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A Nuclear Export Signal Prevents *Saccharomyces cerevisiae* Hsp70 Ssb1p from Stimulating Nuclear Localization Signal-directed Nuclear Transport*

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Hsp70 has been implicated in nuclear localization signal (NLS)-directed nuclear transport. *Saccharomyces cerevisiae* contains distinct *SSA* and *SSB* gene families of cytosolic Hsp70s. The nucleocytoplasmic localization of Ssa1p and Ssb1p was investigated using green fluorescent protein (GFP) fusions. Whereas GFP-Ssa1p localized both to the nucleus and cytoplasm, GFP-Ssb1p appeared only in the cytosol. The C-terminal domain of Ssb1p contains a leucine-rich nuclear export signal (NES) that is necessary and sufficient to direct nuclear export. The accumulation of GFP-Ssb1p in the nuclei of *xpo1-1* cells suggests that Ssb1p shuttles across the nuclear envelope. Elevated levels of *SSA1* but not *SSB1* suppressed the NLS-GFP nuclear localization defects of *nup188*-Δ cells. Studies with Ssa1p/Ssb1p chimeras revealed that the Ssb1p NES is sufficient and necessary to inhibit the function of Ssa- or Ssb-type Hsp70s in nuclear transport. Thus, NES-less Ssb1p stimulates nuclear transport in *nup188*-Δ cells and NES-containing Ssa1p does not. We conclude that the differential function of Ssa1p and Ssb1p in nuclear transport is due to the NES-directed export of the Ssb1p and not to functional differences in their ATPase or peptide binding domains.

The import of proteins into nuclei is mediated by soluble nuclear localization signal (NLS)[1] receptors. SV40 large T-antigen-like NLSs are bound in the cytoplasm by karyopherin α (Kap α), which serves as an adapter to link NLS-cargo to karyopherin β (Kap β). Kap β mediates docking at the cytoplasmic face of the nuclear pore complex (1). *Saccharomyces cerevisiae* contains a single Kap α gene encoded by *SRP1*. Translocation of the NLS-cargo-Kap α/β ternary complex occurs through the central channel of the nuclear pore complex. Once in the nucleus, the NLS-cargo dissociates and Kap α and β are recycled to the cytoplasm (1, 2). Only a portion of the cellular import traffic is mediated by Kap α/β heterodimers. Other classes of NLS-cargo, for example shuttling pre-mRNA binding proteins that display M9-type import signals (3), are transported by Kap β-like factors that bind directly to cognate NLS-cargo without the aid of Kap α adapters (1, 4).

Hsp70/Hsc70s (collectively referred to here as Hsp70s) are conserved molecular chaperones that participate in a variety of cellular functions, including protein folding and transport and the repair of stress-induced damage (5–7). Hsp70s are composed of a 44-kDa N-terminal ATPase domain, an 18-kDa peptide binding domain, and a C-terminal 10-kDa variable domain of unknown function (8, 9). *S. cerevisiae* contains two families of cytosolic Hsp70 genes, *SSA1-4* and *SSB1-2* (10, 11). The yeast Ssa-type Hsp70s are similar to the cytosolic Hsp70s found in other organisms including bacteria. To date, Ssb-type Hsp70s have been identified only in fungi. In *S. cerevisiae*, Ssb1 and Ssb2 are associated with translating ribosomes and can be cross-linked to nascent polypeptides (12, 13).

Hsp70s have been proposed to function in NLS-directed nuclear transport by promoting the formation and stability of NLS-cargo-Kap α complexes (reviewed in Refs. 1, 14, and 15). Thus, the ectopic expression of human Hsp70 in mouse cells rescued the import of a protein carrying a mutant NLS (16). Conversely, the elevated expression of *SSA1* in yeast suppressed a transport defect in *srp1–31* cells (15). Microinjected antibodies against Hsc70 inhibited NLS-directed import (17), and the depletion of Hsp70 from cytosolic extracts inhibited import in cell-free assays (18–20). Finally, the finding that the nuclear localization of Hsp70 is itself dependent on the co-import of NLS-cargo (19) suggests that Hsp70 is imported in association with the NLS-cargo-Kap α/β ternary complex.

In the present study we delineate a functional nuclear export signal (NES) in the C-terminal domain of Ssb1p and show that it is responsible both for the different subcellular localizations of Ssa1p and Ssb1p and for their differential function in NLS-directed nuclear transport.

MATERIALS AND METHODS

Strains, Plasmids, and Culture Conditions—Except where noted, all yeast strains used in this study were based in a W303 (strain WHY12) genetic background (*MAT***a** *ade2-1 leu2-3, 112 his3-11,* and *15 trp1-1 ura3-1 can1-100*). Construction of strains containing *nup188*-Δ (21) and *xpo1-1* alleles (22) and L40 cells (23) were previously described. DNA manipulations were performed using standard protocols (24). The construction of pGAD-NLSGFP and pGFP-URA3 (25), YCpGAL1-SSA1, and YcpGAL1-SSB1, which contain the *SSA1* and *SSB1* genes under the control of the *GAL1* inducible promoter (6), and the *SSA1*/*SSB1* chimeric plasmids (26) were previously described. Chimeras that divided the ATPase domain into two halves according to crystal structure (8) were constructed using an *Eag*I site introduced by polymerase chain reaction at the gene sequence corresponding to amino acid 177 in Ssa1p and amino acid 181 in Ssb1p. A *Bam*HI site introduced by polymerase chain reaction at the N terminus of Ssa1 and Ssb1 was used to clone the chimeras into pCUG2, placing them under *GAL1* control. pCUG2 was constructed by inserting a 690-base pair *Eco*RI/*Bam*HI fragment containing the *GAL1*/*GAL10* promoter into the pRS316 (27). pGFP-N-FUS plasmid (28), containing the *MET25* promoter and GFP, was used to express GFP-Sssa1p/Ssb1p fusions for localization studies. In pGFP-pA-Ca/Cb plasmids, protein A synthetic analog, the so-called Z-domain

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[1] The abbreviations used are: NLS, nuclear localization signal; Kap α and β, karyopherin α and β; NES, nuclear export signal; GFP, green fluorescent protein.

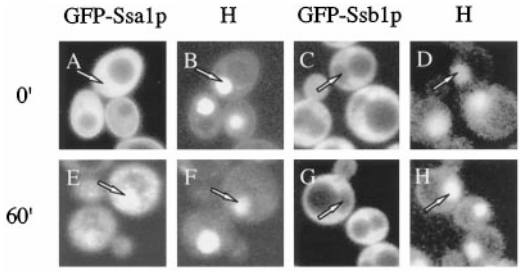

FIG. 1. **Subcellular localization of GFP-Ssa1p and GFP-Ssb1p.** Localization of GFP-Ssa1p (*A, E*) and GFP-Ssb1p (*C, G*) by GFP fluorescence and nuclei (*B, D, F, H*) by Hoechst staining. Cells were maintained at 30 °C (*A–D*) or heat-shocked at 42 °C for 1 h (*E–H*). *Arrows* point to the nuclei.

(29), was inserted between the GFP and C-terminal sequences of *SSA1* and *SSB1*. Appropriate cloning sites were created by high fidelity polymerase chain reaction. Polymerase chain reaction-amplified fragments and vector junctions in completed plasmid constructs were verified by DNA sequencing. Standard complete or selective synthetic media were used as described (30).

NLS-GFP Import Assay, Galactose Induction, and Heat Shock—NLS-GFP kinetic import assays were performed as described (15) with minor modifications as indicated in the text. For heat shock, cells grown at 30 °C were pelleted, resuspended in 39 °C pre-warmed medium, and incubated at 39 °C for 1 h. Control cells were resuspended in 30 °C pre-warmed medium and incubated at 30 °C for 1 h. Induction of protein expression from pGAL1 vectors was achieved by resuspending glucose-grown cells in synthetic medium containing 2% galactose and incubating in a shaking water bath at 30 °C for 2 h.

Fluorescent Conventional and Confocal Microscopy—Only fresh exponentially growing cultures were used for fluorescence microscopy study. Fluorescence microscopy was performed using an Olympus BH-2 microscope, and pictures were taken with a mounted Olympus C-35AD-2 camera. Kodak TMAX 400 film was processed with TMAX developer, Indicator stop bath, and Rapid fixer (Eastman Kodak Co.). Confocal images were obtained with a Leica TCS NT microscope, and digital images were processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA).

RESULTS

Nucleocytoplasmic Localization of GFP-Ssa1p and GFP-Ssb1p—The subcellular localization of Ssa1p and Ssb1p was determined using GFP fusions. GFP-Ssa1p and GFP-Ssb1p fusion proteins were expressed from a *MET25* inducible promoter in WHY12 cells and localized by confocal microscopy. As expected, both Hsp70s were localized in the cytoplasm, but only GFP-Ssa1p accumulated to any significant degree in the nuclei (Fig. 1). Both fusions were excluded from vacuoles. The localization of GFP-Ssa1p in both the cytoplasm and nucleus is consistent with the distribution of Ssa-type Hsp70s in other eukaryotes (31, 32). In contrast, the exclusive cytoplasmic localization of GFP-Ssb1p is unique among known cytosolic Hsp70s.

A feature of the stress response in many eukaryotes is the migration of cytoplasmic Ssa-type Hsp70s from the cytoplasm into the nucleus where they accumulate to high levels (32, 33). To determine whether this phenomenon occurs in *S. cerevisiae*, the localization of GFP-Ssa1p and GFP-Ssb1p was observed following heat shock treatment. After a 60-min heat shock treatment, nuclear GFP-Ssa1p levels increased, relative to cytoplasmic levels, but not to a great extent (Fig. 1). Heat shock had no detectable affect on the localization of GFP-Ssb1p (Fig. 1).

The C Terminus of Ssb1p Directs Cytoplasmic Localization—Hsp70s, including Ssa1p and Ssb1p, can be divided into three structural domains: a 44-kDa ATPase domain, an 18-kDa peptide binding domain, and a 10-kDa C-terminal variable domain (Fig. 2*A*). The ATPase domain can be further divided into two subdomains (8). We constructed chimeric genes containing a

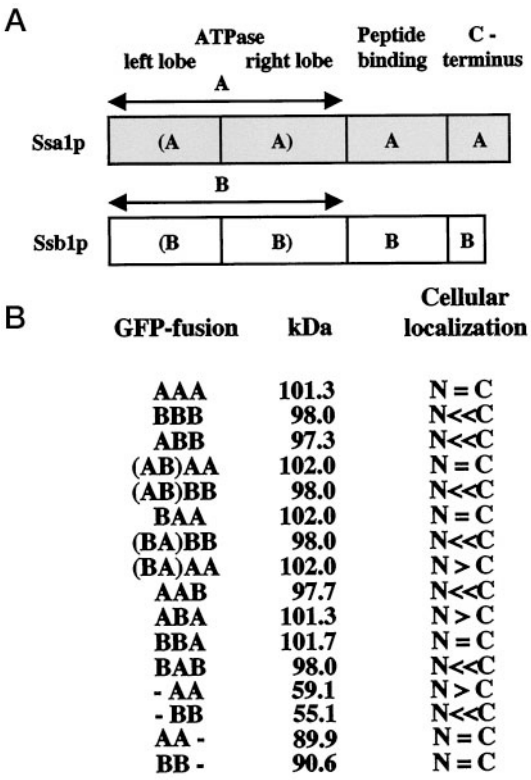

FIG. 2. **Subcellular localization of Ssa1p/Ssb1p chimeras.** *A*, Ssa1p and Ssb1p are divided into three functional domains, the ATPase, peptide binding, and C terminus. Thus, Ssa1p is designated *AAA*. In some constructs the ATPase domain was divided in half such that Ssa1p would be indicated by (*AA*)*AA*. *B*, GFP-Ssa1p/Ssb1p chimeras, their masses, and their cellular localizations: $N \ll C$, exclusion from nuclei; $N = C$, approximate equilibration; $N > C$, obvious nuclear accumulation. *C*, fluorescent images of selected GFP-Ssa1p/Ssb1p fusions and nuclei in the same group of cells positioned by Hoechst staining (column *H*). *Arrows* indicate the positions of nuclei in matched cells.

mixture of Ssa and Ssb structural domains, most of which are designated by a three-letter code (Ref. 26 and this study). For example, "ABA" contains the Ssa ATPase domain, the Ssb peptide binding domain, and the Ssa C-terminal domain. Chimeras that contain subdivided ATPase domains are designated, for example, "(AB)AA" or "(BA)AA." Chimeras that lack particular domains are designated "-AA," which, in this example, lacks an ATPase domain. For the purpose of localization studies, each chimera was expressed as a GFP fusion protein. GFP was not fused to the Hsp70 constructs for the purpose of functional studies (see below).

Localization of the various GFP-Ssa1p/Ssb1p chimeras was

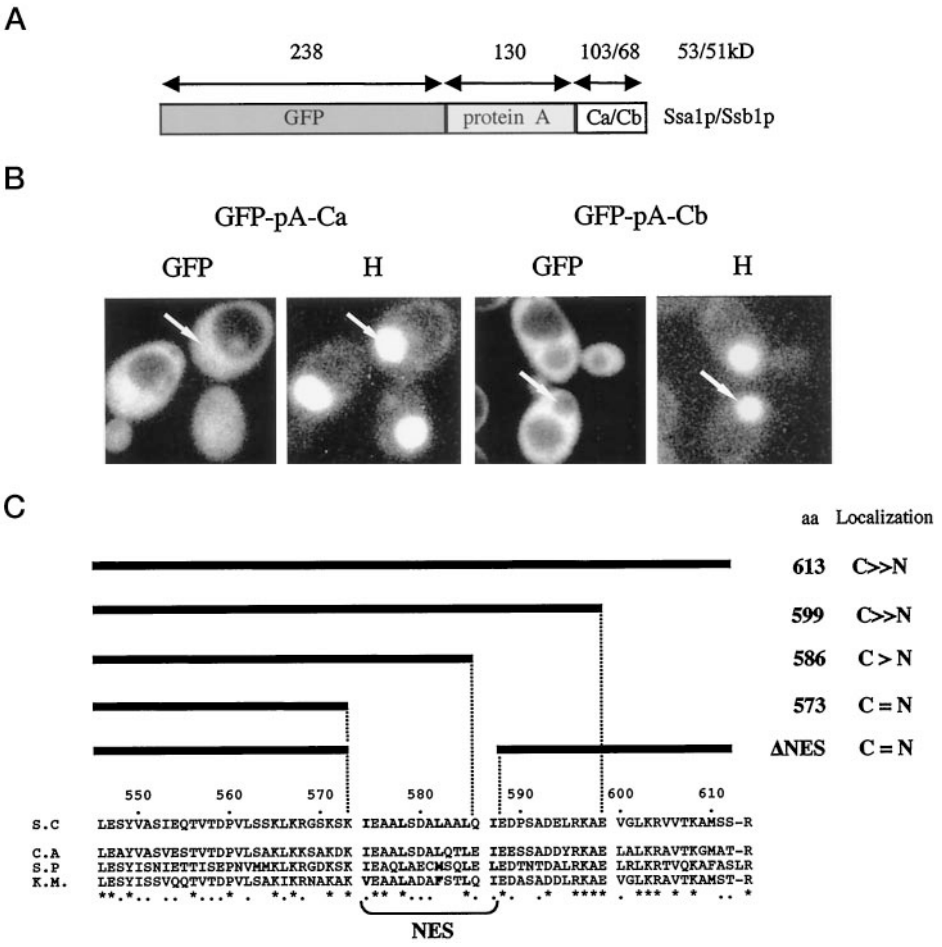

FIG. 3. **Role of Ssb1p C-terminal domain in subcellular localization.** *A*, diagram of GFP-protein A-Ssa1p/Ssb1p C terminus reporter proteins (*GFP-pA-Ca* and *GFP-pA-Cb*). *B*, subcellular localization of GFP-pA-Ca and GFP-pA-Cb (*GFP*) and corresponding nuclei (*H*). *C*, delineation of the Ssb1p cytoplasmic localization sequence. GFP-Ssb1p reporter proteins (full-length Ssb1p contains 613 amino acids (*aa*)) containing the indicated C-terminal truncations and internal deletion were localized by fluorescence microscopy and scored as being completely excluded from nuclei ($C \gg N$), somewhat excluded ($C > N$), or approximately equilibrated ($C = N$). Alignment of putative SSB-type proteins from *Saccharomyces cerevisiae* (*S.C*), *Candida albicans* (*C.A*), *Schizosaccharomyces pombe* (*S.P*), and *Kluyeromyces marxianus* (*K.M.*) shows identical (*asterisks*) and similar (*dots*) residues. Residues that correspond to the consensus NES motif are indicated in *bold*.

determined by conventional and confocal fluorescence microscopy, and the fluorescence in cells was scored as being either equally distributed between nucleus and cytoplasm (N = C), exclusively cytoplasmic (N ≪ C), or significantly more concentrated in the nuclei (N > C) (Fig. 2, *B* and *C*). These data indicate that the Ssb1p C-terminal domain is responsible for the low nuclear levels of GFP-Ssb1p. Thus, ABB, (AB)BB, (BA)BB, AAB, BAB, and -BB all showed background levels of nuclear staining. In contrast, (AB)AA, BAA, (BA)AA, ABA, and BBA localized to both the cytoplasm and the nucleus (Fig. 2*B*). The localizations of selected chimeras are shown in Fig. 2*C*. Immunoblot analysis of cell extracts expressing these GFP fusion proteins indicates that they are stable (data not shown). In addition to these data, GFP fused to AA- and BB- localized to both the cytoplasm and the nucleus (Fig. 2*B*). It is worth noting here that a previous study (34) implicated the C terminus of rat Hsc70 in its bidirectional trafficking.

To determine whether the Ssb1p C terminus can function autonomously to prevent nuclear accumulation, Ssa1p ("Ca") and Ssb1p ("Cb") C-terminal domains were fused to the C terminus of a GFP-protein A (GFP-pA) reporter protein (Fig. 3*A*) and localized in cells by confocal microscopy (Fig. 3*B*). Whereas GFP-pA-Ca (53 kDa) localized both to the nucleus and the cytoplasm, GFP-pA-Cb (51 kDa) did not stain the nuclei (Fig. 3*B*). Immunoblot analysis demonstrated that these fusions were stable *in vivo* (not shown). We conclude that the C terminus of Ssb1p can function autonomously to prevent nuclear localization. The Ssb1p C terminus could function either as a cytoplasmic retention sequence to exclude Ssb1p from the nucleus or as a NES to direct the export of any Ssb1p that reaches the nucleus.

The minimal Ssb1p C-terminal sequence necessary to prevent nuclear localization was delineated by truncation and deletion analysis of a GFP-Ssb1p reporter protein (Fig. 3*C*). These results point to a short sequence that contains a match to the consensus sequence for leucine-rich NESs (35, 36). Ssb1p homologs from other fungi also contain this NES motif (Fig. 3*C*).

Ssbp Contains a Functional NES—An *in vivo* assay for NES function based on the approach of Fritz and Green (37) was developed in the Wente laboratory (Washington University, St. Louis, MO) and was generously provided to us prior to publication. In this assay, *HIS3* is transcribed only when a plasmid-borne transcriptional activator, composed of the LexA DNA binding domain and the pseudorabies activation domain, is expressed in the nucleus. Cells that express this activator grow on medium lacking histidine (Fig. 4). When HIV-1 Rev or Gle1p NESs were inserted between the LexA and pseudorabies domains, the cells failed to grow on selective medium (Fig. 4), presumably because the activator was exported before it could activate *HIS3* transcription. Cells expressing an activator with a mutant Rev NES grew well on selective medium (Fig. 4). Significantly, cells expressing activators containing the entire C-terminal domain of Ssb1p (amino acids 546–613), or just the putative NES (amino acids 574–587), failed to grow on selective medium (Fig. 4). These data support the hypothesis that Ssb1p residues 574–587 function as a NES.

The export of leucine-rich NES-cargo is mediated by a Kap β-like protein called exportin, encoded in yeast by *XPO1/CRM1* (21). *xpo1-1* cells exhibit reduced NES-directed export at nonpermissive temperatures. For example, a protein kinase inhibitor NES-GFP-NLS fusion protein, which is normally efficiently exported from nuclei, becomes almost exclusively nuclear in *xpo1-1* cells shifted to 37 °C (22). Fig. 5 shows the

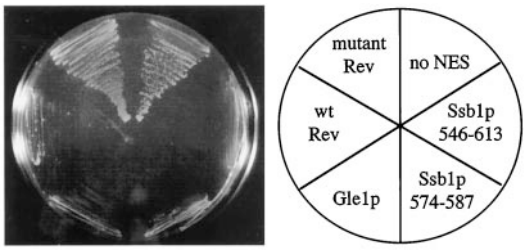

FIG. 4. **Functional inhibition of a transcriptional transactivator by the Ssb1p NES.** Growth of cells on medium lacking histidine is assessed for cells expressing transactivator alone (*no NES*) or transactivator fused to wild-type or mutant Rev NESs, Gle1p NES, Ssb1p C terminus (*546-613*), or Ssb1p NES consensus sequence (*574-587*).

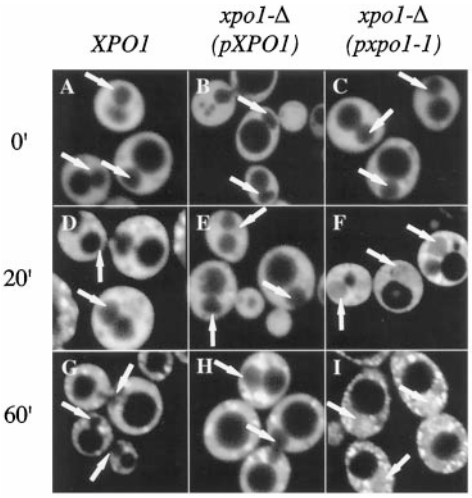

FIG. 5. **Localization of GFP-Ssb1p in temperature-sensitive *xpo1-1* cells.** GFP-Ssb1p was localized by fluorescence microscopy in wild-type and *xpo1*-Δ cells expressing *XP01* or *xpo1-1* at 30 °C (*A, B, C*), after 20 min at 40 °C (*D, E, F*), and after 60 min at 40 °C (*G, H, I*). *Arrows* point to the representative nuclei.

localization of GFP-Ssb1p in *XPO1* and *xpo1-1* cells following a shift from 30° to 40 °C. GFP-Ssb1p was exclusively cytoplasmic in *XPO1* cells after 60 min at 40 °C. In contrast, GFP-Ssb1p fluorescence appeared in the nuclei of *xpo1-1* cells within 20 min at 40 °C and became equilibrated across the nuclear envelope within 60 min (Fig. 5). As expected, the GFP-Ssb1p was exclusively cytoplasmic in *xpo1-1* cells carrying *XPO1* on a plasmid (Fig. 5). We conclude that Ssb1p is exported from nuclei in a Xpo1p-dependent fashion. The accumulation of GFP-Ssb1p in the nuclei of *xpo1-1* cells at a nonpermissive temperature (Fig. 5, *panels F* and *I*) indicates that Ssb1p normally shuttles between the nucleus and the cytoplasm.

NLS-GFP Nuclear Localization Defect of nup188-Δ Cells Is Suppressed by Heat Shock and SSA1 Induction—NLS-GFP is a small nuclear reporter protein that can be used to monitor nuclear transport kinetics in wild-type and mutant yeast cells using method proposed before (15). *nup188*-Δ cells, which are morphologically normal and double at wild-type rates (not shown), accumulate abnormally high cytoplasmic levels of NLS-GFP (Fig. 6*A*, *panel B*). We previously showed that the NLS-GFP import defect of *srp1-31* cells was suppressed either by heat shock or the induction of *GAL1-SSA1*. As shown in Fig. 6*A*, *panel D*, the induction of *GAL1-SSA1* expression also suppressed the steady state NLS-GFP localization defect of *nup188*-Δ cells. In contrast, induction of *GAL1-SSB1* did not suppress the steady state *nup188*-Δ defect (Fig. 6*A*, *panel F*). The level of Hsp70 gene expression under these conditions was determined by immunoblot using specific polyclonal antibodies (26) and revealed that the concentration of Hsp70s increased

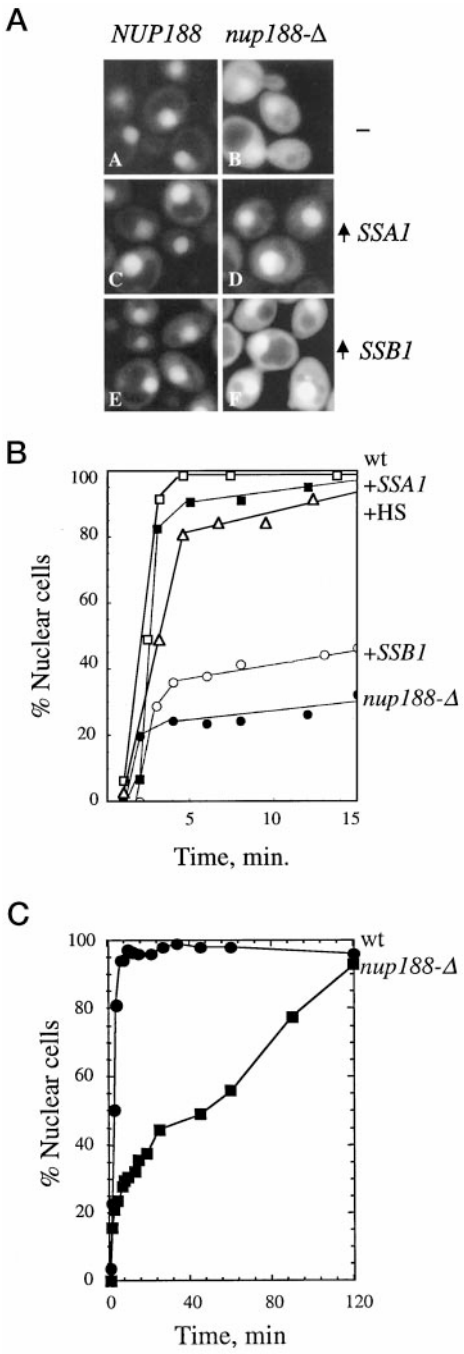

FIG. 6. **Induction of *SSA1* but not *SSB1* suppresses the NLS-GFP nuclear localization defect of *nup188*-Δ cells.** *A*, NLS-GFP expressing normal (*NUP188*) and mutant (*nup188*-Δ) cells were observed before and after induction of *SSA1* and *SSB1* expression (see "Materials and Methods"). *B*, *SSA1* induction stimulates NLS-GFP import kinetics in *nup188*-Δ cells. 37 °C import time courses are shown for NLS-GFP import in *nup188*-Δ cells that were untreated (*closed circles*), heat-shocked (*HS*) for 1 h (*open triangles*), or induced for *GAL1-SSA1* (*closed squares*) or *GAL1-SSB1* expression for 2 h (*open circles*) and wild type, (*wt*) cells (*open squares*). wild- type (*wt*). *C*, extended time course of NLS-GFP import at 37 °C in *NUP188* (*circles*) and *nup188*-Δ (*squares*) cells at 37 °C.

3–4-fold following *GAL1* induction (data not shown). The *SSA1* induction upon heat shock was comparable with *GAL1-SSA1* induction (data not shown).

The effects of heat shock and *GAL1-SSA1* expression on NLS-GFP re-import kinetics in *nup188*-Δ cells grown at 30 °C and assayed at 37 °C are shown in Fig. 6*B*. NLS-GFP import in *NUP188* cells proceeded rapidly and to virtual completion

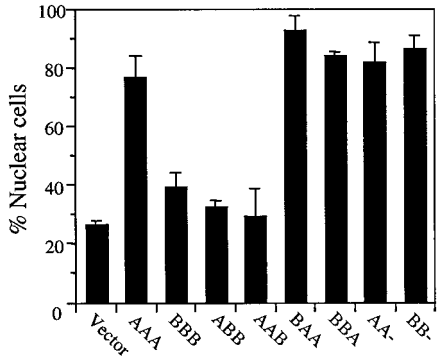

FIG. 7. **Effect of Ssa1p/Ssb1p chimeras on NLS-GFP import in *nup188*-Δ cells.** Various SSA/SSB chimeras, indicated by three-letter codes (see "Results"), were cloned into pCUG2 and expressed in NLS-GFP expressing *nup188*-Δ cells. NLS-GFP import assays were performed at 37 °C as described under "Materials and Methods." The percentages of nuclear cells were scored at $T = 15$ min.

within 5 min. In contrast, NLS-GFP import in *nup188*-Δ cells proceeded with biphasic kinetics, beginning with a "burst" phase, during which 20–40% of the cells rapidly accumulated NLS-GFP, followed by a slower quasi-plateau phase (see Fig. 6C). We do not know whether the biphasic nature of these kinetics are a property of all *nup188*-Δ mutated cells or, alternatively, indicate the presence of different cell populations, perhaps differing in their Hsp70 content. *nup188*-Δ cells that were heat-shocked, or *GAL1-SSA1*-expressing cells, showed wild-type import kinetics (Fig. 6*B*). The induction of *GAL1-SSB1* expression in *nup188*-Δ cells resulted in a slight, but reproducible, increase in the fraction of cells showing good import during the burst phase (Fig. 6B).

nup188-Δ cells grown at 37 °C showed better nuclear accumulation of NLS-GFP than cells grown at 30 °C (not shown). 37 °C is a heat shock temperature, and cells grown at this temperature contain high levels of Ssa-type Hsp70s (17). For this reason we extended the 37 °C NLS-GFP import time course to allow for the full development of a heat shock response. An extended 37 °C time course revealed that after the initial burst phase NLS-GFP nuclear accumulation continued slowly until virtually all of the cells in the culture showed good nuclear localization (Fig. 6*C*). The duration of the slow phase roughly corresponds to the rate at which Hsp70 accumulates in cells during heat shock. When assayed at 30 °C, which is not a heat shock temperature, the kinetics of NLS-GFP import began with a burst but quickly reached a plateau of between 20–40% nuclear cells that was stable for at least 3 h (not shown). These results suggest that the slow increase in NLS-GFP nuclear localization at 37 °C is the result of the heat shock induction of Ssa-type Hsp70 gene expression. We conclude that elevated levels of Ssa1p are sufficient to suppress both the steady state and kinetic NLS-GFP localization defects of *nup188*-Δ cells.

Nuclear Localization of Ssa1p/Ssb1p Chimeras Correlates with Their Ability to Suppress the nup188-Δ Nuclear Transport Defect—The efficient NES-directed export of Ssb1p could provide a basis for the inability of Ssb1p to suppress the NLS-GFP nuclear localization defect in *nup188*-Δ cells. To test this hypothesis we assayed NLS-GFP import kinetics in *nup188*-Δ cells expressing various Ssa1p/Ssb1p chimeras and plotted the "% nuclear cells" after a 15-min re-import (Fig. 7). The Ssa1p/Ssb1p chimeras for this functional analysis were not fused to GFP. As shown in Fig. 7, the expression of chimeras containing the C terminus of Ssb1p (BBB, ABB, and AAB) failed to suppress the NLS-GFP localization defect. In contrast, chimeras containing the C terminus of Ssa1p (AAA, BAA, and BBA), and truncations of Ssa1p and Ssb1p lacking either C-terminal domain (AA- and BB-), suppressed the *nup188*-Δ NLS-GFP import defect. We conclude that the C-terminal domain of Ssb1p, which contains a functional NES, is a dominant inhibitor of Hsp70 function in NLS-directed nuclear transport. Together with the GFP-Ssa1p/Ssb1p localization data shown in Fig. 2*B*, these experiments demonstrate a strong positive correlation between the steady state nuclear localization of Hsp70 and its functions in NLS-directed nuclear transport.

DISCUSSION

The aim of this study was to investigate the role of Hsp70 in NLS-directed nuclear transport and, more specifically, to elucidate the molecular basis for the differential function of Ssa- and Ssb-type Hsp70s. We conclude that an NES in the divergent 8 kDa C-terminal domain of Ssb1p is necessary and sufficient for the low nuclear levels of Ssb1p and for its inability to stimulate NLS-directed nuclear transport. Therefore, the differential function of Ssa1p and Ssb1p in nuclear transport is due to the NES-directed export of the Ssb1p and not to functional differences in their ATPase or peptide binding domains. Previous work showed that certain Ssa1p/Ssb1p chimeras, containing either the ATPase or peptide binding domains of Ssa1p, could rescue phenotypes associated with the double deletion of *SSB1* and *SSB2* (26). Thus, Ssa1p domains can function in Ssb1p-mediated processes. Here, we show that the peptide binding and ATPase domains of Ssb1p can function in a Ssa1p-mediated process. This is not to say that Ssa1p can replace Ssb1p function *in vivo* or vice versa. There are no known examples in which one class of Hsp70 can completely replace the function of a second class. Although a truncated Ssb1p that lacks its NES still functions in nuclear transport, it could not rescue the depletion of all four *SSA* gene products. In this regard, yeast mitochondrial Hsp70, loaded into reconstituted endoplasmic reticulum vesicles, could not replace endoplasmic reticulum Hsp70 in an *in vitro* protein secretion assay (39). The inability of mitochondrial Hsp70 to function in BiP-mediated secretion was attributed to the inability of mitochondrial Hsp70 to associate productively with Sec63p, a BiP-specific DnaJ co-chaperone (39). The possible role of Hsp70 co-chaperones in nuclear transport has not been explored.

The very existence of the Ssb1p NES, and the accumulation of GFP-Ssb1p in the nuclei of *xpo1-1* cells, suggests that Ssb1p normally shuttles between the nucleus and cytoplasm. Although Ssa-type Hsp70s have previously been shown to shuttle between the nucleus and the cytoplasm in *Xenopus* oocytes (40), Ssb-type Hsp70s have been assumed to function exclusively in the cytoplasm (12, 13). These new results suggest that Ssb1p may function in the nucleus as well as in the cytoplasm.

Elevated Levels of Ssa1p but Not Ssb1p Stimulate NLS-directed Nuclear Transport—A 2–3-fold increase in cellular Ssa1p levels suppressed the kinetic and steady state NLS-GFP nuclear localization defects of *nup188*-Δ cells. The severity of the *nup188*-Δ defect, and its complete suppression, provided a clean, specific *in vivo* assay for Hsp70 function in nuclear transport. The NLS-GFP nuclear transport defects of strains containing temperature-conditional mutations in other transport factors, specifically *srp1-31* and *nup82-3* cells (15), were also suppressed by *GAL1-SSA1* induction, but these defects are less severe and are not, therefore, ideally suited to quantification. The present analysis is restricted to the role of Hsp70 in the import of SV40-type NLSs and does not address the potential involvement, or lack thereof, of Hsp70 in the transport of other classes of import or export cargo (4).

The stimulatory effects of *GAL1-SSA1* induction on NLS-GFP import suggest that Hsp70 functions by stimulating at least one rate-limiting step along the nuclear import pathway. This result implies that the concentration of Ssa-type Hsp70s

under non-stress conditions is normally limiting for nuclear transport and that *GAL1-SSA1* or stress induction generates an incipient pool of Hsp70 that is available to interact with the nuclear transport apparatus. Under stress conditions in higher eukaryotes, cytosolic pools of Hsp70 are recruited into the nucleus (31, 33). Therefore, in addition to an increase of Hsp70 levels as a result of new synthesis, there is an additive increase in nuclear levels because of compartment redistribution. In yeast, we observed a minor but reproducible increase in relative nuclear GFP-Ssa1p fluorescence following heat shock. Although this result is consistent with a minor redistribution of Hsp70 in yeast, it is possible that under heat shock conditions nuclear levels remained constant and only appeared to increase because cytoplasmic levels decreased through turnover.

It is not known whether Hsp70 is absolutely required for the import of NLS-cargo or, alternatively, whether it functions as an enhancer of nuclear transport efficiency. Although the literature is undecided on this issue, a strict requirement for Hsp70 in the nuclear transport of selected NLS-cargo was demonstrated using isolated nuclei and permeabilized cell assays, under which circumstances endogenous Hsp70 levels can be more or less completely depleted (17–20).

GFP-Ssa1p Is Localized Both in the Nucleus and the Cytoplasm, Whereas GFP-Ssb1p Is Cytoplasmic—The localization of GFP-Ssa1p to both the nucleus and the cytoplasm is consistent with a large body of evidence on the localization of Ssa-type Hsp70s in a variety of organisms and cells. The exclusive cytoplasmic localization of GFP-Ssb1p is unique for non-organelle Hsp70s. A technical weakness in this analysis of Hsp70 localization is its reliance on the use of GFP fusions, which could produce localization artifacts. The validity of the GFP fusion localization results reported here, however, have been corroborated by two lines of evidence. First, the ability of various Ssa1p/Ssb1p chimeras and truncations to suppress the *nup188*-Δ NLS-GFP nuclear localization defect is correlated perfectly with the localization of the GFP-Ssa1/Ssb1 chimeras. Those chimeras that localized to nuclei suppressed the transport defect, whereas the chimeras that failed to suppress the transport defect also failed to accumulate in nuclei. It is essential to note that the functional assays were performed with Ssa1p/Ssb1p chimeras that were not fused to GFP. Second, the key finding of the GFP-based localization studies was the existence of the Ssb1p leucin-rich NES, which in other experiments was shown to function as an NES out of the context of a GFP fusion protein.

Both genetic and biochemical evidence suggest that Ssb1p and Ssb2p are functionally interchangeable and have primary roles in protein synthesis (13, 41). A large fraction of Ssbp co-sediments with translating ribosomes, and Δ*ssb1* Δ*ssb2* cells are cold- and hygromycin B-sensitive (12). Why then does Ssb1p contain an NES? The presence of an NES in a cytoplasmic protein suggests that the protein shuttles between the nucleus and the cytoplasm and, more interestingly, has a nuclear function. The Ssb1p NES is not required for any known Ssb1p function. An Ssa1p/Ssb1p chimera containing the ATPase and peptide binding domains of Ssb1p and the Ssa1p C-terminal domain, and hence lacking the NES, rescued both Δ*ssb1* Δ*ssb2* phenotypes (26). This experiment did not, however, prevent access of Ssb1p to the nucleus.

Ssb1p could serve any number of functions in the nucleus, a few of which are mentioned here. Because Ssb1p associates with ribosomes (12) and Ssa-type Hsp70s have been observed to localize in the nucleus preferentially to the nucleolus, it is possible that Ssb1p functions in some aspect of ribosome biogenesis. *SSB1* was identified in a high copy number screen for suppressors of a mutation in a proteasome subunit (42, 43). Thus, Ssb1p may participate in proteasome-mediated protein degradation. The proteasome complex is localized both in the cytoplasm and the nucleus (44), so it is possible that Ssb1p interacts transiently with a nuclear proteasome pool. In mammalian cells, a link between the heat shock response and proteasome activity has been noted. Specifically, the expression of a transcriptional regulator of the heat shock response, *HSF2*, is activated in response to proteasome inhibitors (38). Hsp70 has also been implicated in the autoregulation of the heat shock response in mammalian cells. Both Hsp70 and the co-chaperone Hdj1p bind Hsf1p and together repress heat shock gene transcription (45). By analogy, Ssb1p could function in the nucleus as a regulator of gene expression.

With the discovery of the Ssb1p NES, it is apparent now that both Ssa1p and Ssb1p shuttle across the nuclear envelope. Like Kap α and β, Hsp70 would be expected to shuttle during its role in NLS-directed nuclear transport. Thus, it is unclear why the Ssb1p NES would inhibit its function in transport. The induction of *GAL1-SSB1* expression did stimulate a small but reproducible increase in NLS-GFP import in *nup188*-Δ cells (Fig. 6*B*). It is possible that Ssb1p might promote the targeting and translocation of NLS-cargo during its shuttling cycle, but upon entering the nucleus, most of the Ssb1p-associated NLS-cargo complex might be exported before the NLS-cargo could be released to the nucleoplasm. Also, it is possible that a significant steady state concentration of nuclear Hsp70 is required for its function in nuclear transport. For example, Hsp70 may be involved in the recycling of transport factors such as Kap α and Kap β back to the cytoplasm. Normal nuclear levels of Ssb1p may be too low, or simply unavailable, to facilitate factor recycling.

Role of the Hsp70 Chaperone System in NLS-directed Nuclear Transport—Molecular genetic evidence supports the notion that Hsp70 facilitates the formation and stability of the NLS-Kap α complex (15, 16). Cell biological evidence indicates that Hsp70 is co-imported with the NLS-cargo-Kap α/β ternary complex (19, 46). Furthermore, Hsp70s bind with high affinity to *bona fide* NLS peptides (47, 48) and NLS-like peptides (49, 50). Standard models of Hsp70 chaperone action predict that cycles of NLS peptide binding and release could function to minimize nonspecific intermolecular interactions between the NLS and cellular constituents. The chaperone cycle could, in effect, increase the concentration of free NLS-cargo available for Kap α binding. Hsp70s normally work in conjunction with co-chaperones that facilitate and regulate peptide binding/release and ATPase activity, and it will be interesting to investigate the potential role of co-chaperones in nuclear transport.

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