Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae*

Claudio Saavedra,¹ Kuei-Shu Tung,^{2,3} David C. Amberg,^{1,4} Anita K. Hopper,² and Charles N. Cole^{1,5}

¹Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire 03755 USA; ²Department of Biochemistry and Molecular Biology, The Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, Pennsylvania 17033 USA

The response of eukaryotic cells to heat shock and other forms of stress occurs at both transcriptional and post-transcriptional levels. We used *in situ* hybridization to determine whether stress affected the subcellular distribution of poly(A)⁺ RNA in *Saccharomyces cerevisiae*. Following induction of stress by either heat shock (42°C) or addition of a high concentration of ethanol (10%), the nucleocytoplasmic export of most poly(A)⁺ RNA was blocked. *In situ* hybridization indicated that heat-inducible *SSA4* and *SSA1* mRNAs were exported from nuclei under these same conditions. On the other hand, both *GAL1* and *URA3* transcripts expressed from the *SSA4* promoter accumulated in nuclei following heat shock. Sequences within either the 5' 1600 or the 3' 500 nucleotides of *SSA4* mRNA were sufficient to direct *GAL1* mRNA to the cytoplasm during stress. The export of *SSA4* mRNA following stress required functional nuclear pore complexes, as *SSA4* mRNA accumulated in nuclei following heat shock of cells containing temperature-sensitive nucleoporins. However, the selective export of *SSA4* mRNA was maintained in heat-shocked cells carrying temperature-sensitive alleles of *RNA1*, *PRP20*, or an inducible dominant-negative allele of *GSP1*, the *S. cerevisiae* homolog of RAN/TC4. The results reported here suggest that there is selective export of mRNA in yeast.

[Key Words: Nucleocytoplasmic transport; heat shock; yeast; Ran/TC4; protein import; *in situ* hybridization]

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In eukaryotic cells, the biogenesis of mature mRNA requires transcription and several post-transcriptional processing steps that occur within the nucleus before the mature mRNA is exported to the cytoplasm. These processing steps include addition of a cap at the 5' end of the pre-mRNA, cleavage, addition of a poly(A) tail at the 3' end of the mRNA, and splicing to remove introns from the pre-mRNA. The presence of the 7-methyl-guanosine cap at the 5' end of the mRNA enhances mRNA export but is not required for the export of microinjected mRNA [Hamm and Mattaj 1990]. A 3'-end poly(A) tail may enhance mRNA export and probably helps to protect pre-mRNAs and mRNAs from nucleolytic degradation but is not an essential signal for mRNA export [Jarmolowski et al. 1994]. Although transcribed sequences could contain export information, the protein components of hnRNPs are likely to contain signals for export of heterogeneous nuclear ribonucleoprotein (hnRNP), as nuclear export signals have been defined for human hnRNP A1 [Michael et al. 1995] and the HIV-1 Rev protein [Fischer et al. 1995].

Several *trans*-acting components needed for efficient

RNA export have been identified. The *PRP20* [Clark and Sprague 1989; Vijayraghavan et al. 1989; Acbi et al. 1990] and *RNA1* [Hartwell 1967; Hopper et al. 1978] genes encode, respectively, a guanine-nucleotide exchange factor (for review, see Dasso 1993; Sazer 1996) and a GTPase-activating protein [Becker et al. 1995; Bischoff et al. 1995; Corbett et al. 1995] for Gsp1p [Belhumeur et al. 1993], the yeast homolog of the mammalian RAN/TC4 protein. Poly(A)⁺ RNA accumulates in the nuclei of yeast and mammalian cells carrying temperature-sensitive mutations of *PRP20* and *RNA1* [Amberg et al. 1992, 1993; Forrester et al. 1992; Kadowaki et al. 1992], and also in yeast cells overexpressing a dominant-negative form of Gsp1p [Schlenstedt et al. 1995], suggesting that the Gsp1 GTPase cycle is involved in the export of mRNA from the nucleus. An additional important factor shown to be necessary for efficient mRNA nuclear export in budding yeast is Npl3p [Lee et al. 1996]. Some of the structural features of Npl3p are also found in some mammalian hnRNP proteins, including two RRM domains and a carboxy-terminal glycine-rich domain that contains RGGY/F repeats (for review, see Dreyfuss 1986). Moreover, Npl3p has been shown to shuttle between the nucleus and the cytoplasm [Bossie et al. 1992; Russell and Tollervy 1992; Flach et al. 1994; Wilson et al. 1994].

During heat shock or other types of stress, cells redirect their synthetic machinery to produce several fami-

Present addresses: ³Department of Biology, Yale University, New Haven, Connecticut 06510 USA; ⁴Department of Genetics, Stanford University Medical Center, Palo Alto, California 94305 USA.
⁵Corresponding author.

lies of heat shock proteins. Redirection of the synthetic machinery occurs at multiple levels, including transcription, post-transcriptional RNA processing, and RNA turnover. In this study we report that heat shock and other forms of stress lead to the accumulation of poly(A)⁺ RNAs within yeast nuclei, whereas heat shock mRNAs *SSA4* and *SSA1* (encoding *S. cerevisiae* Hsp70) are efficiently exported to the cytoplasm. We examined the heat shock response in cells carrying mutant alleles of *RNA1*, *PRP20*, or *GSP1* and observed that export of most poly(A)⁺ RNAs were blocked but export of *SSA4* mRNA continued following cellular stress. Under the same conditions, however, *SSA4* mRNA was retained in the nuclei of cells carrying temperature-sensitive mutants of nucleoporins *RAT7/NUP159*, *RAT2/NUP120*, and *NUP145*. These results indicate that selective export of heat shock mRNAs occurs following stress, requires functional NPCs, but does not require the *RAN/GSP1* GTPase system. Cellular stress also inhibited nuclear protein import of a karyophilic reporter protein.

Results

Nucleocytoplasmic transport of poly(A)⁺ RNA is blocked following heat stress in yeast

We employed in situ hybridization to compare the subcellular distribution of poly(A)⁺ RNA in *Saccharomyces cerevisiae* under various conditions of growth or stress. This assay uses an oligo(dT)₅₀ probe to which digoxigenin-dUTP is added at its 3' end (Amberg 1992). The sites at which this probe hybridizes to RNA are visualized by using an FITC-conjugated anti-digoxigenin antibody and fluorescence microscopy (Amberg et al. 1992). A typical pattern for cells growing exponentially at 23°C is shown in Figure 1A, and the same field of cells stained with DAPI, to permit visualization of the nuclei, is shown in Figure 1B. As expected, poly(A)⁺ RNA was distributed throughout the cell under conditions of normal growth. However, a strong nuclear accumulation of poly(A)⁺ RNA was seen in all cells after exposing wild-type cells to heat shock stress conditions (42°C or 45°C) for 1 hr (Fig. 1C). The accumulation of poly(A)⁺ RNA was detectable as soon as 5 min after heat shock and was prominent in nearly 100% of the cells within 30 min after heat shock (data not shown). Some poly(A)⁺ RNA was detected in the cytoplasm following heat shock. Most likely, this represents cellular mRNAs that have not been degraded plus those mRNAs induced and exported from nuclei during cellular stress.

*Detection of single species transcripts in yeast: export of *SSA4* (Hsp70) mRNA is not blocked following heat shock*

The heat shock response is regulated both transcriptionally and post-transcriptionally (Morimoto et al. 1994). Therefore, the observation that heat shock induced a block to mRNA export raises an intriguing paradox: How can a stress response be mounted if export of

Figure 1. Poly(A)⁺ RNA accumulates in the nuclei of yeast cells shifted to 42°C for 1 hr. (A) In situ hybridization to detect poly(A)⁺ RNA of wild-type cells incubated at room temperature. (B) DAPI-stained image of the same field of cells as in A. (C) In situ hybridization to detect poly(A)⁺ RNA in wild-type cells shifted to 42°C for 1 hr. (D) DAPI staining of the same field of cells as in C.

poly(A)⁺ RNA from the nucleus is blocked? Perhaps heat shock mRNAs can leave the nucleus by some mechanism while the bulk of poly(A)⁺ RNA would remain in the nucleus following stress. To address this possibility, we modified the in situ hybridization assay to permit detection of individual species of mRNA (see Materials and methods).

SSA4 is one of the four members of the yeast Hsp70-*SSA* gene subfamily. It is expressed at an extremely low level during vegetative growth but is rapidly induced to a high level following heat shock (for review, see Craig 1992). Because of its inducibility, low basal level of expression, and thorough characterization (Boorstein and Craig 1990; Young and Craig 1993), we chose first to detect the *SSA4* transcript. The DNA sequences of the four *SSA* genes are closely related, but the 3'-untranslated region of *SSA4* lacks significant identity with the other three *SSA* genes. To obtain a probe specific for *SSA4* mRNA, we employed PCR to generate a 270-bp DNA corresponding to the 3' end of *SSA4* mRNA.

We introduced a 2-μ plasmid containing the entire *SSA4* gene into wild-type yeast cells. Northern analyses were used to compare the levels of *SSA4* mRNA present under different conditions. There was little *SSA4* mRNA under normal growth conditions, but it was induced rapidly by heat shock (42°C) (Fig. 2A) and to higher levels than in wild-type cells that carry a single copy of *SSA4* (data not shown). Unlike bulk poly(A)⁺ RNA, which was restricted to nuclei under these conditions, *SSA4* mRNA was localized throughout the cell (Fig. 2B, panel C). Little or no fluorescent signal for *SSA4* mRNA was observed in cultures grown at room temperature (Fig. 2B, panel A).

A similar nuclear accumulation of poly(A)⁺, along with induction and export of *SSA4* mRNA, was also seen when wild-type yeast cells were stressed by the addition

Figure 2. *SSA4* mRNA is induced and appears in the cytoplasm following heat shock. (A) Northern blot detection of *SSA4* mRNA in cells transformed with a high-copy plasmid (pEC702) containing the *SSA4* gene. Total RNA was isolated from cells grown at 23°C (lane 1) or shifted to 42°C for 1 hr (lane 2). (B, panel A) In situ hybridization detection of *SSA4* mRNA in wild-type cells incubated at 23°C. (Panel B) DAPI staining of the same field of cells as in panel A. (Panel C) In situ hybridization detection of *SSA4* mRNA in wild-type yeast cells shifted to 42°C for 1 hr. (Panel D) DAPI staining of the same field of cells as in panel C.

of ethanol to a final concentration of 10% (data not shown). We also examined the nucleocytoplasmic distribution of *SSA1* mRNA, encoding another member of the yeast Hsp70 family and found that this mRNA was also efficiently exported to the cytoplasm following either heat or ethanol shock (data not shown).

Transcription from the SSA4 promoter is not sufficient for selective export of mRNAs under its control

The promoters of heat shock genes are primed for rapid response, as evidenced by their nuclease hypersensitivity (Wu 1980) and by the fact that the TATA box-binding protein and heat shock transcription factor are bound to the promoters of heat shock genes in the absence of heat shock (Gross et al. 1990, 1993; Giardina and Lis 1995a,b). This preloading of transcription factors onto the heat shock promoters appears to allow rapid initiation of transcription following stress. A scenario that could account for the differential subcellular localization of bulk poly(A)⁺ RNA and *SSA4* mRNA would be that *SSA4* transcription is activated prior to the onset of the block to export of poly(A)⁺ RNA that develops following heat

stress. If this were the case, heat shock mRNAs would be synthesized and exported extremely rapidly after stress, whereas most mRNAs would progressively accumulate inside the nucleus during the heat treatment.

To address this scenario, we subcloned the *GAL1*-coding region downstream of the *SSA4* promoter in a high copy plasmid, introduced the plasmid into wild-type yeast cells, and located the *GAL1* transcripts by in situ hybridization (Fig. 3). The *SSA4* promoter fragment used for this construct extends from nucleotide –1200 to –40 relative to the *GAL1* transcription initiation site. This fragment contains the TATA box and five canonical heat shock consensus sequences (Fig. 3B).

Upon shifting the cultures from 23°C to 42°C for 1 hr,

Figure 3. *GAL1* mRNA is retained in nuclei following heat shock. Wild-type cells harboring a high-copy plasmid containing *GAL1* sequences under control of the *SSA4* promoter were either incubated at 23°C or shifted to 42°C for 1 hr. (A) Northern blot detection of *GAL1* mRNA. Total RNA was isolated from cells either grown at 23°C (lane 1) or shifted to 42°C for 1 hr (lane 2). (B) Diagram of the *GAL1* construct. Dark area represents the *GAL1* sequences. Vertical arrows show beginning and end of the open reading frame. The open area represents the *SSA4* promoter. Black diamonds show the canonical heat shock elements; the asterisk shows TATA box at –80. (H) *HindIII*; (S) *StuI* sites. (C, panel A) In situ hybridization to detect *GAL1* mRNA in cells grown at 23°C. (Panel B) DAPI staining of the same field of cells as in panel A. (Panel C) In situ hybridization to detect *GAL1* mRNAs in cells shifted to 42°C for 1 hr. (Panel D) DAPI staining of the same field of cells as in panel C.

this hybrid gene was readily induced (Fig. 3C). The sub-cellular distribution of the *GAL1* mRNA was determined by in situ hybridization (see Materials and methods). No signal for *GAL1* mRNA was evident in cells maintained at 23°C (Fig. 3C, panel A). After heat treatment at 42°C, this mRNA (Fig. 3C, panel C) accumulated in the nucleus although some was also detected in the cytoplasm. This indicates that the *SSA4* promoter functioned normally and was induced strongly by heat shock. We also found that a *URA3* mRNA under control of the same *SSA4* promoter accumulated in nuclei under the same stress conditions (data not shown). Therefore, the promoter of the *SSA4* gene and its rapid induction by stress are not sufficient to account for the selective export of an mRNA produced under its control.

If cytoplasmic accumulation of *SSA4* mRNA were attributable solely to rapid transcription of *SSA4* prior to the onset of the block in export of poly(A)⁺ RNA, the *SSA4* mRNA should be transcribed only very early following stress. By this scenario, continued transcription of the *SSA4* gene once the block to export of poly(A)⁺ RNA had developed would be expected to result in nuclear accumulation of *SSA4* mRNA. Therefore, we examined the kinetics of *SSA4* mRNA induction in heat-shocked (42°C) yeast cells (Fig. 4). *SSA4* RNA was virtually undetectable prior to heat shock but was easily detected within 5 min after shifting to 42°C. *SSA4* mRNA synthesis continued and reached a maximum at 30 min after the initial heat shock, at which time accumulation of poly(A)⁺ RNA in the nucleus was readily detected. Subsequently, the levels of *SSA4* mRNA declined. These data suggest that the appearance of *SSA4* mRNA in the cytoplasm following heat shock, and the retention in the nucleus of poly(A)⁺ RNA, reflect selective export of the *SSA4* mRNA rather than extremely rapid induction of its promoter prior to the establishment of a block to export of poly(A)⁺ RNA.

Sequences within the 3'-terminal 500 nucleotides of the SSA4 transcript facilitate export of a heterologous coding region following heat shock

To study whether the *SSA4* mRNA contains sequences

Figure 4. Northern analysis of the kinetics of accumulation of *SSA4* mRNA following heat shock. Wild-type yeast cells in the exponential phase of growth were shifted from 23°C to 42°C. Samples of cells were harvested at the time of the shift (0 min) and at various times after the shift to 42°C. RNA was isolated from cells and subjected to electrophoresis in an agarose gel. A Northern blot was prepared and probed with a digoxigenin-labeled probe for *SSA4* mRNA. (Lane 1) RNA from cells harvested at the time of the shift to 42°C; (lanes 2–6) RNAs harvested at 5, 15, 30, 60, and 120 min after the shift.

Figure 5. Hybrid *GAL1*–*SSA4* mRNAs are exported from the nucleus following heat shock. Hybrid constructs on a high-copy plasmid (2 μ -derived) were prepared that contained both *GAL1* and *SSA4* sequences, expressed from the *SSA4* promoter. (A) The hybrid gene contains nucleotides 1–1276 of *GAL1* upstream of *SSA4* sequences 240–2300, including the 3'-end signals. (B) 1612 nucleotides were deleted from the construct examined in A to yield a construct containing nucleotides 1–969 of *GAL1* and nucleotides 1545–2300 of *SSA4*. Within each half of A and B, panels A show in situ hybridization to detect the hybrid *GAL1*–*SSA4* mRNA in cells cultured at 23°C. The probe used is specific for *SSA4* sequences and is the same probe used for Fig. 2. Panels B show DAPI staining of the same fields of cells shown in panels A. Panels C show in situ hybridization to detect the *GAL1*–*SSA4* hybrid mRNA in cells shifted to 42°C for 1 hr. Panels D show DAPI staining of the same fields of cells shown in panels C.

that facilitate its differential export during heat shock or whether it lacks sequences that promote nuclear retention of most mRNAs during stress, we constructed in a high-copy plasmid a *GAL1*–*SSA4* hybrid gene whose transcription was driven by the *SSA4* promoter. The fusion transcript consisted of the *GAL1*-coding region (1276 nucleotides) minus polyadenylation and transcription termination sequences, followed by most of the *SSA4*-coding region (2060 bp) including its polyadenylation and transcription termination signals (Fig. 5A). When the strain carrying this plasmid was shifted to 42°C for 1 hr, in situ hybridization revealed that the hybrid RNA was located throughout the cell (Fig. 5A, panel C). The same staining pattern was seen using either *GAL1*-specific or *SSA4*-specific probes, as the hybrid mRNA contains sequences complementary to both (data not shown). These results indicate that in this situation, nuclear export is dominant over retention and that *SSA4* mRNA may possess sequences that facilitate export. The signal intensity varied considerably from cell to cell (see Fig. 5A, panel C). This most likely reflects the wide range of plasmid copy numbers in different cells and may also indicate that transcription of the *SSA4* promoter is not induced equally in all cells.

Because *SSA4* sequences were necessary and sufficient to mediate export following heat shock when fused to a

heterologous mRNA that otherwise was retained in the nucleus, we created progressive deletions of the *SSA4*-coding region to identify the *SSA4* sequences involved in export. We began with the *GAL1*-*SSA4* hybrid gene described above and deleted *SSA4* sequences progressively toward the 3' end of the *SSA4* sequences. We were able to remove most of the *SSA4* sequences without significantly affecting the ability of the hybrid transcript to be exported to the cytoplasm following heat shock. The construct containing the smallest amount of *SSA4* is diagrammed in Figure 5B, and the localization of all *GAL1*/*SSA4* hybrid transcript produced from this construct in cells shifted to 42°C for 1 hr is shown in Figure 5B, panel C. Thus, selective export still occurred in a hybrid transcript containing only those *SSA4* sequences derived from the very end of the coding region and extending through to the 3' end of the *SSA4* mRNA.

We also prepared a fusion construct in which the *SSA4* promoter directs production of a hybrid mRNA consisting of the first 1680 nucleotides of *SSA4* mRNA and nucleotides 650–1753 of *GAL1* mRNA, including its signals for 3'-end processing and polyadenylation. The mRNA from this construct was detected primarily in the cytoplasm following heat shock (data not shown), indicating that *SSA4* mRNA contains multiple sequences that facilitate cytoplasmic accumulation of *SSA4* mRNA following heat shock.

Selective export of SSA4 mRNA occurs in yeast strains carrying mutant alleles of the RNA1, PRP20, or GSP1 genes

The yeast small GTPases Gsp1p and Gsp2p (homologs of mammalian Ran/TC4) (Belhumeur et al. 1993), the

GTPase activating protein encoded by *RNA1* (Atkinson et al. 1985; Becker et al. 1995; Bischoff et al. 1995; Corbett et al. 1995), and the guanine nucleotide exchange factor encoded by *PRP20* (Aebi et al. 1990) all are important for import of proteins into nuclei. The export of poly(A)⁺ RNA from the nucleus to the cytoplasm is also blocked in yeast strains carrying mutant alleles of these genes (Amberg et al. 1992, 1993; Forrester et al. 1992; Kadowaki et al. 1992; Melchior et al. 1993; Moore and Blobel 1993; Corbett et al. 1995; Schlenstedt et al. 1995). Because the export of heat shock mRNAs responded differently to stress signals than did the bulk of cellular mRNAs, we tested whether export was dependent on or independent of the Ran/TC4 GTPase system.

Previous studies reported conflicting data concerning the production of heat shock proteins in *rna1-1* cells under temperature conditions (37°C) that we now know prevent export from the nucleus of most poly(A)⁺ RNAs (McAlister and Finkelstein 1980; Miller et al. 1982; Amberg et al. 1992). We have confirmed the data of Miller et al. (1982) and expanded these studies. We found that heat shock mRNAs were transcribed, processed, exported from the nucleus, and translated into heat shock proteins in cells carrying mutant alleles of *GSP1*, *PRP20*, and *RNA1*. We prepared extracts from ³⁵S-labeled wild-type and mutant cells either incubated continuously at 23°C or shifted to 34°C (Fig. 6A), 37°C (Fig. 6B), or 41.5°C (Fig. 6C) for various periods of time. In all cases, proteins were labeled for 15 min beginning at the times indicated in Figure 6.

In wild-type cells, heat shock proteins were synthesized transiently in cells shifted to 34°C or 37°C for 15 min but were no longer synthesized at substantial levels 1 hr following the temperature shift (Fig. 6A,B). A sus-

Figure 6. Analysis of heat shock protein synthesis. Assessment of total protein synthesis in strains EE1b-35 (wild type), EE1b (*rna1-1*), and M3161A (*prp20-7*) after heat shock at 34°C (A), 37°C (B), or 41.5°C (C). Cells were grown at 23°C or were shifted to elevated temperatures for 15, 60, or 90 min, as indicated above each lane, and [³⁵S]methionine was added. After a 15-min labeling period, cell extracts were prepared, and the proteins were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. Particular heat shock proteins are indicated by lowercase letters at the left of each panel. (a) Hsp104, (b) Hsp90, (c) Hsp70s. For extracts prepared from cells shifted to 34°C, 3.3 times more final extract from EE1b cells was loaded compared to EE1b-35 cells. For extracts prepared from cells shifted to 37°C, the autoradiograph of EE1b was exposed eight times longer than that for EE1b-35. The autoradiograph of the extracts prepared from *prp20-7* cells shifted to 37°C was exposed 14 times longer than the one for 34°C.

tained heat shock response in wild-type cells required a shift to 41.5°C. Within 15 min of the temperature shift, heat shock proteins were the most abundant labeled proteins, and this predominance was even greater when cells were labeled 60 min after a shift. This is the expected result wherein a mild stress induces a transient heat shock response followed by recovery and more severe stress induces a sustained heat shock response (Lindquist 1986).

In *rna1-1* and *prp20-7* mutant strains, lower levels of protein synthesis occurred following temperature shift. This most likely reflects the dramatic inhibition of protein import, RNA processing, and RNA export that occur at elevated temperatures. We compensated for this either by loading a greater amount of extract or exposing the autoradiograph for a longer period of time (see legend to Fig. 6). In *rna1-1* cells shifted to 34°C or 37°C (*rna1-1* cells do not grow at temperatures exceeding 30°C), heat shock proteins were detectable in cells labeled following a 15-min temperature shift (Fig. 6A,B). The pattern seen was similar to that seen in wild-type cells. However, whereas inducible heat shock proteins were not synthesized at detectable levels in wild-type cells shifted for 60 min or longer, their synthesis continued in *rna1-1* and *prp20-7* cells 60 and 90 min after the temperature shift. Although the amount of protein synthesized in *rna1-1* or *prp20-7* cells was dramatically reduced 60 min after a shift, the pattern was very similar to that seen in wild-type cells heat-shocked at 41.5°C for 60 or 90 min (Fig. 6, cf. B with C). Thus, the *rna1-1* and *prp20-7* cells mounted a sustained heat shock response at temperatures that led to a transient response in wild-type cells.

Northern analysis was used to compare the induction of *SSA4* mRNA following heat shock in wild-type cells and in the *rna1-1* mutant strain (Fig. 7). Most mRNAs fail to accumulate in *rna1-1* cells, and those that do accumulate are often of an aberrantly large size (Shiokawa and Pogo 1974; Atkinson et al. 1985; Traglia et al. 1989; Forrester et al. 1992). In wild-type cells, as expected, there was a modest induction of *SSA4* RNA following a 15-min shift of wild-type cells to 34°C. This induction was transient, with the level dropping to just above the basal level within 60 min. Stronger induction occurred

Figure 7. Northern analysis of *RNA1* and *SSA4* mRNAs. Total RNA was extracted from EE1b-35 (wild type) (lanes 1–7) or EE1b (*rna1-1*) (lanes 8–12). The temperature and time of incubation for each culture before RNA extraction are indicated. The blots were hybridized with end-labeled oligonucleotide probes for *SSA4* transcripts or with a random primer-labeled *RNA1*-specific probe.

Figure 8. In situ hybridization assessment of *SSA4* mRNA export following heat shock in *rat7-1*, *rna1-1*, and *prp20-1* strains. For each strain, eight panels are shown. The top four represent images from cells incubated continuously at 23°C. The bottom four represent images from cells shifted to 42°C for 1 hr. In all cases, DAPI staining of the same fields of cells as those showing *SSA4* RNA (A,E,I,M,Q,U) or poly(A)⁺ RNA (C,G,K,O,S,W) are shown.

following a 15-min shift of wild-type cells to 37°C, and again, the level dropped substantially by 1 hr after temperature shift. The level of *SSA4* mRNA dropped more slowly from its maximum following a shift of wild-type cells to 41.5°C. In the *rna1-1* strain, the level of *SSA4* mRNA was induced to similar levels by a shift to 34°C or 37°C. In contrast to wild-type cells, in *rna1-1* cells, the induction of *SSA4* mRNA was sustained for at least 1 hr. Thus, *SSA4* mRNA did accumulate in *rna1-1* cells and did not appear to be of an aberrant size (Fig. 7).

The subcellular distribution of poly(A)⁺ RNA and *SSA4* mRNA in heat shocked *rna1-1* and *prp20-1* cells was examined. We also examined the heat shock response in *rat7-1* cells, which carry a temperature-sensitive allele of the *RAT7/NUP159* gene, encoding the Rat7p/Nup159p nucleoporin (Gorsch et al. 1995). Shifting *rat7-1* cells to the nonpermissive temperature of 37°C results in a rapid block to export of poly(A)⁺ RNA (Gorsch et al. 1995). Cultures of exponentially growing cells were shifted to 42°C for 1 hr and then processed for in situ hybridization. In all three cases (*rna1-1*, *prp20-1*, and *rat7-1*), poly(A)⁺ RNA accumulated in nuclei in heat-shocked cells (Fig. 8G,O,W). Following heat shock,

Figure 9. In situ hybridization assessment of *SSA4* mRNA export following heat shock in wild-type cells containing a galactose-inducible *GSP1* gene or a dominant-negative [*GSP1*^{val21}] *GSP1* allele. Wild-type cells (FY86) were transformed with YEp*SSA4* and with either p*GSP1* (wild type) (A–H) or p*GSP1*^{val21} (dominant negative) (I–P). Cells were grown overnight in media containing 2% raffinose and were diluted and induced with 2% galactose during early exponential growth. After 3 hr, cells were shifted to 42°C for 1 hr. (A,I) Localization of *SSA4* mRNA in cells incubated at 23°C. (B,J) DAPI staining of the same fields of cells shown in A and I, respectively. (C,K) Localization of *SSA4* mRNA in cells shifted to 42°C. (D,L) DAPI staining of the same fields of cells shown in C and K, respectively. (E,M) Localization of poly(A)⁺ RNA in cells incubated at 23°C. (F,N) DAPI staining of the same fields of cells shown in E and M, respectively. (G,O) Localization of poly(A)⁺ RNA in cells shifted to 42°C. (H,P) DAPI staining of the same fields of cells shown in G and O, respectively.

SSA4 mRNA was found in the cytoplasm of wild-type cells (data not shown) and accumulated in the nuclei of *rat7-1* cells (Fig. 8E). This indicates that export of *SSA4* mRNA depends on the presence of functional Rat7p/Nup159p and, presumably, functional nuclear pores. We also found that both poly(A)⁺ RNA and *SSA4* mRNA were retained in the nuclei of cells carrying temperature-sensitive alleles of nucleoporins *RAT2/NUP120* and *NUP145* (data not shown). For *rna1-1* cells (Fig. 8M) and *prp20-1* cells (Fig. 8U), *SSA4* was induced and *SSA4* mRNA was distributed throughout the cells. Some accumulation of *SSA4* mRNA in nuclei of heat-shocked *prp20-1* and *rna1-1* cells was also detected, and we believe this represents *SSA4* mRNA bound to fragments of disrupted nucleoli. Although the *PRP20* and *RNA1* gene products are needed for export of poly(A)⁺ RNA from the nucleus, they do not appear to be required for the accumulation of *SSA4* mRNA in the cytoplasm of heat shocked cells.

The distribution of poly(A)⁺ RNA and *SSA4* mRNA

in heat-shocked cells overexpressing either wild-type Gsp1p or a dominant-negative form of Gsp1p (the G21V mutation) was also examined. Induction of the mutant form of Gsp1p (but not the wild-type form) leads to a dramatic block to the export of poly(A)⁺ RNA (Schlenstedt et al. 1995). Plasmids encoding either wild-type Gsp1p or the dominant-negative mutant Gsp1p under control of the *GAL1* promoter were introduced into wild-type cells (FY23). Induction of wild-type Gsp1p did not lead to accumulation of poly(A)⁺ RNA in nuclei (Fig. 9E) nor to the induction of *SSA4* mRNA (Fig. 9A). Induction of overexpression of the mutant form of Gsp1p led to a dramatic accumulation of poly(A)⁺ RNA in nuclei (Fig. 9M), but little or no induction of *SSA4* mRNA occurred (Fig. 9I). When cells that had been overexpressing wild-type Gsp1p for 3 hr were heat shocked, poly(A)⁺ RNA still accumulated in nuclei (Fig. 9G) while *SSA4* mRNA was detected primarily in the cytoplasm (Fig. 9C). In cells overexpressing the mutant form of Gsp1p, poly(A)⁺ RNA was also found mainly in nuclei after heat shock (Fig. 9O) and *SSA4* mRNA was detected predominantly in the cytoplasm (Fig. 9K). Taken together, these data suggest that the selective export of *SSA4* mRNA following heat shock does not require Rna1p or Prp20p and that the dominant-negative form of Gsp1p, which blocks both nuclear protein import and RNA export of bulk poly(A)⁺ RNA, does not interfere with export of *SSA4* mRNA.

Protein import is rapidly inhibited following cellular stress

We analyzed the in vivo subcellular distribution of a green fluorescent protein (GFP) derivative containing a nuclear localization signal (NLS). Although this protein was readily imported into nuclei in unstressed cells (Fig. 10), it accumulated rapidly in the cytoplasm of cells shifted to 42°C. Eight minutes after shifting, 50% of the cells showed cytoplasmic fluorescence. At 12 min after shifting, the GFP–NLS reporter was predominantly cytoplasmic in >90% of the cells. Addition of cycloheximide to cells at the time of shifting to 42°C did not alter the fluorescence distribution nor its signal intensity. Because the reporter protein in this GFP construct is sufficiently small to diffuse out of nuclei (~45 kD), the fluorescent signal seen in Figure 10 reflects primarily reporter protein made prior to the time when cells were shifted to 42°C. We conclude that heat shock leads to an inhibition of nuclear protein import, but it does not affect protein diffusion out of the nucleus.

Discussion

When cells are exposed to higher than normal growth temperatures, the heat shock response is induced. This response is nearly universal, but different organisms display differences in the levels of regulation of the response and its duration (for review, see Lindquist 1986; Lindquist and Craig 1988). In most organisms this response is transient at moderate temperatures, with

Figure 10. Nuclear protein import is inhibited by cellular stress. In vivo fluorescence to examine the subcellular distribution of the karyophilic reporter protein derived from GFP following heat shock. ($t=0'$) The GFP localization at the time of shift (0 min) to 42°C. ($t=8'$, 12', and 20') GFP localization at 8, 12, and 20 min after shifting to 42°C, respectively.

growth resuming after a temporary pause, and the response is sustained at higher temperatures, until cells begin to die slowly (Lindquist 1986). The heat shock response consists of the induction of a group of highly conserved proteins, the heat shock or stress proteins. Conversely, the synthesis of many other proteins is decreased during the heat shock response. In *S. cerevisiae*, for example, a survey of the response of ~500 proteins to a temperature upshift showed 80 proteins to be induced or elevated, 20 to a high level, and >300 to be reduced (Miller et al. 1982).

Decrease in the production of non-heat-shock proteins can result from various mechanisms. For example, for some *Drosophila* mRNAs, this occurs via transcriptional repression (Ashburner and Bonner 1979). However, many studies in mammalian cells, *Drosophila*, *Arabidopsis*, and yeast have also implicated post-transcriptional mechanisms (Yost and Lindquist 1986, 1991; Bond 1988; Sadis et al. 1988; Shukla et al. 1990; Yost et al. 1990; Osteryoung et al. 1993). Pre-mRNA splicing, pre-rRNA processing, poly(A) tail length, nuclear RNA stability, and/or nuclear exit have all been reported to be affected by heat shock. For yeast, the splicing of pre-mRNA encoding actin is blocked by heat shock (Yost and Lindquist 1991), but the mRNA encoding a GTP-binding protein and several mRNAs encoding ribosomal proteins fail to accumulate upon heat shock and therefore are affected either at transcriptional or RNA turnover steps

(Herruer et al. 1988; Yost and Lindquist 1991). We have examined how heat shock affects the levels of *GAL1* and *SSA4* mRNA and find that the half-life of *GAL1* mRNA was reduced from ~20 min to ~10 min following a shift to 42°C, whereas the stability of *SSA4* was largely unaffected by heat shock (data not shown).

Selective nucleocytoplasmic export of mRNA following stress

The studies described in this paper provide evidence that nucleocytoplasmic export is regulated as part of the heat shock response in *S. cerevisiae*. Following heat shock (Amberg 1992) or ethanol-induced stress, poly(A)⁺ RNAs accumulated within the nucleus. A recent report indicates that nuclear accumulation of poly(A)⁺ RNA also occurs in *Schizosaccharomyces pombe* following heat shock (Tani et al. 1995). In contrast, in situ hybridization indicated that *SSA4* mRNA was exported efficiently and accumulated to high levels in the cytoplasm of heat shocked *S. cerevisiae* cells.

In some organisms, including *D. melanogaster*, transcriptional induction of heat shock genes occurs very rapidly after heat shock, owing at least in part to the fact that RNA polymerase is found in a paused complex on heat shock genes (O'Brien and Lis 1991, 1993). Heat shock transcription factor directs the assembly of the transcriptional machinery on heat shock genes and that RNA polymerase becomes arrested following the formation of the first phosphodiester bonds, where it is poised for extremely rapid transcriptional elongation upon stress (Rasmussen and Lis 1993; Giardina and Lis 1995a,b). It is not known whether similar complexes exist in *S. cerevisiae*, but if they do, this could lead to induction of heat shock transcription sufficiently rapidly that a considerable amount of *SSA4* mRNA might be produced and exported prior to the establishment of a block to nucleocytoplasmic export of poly(A)⁺ RNA.

We think this rapid induction prior to a block in mRNA export is unlikely to explain the results we have obtained for two reasons. First, when the mRNAs for *GAL1* and *URA3* were expressed from the wild-type *SSA4* promoter, each accumulated in nuclei following heat shock (Fig. 3B; data not shown). The kinetics of transcriptional induction of these constructs should be very similar to those for the complete *SSA4* gene, yet the pattern of in situ hybridization seen with the *SSA4*-driven *GAL1* mRNA was very different from that seen for *SSA4* RNA (cf. Figs. 2 and 3B). Second, we could detect nuclear accumulation of poly(A)⁺ RNA within 5 min of heat shock. The nuclear signal for poly(A)⁺ RNA was strong within 30 min, a time at which *SSA4* mRNA was continuing to accumulate (Fig. 4).

Could changes in mRNA stability following stress explain the patterns of mRNA distribution described in these studies? If this were a significant factor in our experiments, one would not expect strong nuclear accumulation of poly(A)⁺ RNA in wild-type cells subjected to heat shock (Fig. 1) nor cytoplasmic accumulation of *SSA4* mRNA in mutant strains (*ma1-1*, *prp20-1*, or

GSP1^{val21} (Fig. 8) shown previously to have defects in the export of mRNA. Although some changes in mRNA stability certainly occur and affect the absolute levels of many mRNAs following stress, the most likely consequence of these changes in our experiments is to accelerate the decrease in the cytoplasmic signal for poly(A)⁺ RNA following heat shock. Therefore, the patterns seen are not consistent with a major contribution from altered mRNA stabilities.

In an attempt to begin to define where within *SSA4* mRNA signals for export are located, we deleted progressively beginning at the 5' end of the *SSA4* mRNA. We mapped a region sufficient to permit cytoplasmic export and accumulation of mRNAs containing it following stress to the 3' 500 nucleotides of *SSA4* mRNA. However, there appear to be multiple signals for selective export with *SSA4* mRNA, as another fusion, containing only the 5' 1620 nucleotides of *SSA4*, was also detected primarily in the cytoplasm following heat shock.

Possible mechanisms of selective export

Our data support the idea that heat shock mRNAs contain sequences or structures that are signals for export of these mRNAs under conditions where most mRNA export is blocked. These signals could interact directly with the RNA export machinery, or alternatively, they could bind a protein that itself would interact with the RNA export machinery. It was reported recently that nuclear export signals exist in two RNA-binding proteins, hnRNP A1 and HIV-1 Rev (Fischer et al. 1995; Michael et al. 1995). Because these (and other) RNA-binding proteins shuttle rapidly between the nucleus and the cytoplasm (Piñol-Roma and Dreyfuss 1992; Flach et al. 1994; Meyer and Malim 1994), their nuclear export signals may play an important role in the export of the RNAs to which they bind. Most hnRNP proteins are highly abundant (for review, see Dreyfuss et al. 1993); an RNA-binding protein involved in export of heat shock mRNAs might be comparatively rare, and the spectrum of RNAs to which it bound could determine which mRNAs were exported under heat shock conditions. Just as the HIV-1 Rev protein appears to target an export pathway different from that for export of poly(A)⁺ RNA (Fischer et al. 1995), a protein that bound heat shock mRNAs could target them to a different export pathway from that used by mRNA, thus permitting their export under conditions the lead to nuclear accumulation of most mRNAs. A related possibility is that these sequences act to override a stress-induced block in maturation of mRNA or its packaging into exportable hnRNP. Future studies will be directed at determining the precise mechanism of selective export.

As expected, we found that export of *SSA4* mRNA did not occur following heat stress in cells carrying temperature-sensitive alleles of *RAT7/NUP159*, *RAT2/NUP120*, and *NUP145*, indicating the export through nuclear pore complexes is required for the heat shock response. Surprisingly, however, we found that the selective cytoplasmic accumulation of *SSA4* mRNA did not depend on

functional *RNA1*, *PRP20*, or *GSP1*. Temperature-sensitive mutations of the *RNA1* or *PRP20* genes affect pleiotropically the metabolism of all major classes of RNA in cells shifted to nonpermissive temperatures. Mutations of *RNA1*, *PRP20*, and *GSP1* also lead to a block in nucleocytoplasmic export of poly(A)⁺ RNA and its accumulation in nuclei (Shiokawa and Pogo 1974; Amberg et al. 1992, 1993; Forrester et al. 1992; Kadowaki et al. 1993; Schlenstedt et al. 1995).

One possibility is that the Gsp1p system is the target used to block export of most mRNAs following heat shock. This could occur by either direct or indirect mechanisms. Although mechanistic details are emerging about the roles of Ran/Gsp1p in protein import (Melchior and Gerace 1995; Melchior et al. 1995; Sazer 1996), the precise roles performed by Ran/Gsp1p in the export of poly(A)⁺ RNA are not yet known. However, a direct role for Ran/Gsp1p in RNA export is probable, and thus export of poly(A)⁺ RNA could be directly affected through modulation of the Ran/Gsp1p system.

We think it unlikely that import of all nuclear proteins is blocked following heat shock, as proteins produced in response to stress are probably required in all cellular compartments. However, it is possible that cells contain more than one set of proteins that can play the role in nuclear protein import performed by the Ran/Gsp1p system. These other proteins could act to permit the continued nuclear import of a specific set of proteins and the continued nuclear export of *SSA4* and other stress response mRNAs. Future studies will also focus on defining the gene products required for selective export of heat shock mRNAs and elucidating the mechanism by which export of most mRNAs is inhibited during the stress response.

Materials and methods

Strains and growth conditions

Yeast strains and plasmids are described in Tables 1 and 2, respectively. Except where noted, cells were grown to early exponential phase at 23°C in YPD-rich medium or in synthetic complete medium lacking leucine (SC-Leu) liquid medium (Rose et al. 1989). For induction of the heat shock stress response, cells in the exponential phase of growth (5×10^6 to 2×10^7 cells/ml) were transferred to water baths at the appropriate temperature. The stress response was also induced by the addition of ethanol to 10% (vol/vol).

DNA and RNA manipulations

Yeast transformations were performed by electroporation using an electroporator (Bio-Rad Laboratories, Melville, NY); cells were allowed to recover in rich medium containing 1 M sorbitol at 23°C for at least 1 hr before plating. *Escherichia coli* strain DH5 α was used for most cloning procedures.

To obtain PCR probes for in situ hybridization and Northern blotting, primers were designed by using the Oligo 4.0 software program (National Biosciences, Plymouth, MN) and obtained from Macromolecular Resources (Colorado State University, Fort Collins), the Dartmouth College Macromolecular Facility, or the Pennsylvania State University College of Medicine Macromolecular Core Facility (Hershey). Except as noted, standard PCR techniques were followed as directed by the Perkin-Elmer

Table 1. Yeast strains

Strain	Genotype	Source
M3161A	<i>mata ura3-52 his3Δ200 tyr1 ade2-1 prp20-7</i>	Amberg et al. [1993]
FY86	<i>mata ura3-52 his3Δ200 leu2Δ1</i>	F. Winston (Harvard Medical School, Boston, MA)
EE1b-35	<i>mata ura3-52 ade2-1 tyr1 gal⁻ his3Δ200</i>	Hopper et al. [1990]
EE1b	<i>mata ura3-52 ade2-1 tyr1 gal⁻ his3Δ200 rna1-1</i>	Hopper et al. [1990]
LGY101	<i>mata ura3-52 his3Δ200 leu2Δ1 rat7-1</i>	Gorsch et al. [1995]
CHY104	<i>mata ura3-52 his3Δ200 leu2Δ1 RAT2::HIS3</i>	Heath et al. [1995]
TDY109	<i>mata ura3-52 his3Δ200 leu2Δ1 ade2-1 trp1-1 ade3 nup145-10</i>	T. Dockendorff (unpubl.)
CSY107	<i>mata ura3-52 his3Δ200 leu2Δ1 pYCpGALGSP1</i>	this study
CSY108	<i>mata ura3-52 his3Δ200 leu2Δ1 pYCpGALgsp1val21</i>	this study

Cetus GeneAmp PCR Core Reagents protocol. Digoxigenin-dUTP was incorporated directly into probes during the PCR reaction. The number of digoxigenins incorporated along the probe could be controlled by adjusting the ratio of digoxigenin-dUTP to dTTP in the PCR reaction buffer. Although an increased ratio of dUTP-digoxigenin to dTTP increased the amount of digoxigenin-dUTP incorporated into each molecule of probe, higher ratios of digoxigenin-dUTP/dTTP decreased the efficiency of the PCR reaction. Because an antibody molecule is much larger than a nucleotide pair, the maximum fluorescent signal possible will be achieved before all dTTPs have been replaced by digoxigenin-dUTP. A ratio of 1:4 [digoxigenin-dUTP/dTTP] provided a suitable compromise between high probe yield and maximum probe sensitivity (40 μ M digoxigenin-dUTP; 160 μ M dTTP final concentration).

To synthesize the *SSA4* probe, a 270-bp fragment was amplified using plasmid EC702 (a gift from E. Craig, University of Wisconsin, Madison) as a template and primers 5'-CAAAC-CCCATTTATGAGTAA and 5'-TGGCTTATGACGATGAGAA. To synthesize *GAL1* probes from template pBM47 (kindly provided by Dr. Mark Johnston, Washington University Medical Center, St. Louis, MO), the following primers were included in a single PCR reaction: 5'-GAAAAGTGCCCCGAGCATAA and 5'-ACCGAGAAGTCACAATAATCA; 5'-AAAAATCCATCCATTACCTTA and 5'-TCCGGTGCAAGTTTCTTTA; 5'-TTAGCTGTTGTTAAAGCGAATA and 5'-TGATCCATACCGC-CATTGT; 5'-CGAACACCCTTGTTGTATCTA and 5'-GCTCGATCCTTCTTTTCC; 5'-TGGAACGGCGATATTGAA and 5'-TTAAGACTTGAAATCTCACTGGA. To subclone the *GAL1* gene downstream of the *SSA4* promoter, the *GAL1* gene was

PCR-amplified from pBM47 using primers 5'-TTTCGGTT-TGTGTTACTTC and 5'-GTGTTACTACTCGTTATTATTGT in a reaction that included UITma DNA polymerase Kit (Perkin-Elmer, Branchburg, NJ). The 1.7-kb DNA fragment was ligated to the *StuI*-linearized EC702 using standard procedure [Sambrook et al. 1989].

Northern blotting

Most Northern blot analyses were performed by preparing total yeast RNA as described [Guthrie and Fink 1991], and RNA was detected by chemiluminescence using a Genius 7 Luminescent Detection Kit (Boehringer Mannheim). For the Northern blot in Figure 7, total RNAs were isolated from yeast cells as described [Najarian et al. 1987] and 20 μ g of each sample was subject to electrophoresis on 1% agarose gels containing formaldehyde [Sambrook et al. 1989]. The gels were blotted to MSI MAGNA nylon membrane by capillary action. The end-labeled oligonucleotide, 5'-GTTGTACCTAAATCAATACC-3', which is complementary to nucleotide positions 16–35 within the *SSA4* open reading frame, was used to detect *SSA4* RNA. A 0.9-kb *Bam*HI-*Xba*I fragment of the *RNA1* gene was used to detect *RNA1* transcripts. It was purified from plasmid pRU35 [Taglia et al. 1989] and labeled using random primers and ³²P-labeled deoxycytidine-triphosphate.

In situ poly(A)⁺ RNA localization assay

Cells shifted to 42°C or 37°C for specified times were fixed by adding formaldehyde to 4% and incubated for 90 min on a ro-

Table 2. Plasmids

Plasmid	Markers	Comments	Source
EC702	2 μ <i>LEU2</i> Amp ^r	<i>SSA4</i> gene in YEp351	E. Craig (University of Wisconsin, Madison)
EC703	2 μ <i>LEU2</i> Amp ^r	<i>SSA1</i> gene in YEp351	E. Craig (University of Wisconsin, Madison)
YCpGAL-GSP1	<i>CEN4 URA3</i> Amp ^r	<i>GSP1</i> under GAL control in YCp50	P. Silver (Harvard Medical School)
YCpGAL-GSP1 ^{val21}	<i>CEN4 URA3</i> Amp ^r	<i>GSP1^{val21}</i> under GAL control in YCp50	P. Silver (Harvard Medical School)
CSY109	2 μ <i>LEU2</i> Amp ^r	<i>SSA4</i> 3'Δ240 in YEp351	this study
CSY110	2 μ <i>LEU2</i> Amp ^r	<i>SSA4</i> 3'Δ685 in YEp351	this study
CSY111	2 μ <i>LEU2</i> Amp ^r	<i>GAL1</i> - <i>SSA4</i> hybrid in YEp351	this study
CSY112	2 μ <i>LEU2</i> Amp ^r	<i>GAL1</i> - <i>SSA4</i> Δ747-2300	this study
CSY113	2 μ <i>LEU2</i> Amp ^r	<i>GAL1</i> - <i>SSA4</i> Δ1600-2300	this study
CSY114	2 μ <i>LEU2</i> Amp ^r	<i>SSA4</i> 1-1680-GAL1 650-1753	this study
CSY115	2 μ <i>LEU2</i> Amp ^r	<i>SSA4^{GAL1}</i> in YEp351	this study
CSY116	2 μ <i>LEU2</i> Amp ^r	<i>SSA4^{URA3}</i> in YEp351	this study
pGFP-NLS	2 μ <i>URA3</i> Amp ^r	ADH _P , Gal4 AD, SV40 NLS, GFP	D. Goldfarb (University of Rochester, NY)

tator at room temperature. All other procedures for in situ hybridization to detect poly(A)⁺ RNA were carried out as described previously (Amberg et al. 1992; Gorsch et al. 1995). For detection of individual species of mRNA, probes (SSA4 or *GAL1*) were denatured in boiling water for 10 min, immediately placed on dry ice, and added to prehybridization solution to a final concentration of 20 ng/ml. Cells were incubated for 18 hr at 37°C in a humid chamber.

In vivo protein labeling

In vivo labeling of heat shock proteins was performed as described by Nicolet and Craig (1991). Cells grown in SC-Met medium were transferred from 23°C to heat shock temperatures for indicated times, and then 1.5 ml of the culture was incubated with 150 μ Ci of a mixture of [³⁵S]methionine and [³⁵S]cysteine (Translabel, ICN Biomedicals) at the same temperatures in a prewarmed microfuge tube for 15 min. The pre-heat shock control cultures were grown and labeled for 15 min at 23°C. Total yeast protein extracts were prepared as described previously (Schultz et al. 1987). The protein concentrations were determined by the method of Peterson (1977). Protein extracts were resolved on a 5% stacking/8% separating SDS-polyacrylamide gel and transferred to nitrocellulose by electroblotting.

Protein import assay

A high-copy (2 μ) plasmid, pGFP-URA, containing the in vivo fluorescent protein GFP gene engineered with an SV40 NLS (constructed by Paul Roberts and obtained from David Goldfarb, University of Rochester, Rochester, NY) was introduced into wild-type yeast strain FY86. The GFP in this plasmid also contains sequences from the *GAL4* activation domain and has a molecular mass of ~45 kD. For protein import assays, cells were incubated at 23°C and grown to early log phase (0.1–0.2 OD₆₀₀) in SC-Ura medium. Cells were collected by centrifugation and resuspended in residual supernatant in a 1.5-ml Eppendorf tube. At time $t=0$ min, the culture was shifted to 42°C, or ethanol was added to a final concentration of 10%. Thereafter, at 2-min intervals, an aliquot (1 μ l) was removed from the culture and placed on a glass slide to be observed immediately by microscopy for the location of green fluorescence. To inhibit additional protein synthesis, cycloheximide was added at $t=0$ to a final concentration of 10 μ g/ml. A Zeiss Axiophot photomicroscope equipped with a 100 $\times$ oil-immersion lens and filters for FITC fluorescence were used. The percentage of cells showing a nuclear signal was recorded at each time and photographed.

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C Saavedra, K S Tung, D C Amberg, et al.

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