 1.0 mL of NET-2 buffer (50 mM Tris HCl, pH 7.4, 0.05% Nonidet P-40, 150 mM NaCl). The pellets were then washed three times with 1 mL NET-2 buffer. Five hundred micrograms of total splicing extract were incubated with the antibody-bound beads in 1 mL of fresh NET-2 buffer for 1.5 h at 4 °C with gentle mixing. The resin was then washed six times with 1-mL amounts of NET-2. Hsp70 was immunoprecipitated with anti-Ssa1 antibody (C1ΔB; a kind gift from Professor E. Craig, University of Wisconsin) using this same protocol. Pellet fractions from the immunoprecipitation were separated on 10% SDS-polyacrylamide gels and electroblotted to Protran nitrocellulose membranes (Schleicher & Schuell) in 1× transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine). The membranes were blocked in 100 mM maleic acid, 150 mM NaCl, 1.0% casein (MAB-casein) for 1 h and subsequently incubated with anti-Ssa1 antibody in MAB-casein for 2 h at a dilution of 1/5,000. Membranes were then washed in MAB-Tween (100 mM maleic acid, 150 mM NaCl, 3.0% Tween-20) and then incubated with horseradish peroxidase-conjugated anti-rabbit antibody (Sigma Chemical Co.) at a dilution of 1/3,000 in MAB-casein. Following washing in MAB-Tween, membranes were detected with the chemiluminescent substrate, Supersignal (Pierce Chemicals) according to manufacturer's instructions.

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