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Heat Shock Proteins Affect RNA Processing during the Heat Shock Response of *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae*, the splicing of mRNA precursors is disrupted by a severe heat shock. Mild heat treatments prior to severe heat shock protect splicing from disruption, as was previously reported for *Drosophila melanogaster*. In contrast to *D. melanogaster*, protein synthesis during the pretreatment is not required to protect splicing in yeast cells. However, protein synthesis is required for the rapid recovery of splicing once it has been disrupted by a sudden severe heat shock. Mutations in two classes of yeast hsp genes affect the pattern of RNA splicing during the heat shock response. First, certain *hsp70* mutants, which overproduce other heat shock proteins at normal temperatures, show constitutive protection of splicing at high temperatures and do not require pretreatment. Second, in *hsp104* mutants, the recovery of RNA splicing after a severe heat shock is delayed compared with wild-type cells. These results indicate a greater degree of specialization in the protective functions of hsps than has previously been suspected. Some of the proteins (e.g., members of the hsp70 and hsp82 gene families) help to maintain normal cellular processes at higher temperatures. The particular function of hsp104, at least in splicing, is to facilitate recovery of the process once it has been disrupted.

When cells of virtually any organism examined to date are exposed to temperatures slightly higher than their normal growth temperature or to a variety of other physical or biochemical stresses, a small number of polypeptides, known as the heat shock proteins (hsps), are induced. These highly conserved proteins appear to protect cells from a variety of injuries. The specific functions of individual hsps and of closely related proteins expressed at normal temperatures (cognates) are currently under intense investigation (for review, see references 24 and 33). We have previously shown that one of the effects of heat shock in *Drosophila melanogaster* cells is the disruption of RNA splicing (40). This was initially discovered because this disruption reduces the expression of *HSP82*, an intron-containing gene, at high temperatures. Since other *Drosophila hsp* genes do not have introns, their expression is not affected. The block in splicing is not specific to *HSP82* transcripts; it also prevents the splicing of transcripts from non-heat shock genes (40, 41). The precise nature of the block is not known, but it occurs early in the splicing pathway. Transcripts that are blocked from splicing appear to have normal 5′ ends and are uncut at either splice site (41).

RNA processing is impaired by severe heat shock and other cellular stresses in a variety of other organisms and cells, including other species of *Drosophila* (for review, see reference 42), *Trypanosoma* (28), *Dictyostelium* (25), and cultured chicken and mammalian cells (1, 2, 19).

Mild heat pretreatments sharply reduce the frequency of developmental abnormalities and greatly increase survival after a severe heat shock in a variety of organisms. With few exceptions, the protective effects of pretreatment are closely correlated with the appearance of heat shock proteins (24). The hsps appear to protect many vital processes from the toxic effects of heat. We have investigated their effects on splicing. When *Drosophila* cells are given a mild heat treatment before they are exposed to more severe temperatures, RNA splicing is protected. Hsps induced and synthesized during the pretreatment appear to be involved in the protection of RNA splicing, since the protective effect of the pretreatment is eliminated when cycloheximide is administered before the pretreatment. Cycloheximide does not block protection if it is added after heat shock proteins have accumulated (40).

All eucaryotic organisms studied to date produce many of their mature mRNAs via posttranscriptional RNA splicing. Although the mechanism of pre-mRNA splicing appears to be similar in metazoans (6, 15, 27, 30, 36) and in the yeast *Saccharomyces cerevisiae* (4, 5, 16) there are notable differences. First, relatively few yeast genes contain intervening sequences, and the vast majority of those that do have only one. Second, in metazoans the ligation of alternate exons results in expression of different polypeptides from the same primary sequence. There is no example of an alternate splicing mechanism in yeast cells. Third, and most significantly, the splicing machinery and sequence requirements in the transcripts differ substantially. RNAs from metazoan genes usually cannot be spliced in *S. cerevisiae*.

These differences in RNA splicing in *S. cerevisiae* and in metazoans led us to examine the effects of heat and hsps on RNA splicing in the former. Here, we report that the splicing of yeast actin mRNA precursors is disrupted at high temperatures and that mild heat pretreatments protect splicing from disruption. There are four major classes of heat shock genes in *S. cerevisiae* (*hsp104*, *hsp83*/*hsc83*, *hsp26*, and the nine-member *hsp70* multigene family). Using both cycloheximide treatment and yeast strains carrying mutations in members of each major class of heat shock genes, we investigated what factors are involved in the protection and recovery of RNA splicing.

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MATERIALS AND METHODS

Yeast strains and culture. W303-1B (*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was kindly provided by Rodney Rothstein. Deletions in this strain of *HSP26* (strain L12: *MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [*hsp26*::*HIS3*]) and of *HSP104* have been described previously (32, 34). Strains T-127 (*MATα ade2-101 leu2-3,112 lys2 trp1 ura3-52*) and MW109 (*MATa his3-11,15 leu2-3,112 lys2 trp1 ura3-52*) and the corresponding *HSP70* double mutant strains derived from the same tetrads, T-128 (*MATα ade2-101 his4-713 lys2 trp1 ura3-52* [*ssa1*::*LEU2*] [*ssa2*::*LEU2*]) and MW123 (*MATa his3-11,15 leu2-3,112 lys2 trp1 ura3-52* [*ssa1*::*HIS3*] [*ssa2*::*LEU2*]) were kindly provided by Elizabeth Craig (7, 39). FYH6 (*MATα his3 leu2-3 trp1 ura3-53*) and the corresponding strain in which *HSP82* is disrupted, 10:1-16 (*MATα his3 leu2-3 trp1 ura3-53* [*hsp82*::*LEU2*]), were kindly provided by David Finkelstein (11, 12).

Yeast cultures were grown to the logarithmic phase by vigorous shaking at 25°C in rich medium (YPDA) to 5.0×10^6 cells per ml. Aliquots (50 ml) were distributed to 250-ml flasks warmed to the indicated temperatures in a shaking waterbath (New Brunswick). When indicated, cycloheximide (10 μg/ml) was added 5 min before the temperature shift.

RNA and protein analysis. Cells were collected by centrifugation, resuspended in cold YREB (0.1 M Tris [pH 7.5], 0.1 M LiCl, 20 mM dithiothreitol) and lysed in phenol-chloroform by agitation with glass beads on a Vortex mixer for 5 min, as described previously (21). Total cellular RNAs (2 μg per lane, as determined by optical density) were either electrophoretically separated on 1.2% agarose gels containing 5 mM methylmercury hydroxide (Alfa Products, Danvers, Mass.) and stained with ethidium bromide to confirm equal sample loading, or glyoxylated and electrophoretically separated on 1.4% agarose gels (26). The separated RNAs were blotted to nitrocellulose or Nytran (Schleicher & Schuell). Blots were baked at 80°C for 2 h, prehybridized at 42°C for at least 6 h (50% formamide, 5× Denhardts' solution, 100 μg of single-stranded DNA [ssDNA] per ml, 0.1% sodium dodecyl sulfate [SDS], 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) and hybridized for at least 12 h (50% formamide, 100 μg of ssDNA per ml, 0.1% SDS, 1.25× Denhardts' solution, 4× SSC) with ^{32}P-labeled DNA containing the yeast actin and *YPT1* genes (13, 14, 29) or subclones, as indicated. RNA blots were washed four times at 42°C (0.2× SSC, 0.5% SDS) and exposed at −80°C to Kodak XAR-5 film with a single intensifying screen. Yeast proteins were extracted by the method of Kurtz et al. (20), electrophoretically separated on 10% polyacrylamide–SDS gels (22), and visualized by Coomassie staining and fluorography.

RESULTS

Yeast mRNA precursor splicing is blocked by heat shock and is protected by mild heat pretreatments. To examine the effects of heat shock on mRNA precursor splicing in *S. cerevisiae*, logarithmically growing cells were incubated at various temperatures for 1 h. Total cellular RNAs were extracted, electrophoretically separated, and hybridized with a radiolabeled probe for the yeast actin gene and a neighboring gene for a GTP-binding protein, *YPT1* (13, 35). The yeast actin gene contains a single intron; the *YPT1* gene has none. With RNAs from cells grown at 25°C or given a mild heat shock at 37°C for 1 h, only mature actin mRNAs and *YPT1* mRNAs were detected (Fig. 1A, lanes 1 and 2). The concentration of both RNAs was roughly similar in cells grown at 25°C or shifted to 37°C.

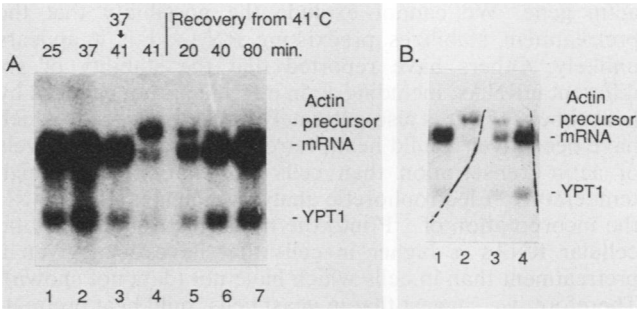

FIG. 1. Analysis of actin and *YPT1* RNAs from wild-type cells incubated at various temperatures. (A) Cells of strain W303-1B were grown at 25°C and maintained at 25°C (lane 1) or shifted to 37°C (lane 2) or 41°C (lane 4) for 1 h, or pretreated at 37°C for 45 min and then shifted to 41°C for 1 h (lane 3). For analysis of RNAs accumulating during recovery from a severe heat shock, cells were shifted directly to 41°C for 1 h and returned to 25°C for 20, 40, or 80 min (lanes 5, 6, and 7, respectively). (B) A lighter exposure of lanes 3, 4, 5, and 6 from panel A. In separate experiments, the upper band observed in 41°C-heated cells reacted with an actin intron probe. The positions of unspliced actin RNAs, actin mRNAs, and *YPT1* mRNAs are indicated at the right. Since no other bands appeared on the autoradiograms, only the relevant portion is shown.

In cells that had been given a severe heat shock at 41°C for 1 h, substantial quantities of larger RNAs were detected (Fig. 1A, lane 4, and 1B, lane 2). These RNAs were of the size expected for actin intron-containing mRNA precursors (14, 29). They hybridized both to 3′ exon-specific probes and to single-stranded antisense probes that spanned the intron-3′ exon junction of the actin gene. Free 5′ exons were not detected on Northern (RNA) blots of low-molecular-weight RNA (data not shown). Although we have not further analyzed the structure of the larger actin RNA, these results suggest that higher temperatures disrupt RNA splicing before cleavage of the 5′ splice site in yeast cells, as in *Drosophila* cells (41).

Consistent with the short half-lives of yeast mRNAs (reference 17 and references cited therein), preexisting actin and *YPT1* mRNA concentrations were greatly decreased at 41°C. The disappearance of *YPT1* mRNAs is most likely due to reduced transcription of the gene at high temperatures. It is also possible that there is an increase in the rate of degradation of *YPT1* mRNA at severe temperatures.

In *D. melanogaster*, mild heat treatments protect RNA splicing from disruption by a subsequent severe heat shock (40). To test whether mild heat treatments have a similar effect in *S. cerevisiae*, cells were incubated at 37°C for 45 min before the 41°C heat shock. In these cells, unspliced actin RNAs did not accumulate at 41°C, and actin mRNA concentrations remained high (Fig. 1A, lane 3, and 1B, lane 1).

The most likely explanation for this effect is that pretreatment protects the processing of mRNA precursors in yeast cells, resulting in the continued accumulation of mRNAs instead of the accumulation of unspliced RNAs. Such protection has been demonstrated directly for transcripts of the *HSP82* gene in *D. melanogaster* (40). An alternative explanation for the presence of mRNAs instead of precursor RNAs is that the pretreatment stabilizes preexisting actin mRNAs and at the same time inactivates transcription of the

actin gene. We cannot exclude the possibility that the pretreatment stabilizes preexisting RNAs, but it appears unlikely. Others have reported that the stability of six different mRNAs, including actin mRNAs, is not changed by heat shock (17). It is also extremely unlikely that cells which have been given a mild heat pretreatment have lower levels of actin transcription than cells shifted directly to high temperature. Electrophoretic analysis indicates that at 41°C the incorporation of ^{32}P into the broad distribution of total cellular RNAs is higher in cells that have been given a pretreatment than in cells which have not (data not shown). Therefore, we suggest that in yeast cells, mild heat pretreatments protect mRNA precursor splicing from disruption by severe heat shock.

Actin mRNA concentrations increase rapidly during recovery from heat shock. Although the 41°C heat treatment produces a complete disruption of actin mRNA splicing, the effect is rapidly reversed when cells are returned to normal temperatures. In cells incubated at 41°C for 1 h, the concentration of actin mRNAs increased greatly within 20 min of the return to 25°C. By 40 min, message levels had returned to near normal (Fig. 1A, lanes 5 to 7). As shown in the lighter exposure of Fig. 1B, previously accumulated, unspliced transcripts disappeared (lanes 2 to 4). We have not directly addressed the fate of unspliced transcripts and do not know if they contribute to the pool of new messages, but it seems unlikely. In *D. melanogaster*, messages produced during recovery are derived from new transcripts; unspliced transcripts escape the nucleus and enter the cytoplasm (41). In *S. cerevisiae*, the location of unspliced, heat-blocked transcripts has not been determined, but transcripts that are blocked from splicing for other reasons escape the nucleus and enter the cytoplasm (23). It seems unlikely that cytoplasmic transcripts would return to the nucleus for splicing.

The rapid recovery of splicing in yeast cells is notable, because it presents a point of contrast with *Drosophila* cells. When *Drosophila* cells are exposed to a heat shock that disrupts splicing, it remains disrupted for nearly 2 h after the cells are returned to normal temperatures (40, 41). This slower recovery in *Drosophila* cells is observed even when the disrupting treatment is carefully titrated to be just sufficient to provide disruption (38°C for 30 min).

Cycloheximide does not block the protection of RNA splicing in yeast cells. To determine whether proteins synthesized during the pretreatment were required for the protection of RNA splicing in *S. cerevisiae* as they are in *D. melanogaster*, yeast cells were treated with cycloheximide at 10 μg/ml, a concentration that very greatly reduced incorporation of [^3H]leucine into total cellular protein and completely inhibited the induction of hsps, as assessed by immunological assays with hsp26 antibody, Coomassie staining, and fluorography (data not shown). After incubation with cycloheximide at various temperatures for 1 h, RNAs were extracted and actin transcripts were analyzed.

No actin mRNA precursors were detected in cells incubated at temperatures ranging from 25 to 39°C, and actin mRNAs were present in approximately equal concentrations (Fig. 2, lanes 1 to 3). In cells incubated at 41°C, the concentration of mature actin mRNAs decreased and unspliced actin RNAs accumulated (Fig. 2, lane 4), as they had in the absence of cycloheximide (Fig. 1). Thus, cycloheximide does not block transcription of the actin gene or splicing of actin RNA, nor does it prevent the disruption of splicing or the accumulation of unspliced RNAs at severe temperatures.

These results are consistent with the results of similar

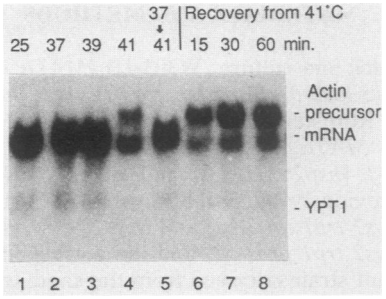

FIG. 2. Analysis of RNAs from wild-type cells treated with cycloheximide and incubated at various temperatures. Cells of strain W303-1B were grown at 25°C, and cycloheximide (10 μg/ml) was added 5 min before the culture was divided. Aliquots were maintained at 25°C (lane 1), shifted for 1 h to 37°C (lane 2), 39°C (lane 3), or 41°C (lane 4), or pretreated at 37°C for 45 min and then shifted to 41°C for 1 h (lane 5). For analysis of RNAs accumulating during recovery from severe heat shock in the presence of cycloheximide, cells were shifted to 41°C for 1 h and returned to 25°C for 15, 30, or 60 min (lanes 6, 7, and 8, respectively). The level of *YPT* RNA in this autoradiograph indicates that this is a lighter exposure than Fig. 1.

experiments with *D. melanogaster*. However, the two organisms differ sharply in another respect. When cells were treated with cycloheximide to block the induction of heat shock proteins immediately before they were given a mild heat pretreatment, protection was still observed. No unspliced actin RNAs were detected when the cells were shifted to 41°C. Furthermore, the concentration of mature actin mRNAs was much higher in cells which had been given a pretreatment (Fig. 2, compare lanes 4 and 5). Thus, mild heat pretreatments provide protection of RNA splicing at high temperatures in yeast cells even in the absence of new protein synthesis. In contrast, in *Drosophila* cells, cycloheximide completely blocks the protective effect of the pretreatment.

We next asked whether proteins synthesized during recovery might help to restore RNA splicing once it has been blocked by a sudden severe heat shock. Yeast cells were treated with cycloheximide, shifted directly to 41°C for 1 h to disrupt splicing and then returned to 25°C. During recovery, actin message concentrations increased only slightly, while unspliced RNA concentrations increased greatly. (Compare the ratios of precursor to message in the recovery lanes of Fig. 2 [lanes 6 to 8] with those of Fig. 1 [lanes 5 to 7].) Thus, in the absence of protein synthesis, splicing was not effectively restored and unspliced RNAs continued to accumulate for at least 1 h. This is in contrast to the rapid recovery of splicing at 25°C when protein synthesis was not inhibited (Fig. 1, lanes . to 7). In summary, mild heat pretreatments protect RNA splicing during a second more severe heat shock, even in the absence of protein synthesis. However, once RNA splicing has been disrupted by a sudden severe heat shock, protein synthesis is required for the rapid restoration of RNA splicing.

Certain hsps are not required for the protection or recovery of RNA splicing. One explanation for the different effects of cycloheximide in *Saccharomyces* and *Drosophila* cells is that the yeast hsps are not required for the protection of RNA splicing. That is, some other protective mechanism might be activated by the mild heat pretreatment. Alternatively, hsps may be required for the protection of splicing in both organisms, but in yeast cells the critical proteins might

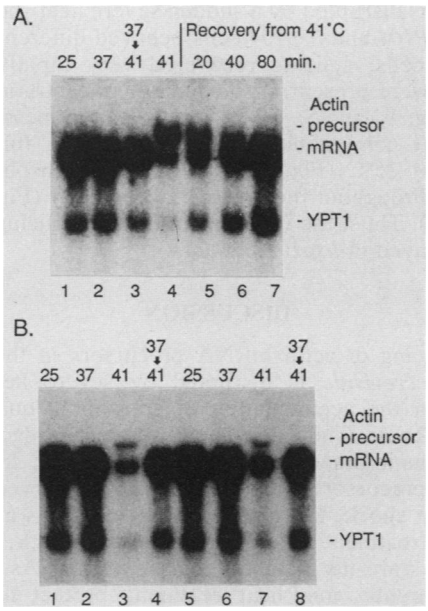

FIG. 3. Analysis of RNAs from wild-type and *hsp26* and *hsp82* mutants. (A) Cells of strain LP12 (*hsp26*) were grown at 25°C and maintained at 25°C (lane 1) or shifted for 1 h to 37°C (lane 2) or 41°C (lane 4), or pretreated at 37°C for 45 min and then shifted to 41°C for 1 h (lane 3). For analysis of RNAs during recovery from severe heat shock, cells were shifted to 41°C for 1 h and returned to 25°C for 20, 40, or 80 min (lanes 5, 6, and 7, respectively). Results obtained with wild-type cells (W303-1B) treated in parallel with the mutants are shown in Fig. 1. (B) *hsp82* mutant cells (strain 10:1-16, lanes 1 to 4) and the corresponding wild-type cells (FYH6, lanes 5 to 8) grown at 25°C were maintained at 25°C (lane 1 and 5) or shifted for 1 h to 37°C (lane 2 and 6) or 41°C (lane 3 and 7) or pretreated at 37°C for 45 min and then shifted to 41°C for 1 h (lane 4 and 8).

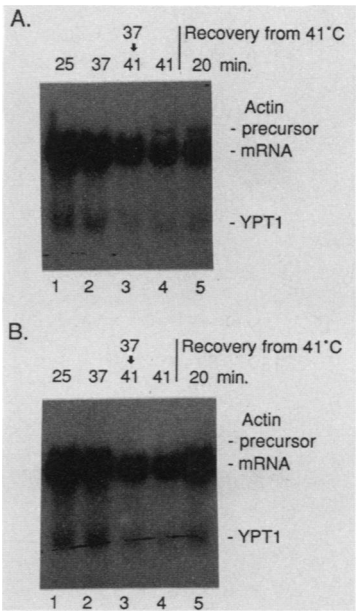

FIG. 4. Analysis of RNAs from wild-type and *ssa1 ssa2* double mutant cells. Cells of strain T127 (A) and T128 (B) were grown at 25°C and either maintained at 25°C (lane 1) or shifted for 1 h to 37°C (lane 2) or 41°C (lane 4), or pretreated at 37°C for 45 min and then shifted to 41°C for 1 h (lane 3). For analysis of RNAs during recovery from severe heat shock, cells were shifted to 41°C for 1 h and returned to 25°C for 20 min (lane 5).

be present in substantial quantities prior to heat shock and only require activation by the pretreatment. For example, certain members of the hsp70 family (encoded by *SSA1* and *SSA2*) that are functionally equivalent to heat-inducible members of the family (*SSA3* and *SSA4*) are abundant in yeast cells at normal temperatures (8–10, 18, 39).

To address this question, we examined RNA splicing in strains carrying mutations in various hsp genes. Note that the temperature which produces the maximum block in splicing varies slightly for different strain backgrounds. For any given strain the effects of temperature are very reproducible. For simplicity, all experiments reported here were performed at 41°C. In each case the isogenic wild-type strain provides the basis for assessing the effects of the mutations. No significant differences were found between strains carrying a deletion of *HSP26* (Fig. 3A), a disruption of *HSP82* (Fig. 3B), or a disruption of *HSC83* (not shown) and their respective wild-type strains. Actin mRNAs accumulated, without detectable accumulation of unspliced RNAs, at 25 and 37°C. At 41°C, the concentration of actin mRNAs decreased greatly and unspliced actin RNAs accumulated. Slight differences in overall RNA accumulation may occur, but in both the *hsp83* mutant and wild-type strains, precursor RNA accumulates at 41°C. For all strains, when cells were first given a mild pretreatment, no unspliced RNAs accumulated at 41°C. Furthermore, there was no difference between *hsp26* wild-type and mutant cells in the rate at which RNA splicing recovered after a 41°C heat shock (compare Fig. 3A, lanes 5 to 7, *hsp26* cells and Fig. 1, lanes 5 to 7, the corresponding isogenic wild-type strain.) In summary, *hsp26*, *hsp82*, and *hsc83* are not individually required for mRNA splicing at normal temperatures or at intermediate heat-shock temperatures nor for the protection of RNA splicing at severe temperatures by a mild heat pretreatment.

Strains carrying mutations in various members of the *hsp70* gene family were also tested. In strains carrying mutations in either of the two strongly heat-inducible members of this family, *SSA3* and *SSA4*, the patterns of RNA splicing at various temperatures were indistinguishable from those of the corresponding wild-type strains. RNA splicing was blocked at 41°C and above and was protected by a mild heat pretreatment (not shown). Thus, as with the other heat-inducible hsps discussed above, *hsp70 SSA3* and *hsp70 SSA4* are not individually required for the protection of RNA splicing by mild heat pretreatment or for the stability of precursor RNAs at high temperatures.

Protection of RNA splicing is constitutive in some *hsp70* mutants. Yeast strains carrying mutations in two other members of this *hsp70* gene family, *SSA1* and *SSA2*, were of special interest for two reasons. First, these double mutants are 100-fold more thermotolerant than wild-type cells, as determined by survival after sudden exposure to extreme temperatures. In fact, the killing curves for these cells look very much like the killing curves for wild-type cells that have been given a mild heat pretreatment. Second, at normal temperature, these strains produce other hsps, including other members of the hsp70 family (SSA4 and possibly SSA3), hsp104, hsp83, and hsp26, at a much higher level than wild-type cells (7, 39; unpublished observations). These are the only hsp mutants known to express high levels of other hsps at normal temperatures.

RNA splicing was examined in the *ssa1 ssa2* double

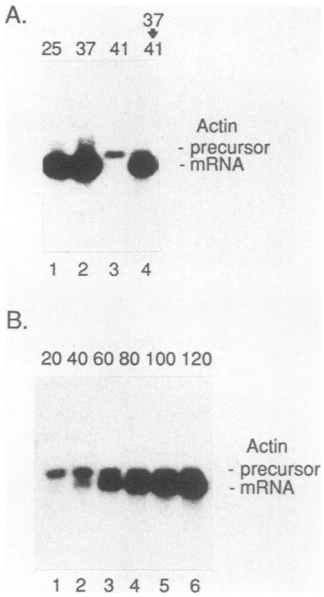

FIG. 5. Analysis of RNAs from *hsp104* mutant cells. (A) Cells of strain δ104 grown at 25°C were maintained at 25°C (lane 1) or shifted to 37°C (lane 2) or 41°C (lane 3) for 1 h, or given a pretreatment at 37°C for 45 min and then shifted to 41°C for 1 h (lane 4). (B) For analysis of RNAs during recovery from severe heat shock, *hsp104* cells were shifted to 41°C for 1 h and returned to 25°C for 20, 40, 60, 80, 100, or 120 min (lanes 1 to 6, respectively).

mutant under various conditions (Fig. 4B), in parallel with wild-type isogenic strains (Fig. 4A). Actin mRNAs accumulated normally at 25 and 37°C. As with wild-type cells, when *ssa1 ssa2* cells were pretreated at 37°C for 45 min and then shifted to 41°C for 1 h, mRNAs were present and unspliced RNAs were not detected. However, in contrast to the wild-type cells, unspliced RNAs never accumulated in *ssa1 ssa2* cells shifted directly to 41°C (Fig. 4B, lane 4). Furthermore, unspliced RNAs were not detected through 80 min of recovery from a 1-h heat shock at 41°C (lane 5, and not shown). Apparently, RNA splicing was protected in *ssa1 ssa2* cells even without administration of a mild heat pretreatment. Preinduced protection of RNA splicing was very reproducible and highly specific to this strain. It was never observed in strains carrying mutations of other closely related members of the same *hsp70* gene subfamily, *SSA3* or *SSA4*.

Recovery of RNA splicing is delayed in *hsp104* mutants. The single-copy gene encoding hsp104 has recently been cloned and characterized. Cells in which *hsp104* has been eliminated by site-directed mutation grow at the same rate as wild-type strains at 25 and 37.5°C. They also die at the same rate as wild-type cells when exposed directly to extreme temperatures. However, in contrast to wild-type cells, *hsp104* mutants are deficient in induced thermotolerance (34); that is, when treated at 37°C before exposure to extreme temperature, wild-type cells survive much longer than the mutants.

Comparison of actin RNA splicing under various conditions in isogenic *HSP104* (Fig. 1) and *hsp104* (Fig. 5) cells revealed another important difference. In both wild-type and mutant cells, RNA splicing was normal at 25 and 37°C, blocked at 41°C, and protected by a 37°C pretreatment (Fig. 1, lanes 1 to 4, and Fig. 5A, lanes 1 to 4). However, when splicing was disrupted by a sudden severe heat shock (1 h at 41°C), *HSP104* and *hsp104* cells behaved differently. In the wild-type cells, splicing recovered very rapidly. Mature messages were present in substantial concentrations within 20 min (Fig. 1, lane 5). In the mutant, actin messages were detected at comparable levels only after a full hour of recovery at 25°C (Fig. 5B, lane 3), and unspliced RNA persisted throughout the first 2 h of recovery (Fig. 5B, and not shown). Thus, the recovery of mRNA splicing is significantly delayed in *hsp104* mutants.

DISCUSSION

The splicing of actin mRNA precursors in the budding yeast *S. cerevisiae* is disrupted by severe heat shock, resulting in the accumulation of substantial quantities of unspliced RNAs. Thus, consistent with a growing number of observations in other laboratories (1, 2, 19, 25, 28), a block in mRNA precursor splicing is a highly conserved effect of severe heat shock. Moreover, when yeast cells are given a mild heat treatment before a severe heat shock, unspliced actin RNAs are not detected and actin mRNAs are abundant. Apparently, mild heat treatments protect mRNA precursor splicing from disruption in yeast cells as they do in *Drosophila* cells.

However, we note two important differences between the two organisms. The first concerns a difference in the requirement for protein synthesis during the induction of protection. In *D. melanogaster*, the protective effect of a mild heat pretreatment is inhibited when cycloheximide is administered before the pretreatment, blocking the induction of heat shock proteins (40). This is not an effect of the drug per se, because cycloheximide does not block protection if it is administered after the treatment, when heat shock proteins have already been produced. Since the pretreatment greatly reduces the synthesis of all proteins except the heat shock proteins, these results suggest that heat shock proteins are required for the protection of RNA splicing in *D. melanogaster*. In contrast, in yeast cells, the protective effect of a mild heat pretreatment is not inhibited by cycloheximide and is apparently independent of new protein synthesis.

A second difference between the two organisms is apparent in the absence of a protective pretreatment, when splicing is disrupted by a sudden shift to high temperature. In *Drosophila* cells, even after short treatments at the lowest temperature which will disrupt splicing, recovery is slow, requiring at least 90 min. In yeast cells, recovery of splicing is extremely rapid; under normal circumstances, splicing is restored within 20 min.

An explanation for the differences we observed between *D. melanogaster* and *S. cerevisiae* assumes that differences in the patterns of heat shock protein synthesis in the two organisms account for their idiosyncrasies. This is based, in part, on three characteristics of the yeast *ssa1 ssa2* double mutants. First, in these mutants, hsp104, hsp82, hsp26, and certain members of the hsp70 family are constitutively synthesized at abnormally high levels (7, 39; unpublished observations), perhaps because *SSA1* and *SSA2* are involved in the regulation of heat shock gene expression. Second, the double mutants are 100-fold more thermotolerant than the wild-type cells (7). Apparently, the mechanisms that are normally activated by mild heat pretreatment to confer increased survival at high temperature are already activated at normal temperature in the double mutants. Third, as shown here, protection of RNA splicing, which normally requires activation by mild heat pretreatments, is already

activated at normal temperature in the double mutants. Thus, in these mutant strains, high constitutive expression of heat shock proteins at normal temperature is correlated with both the ability to survive exposure to severe temperatures and the ability to protect RNA splicing at high temperatures.

Together with the effects of cycloheximide and of other *hsp* mutations on RNA splicing, these results suggest that at least two conditions are necessary for the protection of RNA splicing. First, the protective factors, including most probably the heat shock proteins, must be present in requisite quantities. In yeast cells these factors are present in appreciable concentrations at normal temperature, whereas in *Drosophila* cells one or more of them must be induced by heat treatment. A possible candidate is hsp70. In yeast cells, the pattern of expression of this protein is different than in *Drosophila* cells. In particular, there is considerable overlap in the expression of functionally equivalent members of the family. One protein is highly expressed at normal temperatures (*SSA2*), one is expressed at an appreciable level at normal temperatures and is further induced with heat (*SSA1*), while two others are strongly induced by heat (*SSA3* and *SSA4*). In *D. melanogaster*, the protein which predominates after heat shock, hsp70, is not expressed at normal temperatures (38). It is not known if those members of the hsp70 family which are expressed at normal temperatures are functionally equivalent to hsp70. However, the more extreme discontinuity in the expression of members of the *Drosophila* family compared with the yeast family (8, 9) suggests a greater discontinuity in function. That is, the functions of *Drosophila* hsp70 may be more specifically tuned to conditions of physiological stress. Indeed, in separate experiments, we have found that expression of hsp70 at normal temperatures in *D. melanogaster* blocks cell growth (36a).

A second condition for the protection of RNA splicing is that the protective factors must be in an activated state. Such activation may proceed through a biochemical modification or subcellular relocation. To continue with the example of hsp70, in *D. melanogaster* this protein is concentrated in the nucleus and along membranes during heat shock but is more concentrated in the cytoplasm during recovery. Upon a second heat shock, hsp70 reconcentrates in the nucleus, where presumably at least some of its protective functions are localized (37).

In the yeast double mutants, regulation of the heat shock response may be altered so that both conditions occur at normal temperatures, thereby providing protection of RNA splicing when cells are suddenly shifted to high temperatures. In wild-type yeast cells, we suggest that hsps are present at normal temperatures in sufficient quantities to provide protection, but still require activation by a mild heat pretreatment. In *Drosophila* cells, the heat treatment would be required both to induce the synthesis of the hsps and to activate them.

A role for hsp70 in protecting RNA splicing at high temperatures is consistent with its subcellular localization and with other known functions of the protein family in the assembly and disaggregation of multiprotein complexes (24, 31, 33). Nevertheless, because of the pleiotropic effects of the *hsp70* mutations in *S. cerevisiae*, the arguments remain indirect. A role for hsp104 in facilitating the recovery of splicing after a severe heat shock is directly supported by the phenotype of the *hsp104* mutation. This mutation has no detectable effect on the expression of other hsps in yeast cells or on growth at either 25 or 37°C (34). The mutation does not affect the block in splicing when cells are suddenly shifted to high temperatures, nor does it affect the protection that is induced by a mild heat pretreatment. However, if splicing is disrupted by a sudden severe heat shock, the recovery of splicing is delayed in the mutant.

The effects of the *hsp104* mutation are exactly like the effects of cycloheximide treatment; that is, cycloheximide does not block the protection of splicing by mild heat pretreatments. However, once splicing is disrupted by a sudden severe heat shock, cycloheximide delays its recovery. hsp104 is not present at normal growth temperatures in yeast cells but is strongly induced by heat shock (34). These results are consistent with the supposition that the effects of cycloheximide on splicing are the result of its ability to block hsp104 expression.

Other findings aid in the interpretation of this result. The products of four other yeast genes, *SSA3*, *SSA4*, *HSP82*, and *HSP26*, are also strongly induced by heat shock. However, mutations in these genes do not affect the recovery of splicing during mild heat shock. Apparently, hsp104 facilitates, directly or indirectly, the restoration of RNA splicing once it has been disrupted. Strikingly, this delayed recovery of RNA splicing after disruption by sudden severe heat shock is also observed in *Drosophila* cells (40). *D. melanogaster* is one of the few organisms which appears to lack a heat shock protein in the *HSP104* class (24).

Finally, our studies with cycloheximide and yeast *hsp* mutants demonstrate that the mechanisms which protect RNA splicing from disruption at high temperatures are distinct from those that facilitate the recovery of splicing after it has been disrupted. Together with our earlier observations on *D. melanogaster*, distinct classes of hsps appear to be involved in each of these processes.

These results are consistent with the distinct phenotypes of *hsp* gene mutations in yeast cells. Cells harboring mutations in the genes encoding hsp82 and certain members of the hsp70 family are temperature sensitive for growth but have high rates of survival after exposure to extreme temperature (3, 7). Cells harboring a mutation in the gene encoding hsp104 are not temperature sensitive for growth but show greatly reduced survival after exposure to extreme temperature (34). Thus, hsp70 and hsp82 help to maintain vital cellular processes at the upper end of a cell's normal temperature growth range. hsp104, on the other hand, helps cells to recover from the damage caused by a sudden severe heat shock.

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