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Identification of Novel *Saccharomyces cerevisiae* Proteins with Nuclear Export Activity: Cell Cycle-Regulated Transcription Factor Ace2p Shows Cell Cycle-Independent Nucleocytoplasmic Shuttling

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Nuclear export of proteins containing leucine-rich nuclear export signals (NESs) is mediated by the NES receptor CRM1/Crm1p. We have carried out a yeast two-hybrid screen with Crm1p as a bait. The Crm1p-interacting clones were subscreened for nuclear export activity in a visual assay utilizing the Crm1p-inhibitor leptomycin B (LMB). This approach identified three *Saccharomyces cerevisiae* proteins not previously known to have nuclear export activity. These proteins are the 5′ RNA triphosphatase Ctl1p, the cell cycle-regulated transcription factor Ace2p, and a protein encoded by the previously uncharacterized open reading frame YDR499W. Mutagenesis analysis show that YDR499Wp contains an NES that conforms to the consensus sequence for leucine-rich NESs. Mutagenesis of Ctl1p and Ace2p were unable to identify specific NES residues. However, a 29-amino-acid region of Ace2p, rich in hydrophobic residues, contains nuclear export activity. Ace2p accumulates in the nucleus at the end of mitosis and activates early-G_1-specific genes. We now provide evidence that Ace2p is nuclear not only in late M-early G_1 but also during other stages of the cell cycle. This feature of Ace2p localization explains its ability to activate genes such as *CUP1*, which are not expressed in a cell cycle-dependent manner.

Intracellular movement between the nucleus and the cytoplasm takes place through large proteinaceous structures called nuclear pore complexes (NPCs [7, 51]). NPCs are embedded in the nuclear membrane and provide aqueous channels through which macromolecules can cross. In the budding yeast *Saccharomyces cerevisiae*, these large protein structures are composed of ~50 different proteins called nucleoporins, which often contain phenylalanine-glycine (FG) repeats. Several nucleoporins contain coiled coil and leucine zipper domains, involved in protein-protein interactions (35).

Active transport through NPCs is a signal-mediated process (32, 35). Transport cargos travelling from the cytoplasm to the nucleus contain nuclear localization signals (NLSs), whereas nuclear export signals (NESs) direct cargos from the nucleus to the cytoplasm. These localization signals are recognized by transport receptors which belong to the importin-β family of proteins (32). The genome of *S. cerevisiae* encodes 14 transport receptors recognized by sequence homology (62). They are all of similar size (90 to 130 kDa) and share an N-terminal domain involved in binding the small GTPase Ran, which has been shown to be a main player in nuclear transport processes (11, 16, 22). Transport receptors are thought to mediate the directional movement of their respective cargos through the NPC, via interactions with FG-repeat containing nucleoporins as well as with Ran. Like other GTPases, Ran exists in both a GTP form and a GDP form. An essential requirement for nuclear transport events is the establishment of a gradient of the nucleotide bound state of Ran, with RanGDP residing in the cytoplasm and RanGTP residing in the nucleus (22). This gradient is facilitated by the compartmentalization of the Ran regulatory proteins.

The first characterized NESs were initially identified in the inhibitor of cyclic-AMP-dependent protein kinase (PKI) and in the viral protein Rev (9, 60). Subsequently, NES export was shown to be mediated by the importin β family member CRM1 (exportin 1) (Crm1p or Xpo1p in yeast [14, 38, 41, 50]). NESs, dependent on CRM1 for their activity, have now been identified in many eukaryotic proteins. Mutagenesis and randomization-selection analysis of these NESs have shown that they are short sequences (~10 amino acids) with critically spaced hydrophobic residues essential for export activity (2, 24, 29, 30, 60). Since leucine is a preferred residue in this type of NES, it is often termed a leucine-rich NES; to date, most CRM1-dependent NESs are of this type. However, atypical NESs have been found in Rev-like proteins from feline immunodeficiency virus and equine infectious anemia virus (12, 31). In these proteins hydrophobic residues play a role in NES activity, but their spacing is altered compared to the conventional NES. Furthermore, it was recently shown that the mammalian m^3G-cap receptor snurportin1 is a target for CRM1-mediated export and that a rather large domain of snurportin1 is required for a CRM1 interaction (43). Therefore, signals other than small hydrophobic ones are utilized for CRM1-dependent nuclear export.

A powerful tool in the discovery of CRM1 as the export receptor for leucine-rich NESs was the *Streptomyces* metabolite leptomycin B (LMB). LMB inhibits NES-mediated export in mammalian cells and in *Schizosaccharomyces pombe* by binding directly to CRM1 and disrupting the trimeric NES-CRM1-RanGTP export-competent complex (1, 10, 25, 26, 61). In contrast to mammalian cells and to *S. pombe*, *S. cerevisiae* is

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resistant to LMB. However, a single amino acid change (threonine^{539} to cysteine [T539C]) in *S. cerevisiae* Crm1p converts the organism from resistant to highly LMB sensitive (39). This can be attributed to the fact that *S. cerevisiae* Crm1p does not interact with LMB, whereas the T539C mutant protein binds LMB with an affinity comparable to that of *S. pombe* and human CRM1. Thus, in a strain harboring the T539C substitution in Crm1p (Crm1T539C), treatment with LMB results in rapid inhibition of Crm1p-mediated nuclear export (39).

To date, only a few NESs have been identified in *S. cerevisiae* (8, 48, 63). Although all conform to the leucine-rich type, more *S. cerevisiae* NESs are necessary to evaluate whether a typical yeast NES resembles NESs from metazoans. The most thoroughly characterized yeast export signal has been identified in the AP1-like transcription factor Yap1p (63), which is known to activate the expression of genes in response to oxidative stress (28, 47). Under normal conditions, Yap1p is cytoplasmic due to rapid Crm1p-dependent nuclear export. Under oxidative conditions, the Yap1p-Crm1p interaction does not occur, leading to nuclear accumulation of Yap1p and target gene upregulation (27, 63).

Regulated localization of transcription factors has emerged more generally as a major means of regulating gene expression (17). In *S. cerevisiae* the *ACE2* and *SWI5* genes encode transcription factors that show extensive homology, with 95% similarity in the DNA binding zinc finger domains and 37% similarity over their entire lengths (3). Both *ACE2* and *SWI5* have mitotic cell cycle-regulated expression and subcellular localization. Transcription of the *ACE2* and *SWI5* genes increases during S phase and peaks at the G_2-M transition, after which RNA levels decrease dramatically (5, 37). Throughout G_2 newly synthesized Swi5p is cytoplasmic. Swi5p moves into the nucleus after cells enter mitosis and then activates transcription of G_1-specific target genes, after which the protein is rapidly degraded (36). Ace2p localization has been suggested to be regulated in a similar fashion (5). Although Ace2p and Swi5p activate common G_1 genes, including *ASH1*, *CDC6*, *EGT2*, *PCL2*, *PCL9*, *RME1*, and *SIC1* (33), they also regulate distinct genes. Swi5p is an activator of the *HO* gene involved in mating-type switching, and Ace2p is required for expression of *CTS1*. This gene encodes chitinase, an enzyme that removes the chitin septum between mother and daughter cells after cell division (5). Both *HO* and *CTS1* show a G_1-specific expression pattern. Ace2p is also involved in basal level transcription of *CUP1*, which encodes a metallothionein involved in the protection of yeast cells against heavy metal toxicity (3). *CUP1* transcription is not cell cycle regulated (49), creating an apparent paradox with the presumed late-M–early-G_1-specific nuclear localization of Ace2p.

In this study, we describe attempts to expand the number of known NESs in *S. cerevisiae*. Using *S. cerevisiae* Crm1p as a bait, we performed a yeast-two hybrid screen. Based on the LMB sensitivity of the Crm1(T539C) strain, we subscreened the Crm1p-interacting clones and identified three new proteins with export activity (named Cip1 to Cip3, for Crm1p interacting proteins 1 to 3). Characterization of the Cip1p NES revealed a small domain corresponding to the leucine-rich NES consensus sequence. Cip3p is the cell cycle-regulated transcription factor Ace2p. The Ace2p fragment isolated in our two-hybrid screen harbors both NES and NLS activity, and we show that Ace2p is able to enter the nucleus at all stages of the cell cycle. Our data thus strongly suggest that the protein continuously shuttles between the nucleus and the cytoplasm in a cell cycle-independent manner.

MATERIALS AND METHODS

Plasmid constructions. Construction of the following two-hybrid fish plasmids have been described elsewhere: yRip(FG), hCAN(FG), Yap1-CRD (39, 55, 63). The fish construct pJG4-5-Gsp2 was made by ligating a PCR-generated *Eco*RI/*Xho*I *GSP2* fragment into compatible sites of pJG4-5. The bait constructs pEG202-Ace2(amino acids [aa] 42 to 242) and pEG202-Ctl1 (aa 77 to 160) were generated by ligating PCR-generated *Eco*RI/*Xho*I fragments of the relevant regions into compatible sites of pEG202. pEG202-Cip1-NES was constructed by annealing two oligonucleotides containing the appropriate sequence and restriction site overhangs and then ligating this double-stranded DNA fragment into pEG202 digested with *Eco*RI and *Xho*I.

Constructs for the expression of green fluorescent protein (GFP) fusion proteins were made by PCR amplification of complete open reading frames (ORFs) or relevant coding fragments from genomic DNA template using primers containing appropriate restriction sites. For the creation of N-terminal fusions to GFP, the PCR fragments were inserted into compatible sites of JH23; for C-terminal fusions to GFP, fragments were inserted into compatible sites of pPS808, and for creation of fusions to NLS-2XGFP, fragments were inserted into compatible sites between NLS and 2XGFP sequences of pPS1372. pJH23, pPS808, and pPS1372 are all *URA3* and 2μm vectors. pPS808 and pJH23 carry a galactose-inducible promoter, whereas pPS1372 carries the constitutive ADH promoter.

Constructs for the expression of GST fusion proteins were made by PCR amplification of relevant fragments from genomic DNA using primers with appropriate restriction sites. DNA fragments were inserted into either *Bam*HI/*Eco*RI or *Eco*RI/*Xho*I sites of the pGEX-4T-1 vector (Pharmacia). The plasmid expressing the PKI-NES fused to glutathione *S*-transferase (GST) has been described previously (63). All constructs were devoid of sequence errors as verified by DNA sequencing.

GST pulldown experiments. Bacterial lysates containing the various GST fusion proteins were prepared according to the description of the manufacturer (Pharmacia). In vitro-translated Crm1T539Cp protein was synthesized from a T7-promoter containing PCR fragment template as described previously (39). For binding reactions GST fusion proteins were first prebound to glutathione-Sepharose beads: 1 ml of bacterial lysate in phosphate-buffered saline containing 10 to 20 μg of the relevant fusion protein (40 to 90% pure as determined by Coomassie blue staining) was incubated on a spinning wheel for 1 h at 4°C with 50 μl of a 50% slurry of glutathione-Sepharose beads in binding buffer B (50 mM HEPES-KOH, pH 7.0; 200 mM NaCl; 5 mM $MgCl_2$; 0.1% Tween 20, 1 mM dithiothreitol, 5 μg of leupeptin per ml, 5 μg of aprotinin per ml). Beads were then washed three times in 500 μl of buffer B and resuspended in 200 μl of buffer B, and then 70 μl of the Crm1T539Cp translation reaction was added. Reactions were split in two, and to half of the reactions LMB was added to a final concentration of 0.5 μM. Binding was carried out on a spinning wheel for 2 h at 4°C, after which beads were washed five times in 500 μl of buffer B. Proteins were eluted from beads by boiling for 10 min in sodium dodecyl sulfate (SDS) sample buffer and fractionated on a 4 to 20% protein gradient gel (Bio-Rad), and bound Crm1T539Cp protein was visualized using a GS-363 Molecular Imager System (Bio-Rad).

Yeast two-hybrid screen. The *S. cerevisiae* CRM1-bait construct used for the screen was generated by PCR amplification of the *S. cerevisiae CRM1* ORF using 5′ and 3′ primers containing *Bam*HI and *Pst*I sites, respectively. This PCR fragment was cloned into pGBT9 (Clontech), generating an in-frame fusion with the GAL4 DNA-binding domain. The resulting plasmid pCRM1-bait was confirmed to be free of PCR-induced mutations by sequence analysis. A detailed description of the yeast genomic library (FRYL library) construction and two-hybrid strategy have been described (13). A frozen aliquot of yeast strain Y187 (Clontech) (59) transformed with the FRYL library was thawed, and cells mixed with CG1945 cells (Clontech) containing the pCRM1-bait plasmid. Diploids that were able to activate the His reporter were selected by growth on Leu^- His^- Trp^- media at 30°C for 3 days. $LacZ^+$ activation was determined by a X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) lift assay. A total of 5,000 colonies were His^+; 2,000 colonies were $LacZ^+$; for the 320 strongest interactions (determined by intensity of blue on X-Gal plates), library plasmids were rescued in *Escherichia coli*, and insert junctions were determined by sequence analysis. Pairwise yeast two-hybrid interaction assays were carried out as described previously (38).

Expression and analysis of GFP fusion proteins. Plasmids encoding full-length proteins from the two-hybrid screen fused to either the N or the C terminus of GFP, and plasmids encoding the various GFP fusion constructs were transformed into the Crm1T539C strain. For galactose-inducible protein expression, the GFP fusion proteins were localized as follows: cells were grown overnight at 30°C in 10 ml of Ura^- medium containing 2% lactate, 2% glycerol, and 0.05% glucose to early to mid-log phase. Then, 2 ml of 20% filter-sterilized galactose was added for 2 to 4 h. At this time GFP expression was confirmed by fluorescent microscopy. Cells were washed in H_2O, resuspended in 10 ml of Ura^- medium containing 2% glucose, and incubated for 1 to 2 h at 30°C. Cells were divided in half and either treated with 100 ng of LMB per ml or left untreated and allowed to continue growing at 30°C. At relevant time points after LMB treatment, 0.5 ml of cells were removed from each half (with or without LMB) and concentrated prior to analysis by fluorescent microscopy. For expression of pPS1372-derived

constructs carrying the ADH promoter, cells were grown in Ura$^-$ medium containing 2% glucose to early to mid-log phase, and the localization of GFP fusion proteins was examined (with or without LMB) as described above.

Site-directed mutagenesis. The QuickChange Site-Directed Mutagenesis Kit from Stratagene was used to generate all site-directed mutations according to the instructions of the manufacturer. The concentration of the plasmid templates was 50 ng/ml. Correct mutations were confirmed by sequence analysis.

Primer extension analysis. To analyze the effect of LMB on the abundance of individual transcripts, the Crm1T539C strain was grown to logarithmic phase and either treated with 100 ng of LMB per ml for 30 min or left untreated. Total RNA purification and primer extension analysis was done as previously described (44). The following four oligonucleotides were used for primer extension analysis: for CTS1, 5′-GGCAGTAGTAAGAATTGTGTGAATAGAAGA; for CUP1, 5′-GGCATTGGCACTCATGACCTTCA; for TRX2, 5′-CAACATCCA ACTTGTAAAAAGCAGCG; and for U2snRNA, 5′-GCCAAAAAATGTGTA TTGTAAC.

Induction of cell cycle arrest. To induce cell cycle arrest, cells were grown to an optical density at 600 nm of 0.2 and washed with sterile water. For arrest in S phase cells were grown for 3.5 h in medium containing 100 mM hydroxyurea, and for arrest in early G_1 phase cells were grown for 3.5 h in medium containing 2.5 μg of alpha-mating factor. When examining GFP-Ace2p localization in S phase, cells were kept in galactose-containing medium during the entire experiment.

RESULTS

Crm1p yeast two-hybrid screen. To search for Crm1p interacting proteins, we performed a yeast two-hybrid screen using *S. cerevisiae* Crm1p as a bait. We utilized a highly selective procedure (13) to screen a yeast genomic DNA two-hybrid library (11); 76×10^6 interactions were tested, corresponding to approximately 5 times library coverage. Of the strongest interactions (see Materials and Methods), 320 were sequenced.

Table 1 summarizes the different clones isolated in the screen. The 320 sequences corresponded to fragments of 29 different yeast ORFs. These ORFs have all been classified based on previously established criteria (A1, A2, A3, and A4; see legend to Table 1 and reference 13). The screen was near saturation, since relatively few ORFs were represented and many of the A1 candidates were highly redundant.

Crm1p interacts with NPC components. Two A1 ORFs corresponded to the known NPC components Rip1p and Nup159p. Five different overlapping fragments of Rip1p represented 18.4% (59 of 320) of the clones, and seven overlapping fragments of Nup159p were isolated 5% (16 of 320) of the time. These two nucleoporins belong to a subgroup that has previously been shown to interact with Rev in the yeast two-hybrid assay (55), an interaction believed to be bridged by Crm1p (38); therefore, their presence was expected. Both Rip1p and Nup159p contain FG repeat domains, which have been classified as XXFG. However, most of the repeats in both proteins fit either a SA/PFG or a PS/AFG motif. This kind of FG repeat is not characteristic of any other yeast nucleoporin. The isolation of overlapping fragments of Rip1p and Nup159p constitute a Crm1p interaction domain mapping, which is illustrated in Fig. 1A. The minimally defined fragments necessary for a Crm1p interaction demonstrate clear specificity for the FG repeat domains. It has previously been noted that within the FG repeat domain of Nup159p reside four nearly perfect 26-aa repeats (18; Fig. 1B). Interestingly, the minimally defined Crm1p-interacting region of Nup159p contains almost exclusively all four repeats. Within the minimal Crm1p-interacting portion of Rip1p, three of six potential 10-aa repeats resemble a core region of the Nup159p repeats (Fig. 1B). This core sequence is specific to Nup159 and Rip1p and suggests a major role for these sequences in Crm1p-mediated export.

Identification of novel proteins with export activity. The major aim of the screen was to identify novel yeast NES-containing proteins. Therefore, the transcription factor Yap1p was expected to be identified. A single *YAP1* clone was isolated twice (A3 category; Table 1). Given the number of interactions tested and the number of positive clones, the sequencing of 320 candidates represents approximately one full library coverage; biologically significant interactions are therefore potentially represented only once. The relatively small number of isolated *YAP1* clones is therefore not surprising.

We used a GFP-localization assay and the LMB-sensitive Crm1T539C strain to subject most of the isolated clones to a secondary screen for NES activity (Fig. 2A). In this strain, inactivation of Crm1p with LMB results in total nuclear accumulation of a NLS-NES-GFP reporter protein in approximately 15 min (39). Full-length proteins of the isolated clones were expressed as either N- or C-terminal GFP fusions in the Crm1T539C strain, and localization was determined before and after LMB inhibition of Crm1p-mediated export. The sensitivity of the subscreen depends to some extent on the initial steady-state localization of the candidate GFP fusion proteins. NES-containing substrates that are predominantly nuclear at steady state are ignored, since relocalization is not detectable. Of the candidates analyzed (see GFP column in Table 1), three proteins scored positive in the screen. These proteins have been named Crm1p interacting proteins 1, 2, and 3 (Cip1 to Cip3).

TABLE 1. *S. cerevisiae* Crm1p two-hybrid screen

ORF[a]	No. of clones[b]	Category[c]	Gene[a]	GFP[d]
YAL047C	12 (2)	A1	*SPI6*	+
YBL031W	2	A2	*SHE1*	+
YBR235W	86 (20)	A1		+
YCR077C	5	A2OOF	*PAT1*	ND
YDR192C	59 (5)	A1	*Rip1/NUP42*	ND
YDL189W	13	A4		+
YDR229W	12	A2/A3	*BFR2*	+
YDR499W	1	A4		+
YIL115C	16 (7)	A1	*RAT7/NUP159*	ND
YER008C	7 (2)	A1	*SEC3*	ND
YEL043W	21 (3)	A1		+
YHR082C	6 (3)	A1	*KSP1*	+
YHR108W	1	A4	*GGA2*	+
YJL074C	2	A3	*SMC3*	ND
YJR134C	11 (2)	A1		+
YJR061W	1	A4		+
YKL045W	8	A4	*PRI2*	+
YLL060C	1	A4	*GTT2*	+
YLR131C	3	A4	*ACE2*	+
YMR180C	1	A4	*CTL1*	+
YML117W	1	A2/A3		+
YML007W	2	A3	*YAP1*	+
YMR124W	3	A4		+
YNL196C	9 (4)	A1	*SLZ1*	+
YNL118C	9 (2)	A1	*PSU1*	+
YOR326W	1	A4	*MYO2*	ND
YOR371C	2	A4		+
YPL120W	1	A2/A3	*VPS30*	+
YPR164W	1	A3	*KIM3*	ND

[a] ORF name and gene name of the 29 ORFs identified in the two-hybrid screen are given.

[b] Number of different clones isolated in the screen. The numbers in parentheses denote the number of overlapping clones.

[c] ORFs are categorized based on previously established criteria (13): A1, distinct overlapping fragments; A2, single fusion which starts close to initiation codon; A3, single fusion consisting of a large coding insert; and A4, all other candidates. The heuristic value of these categories has been suggested to be: A1 > A2 = A3 > A4. OOF refers to fusions out of frame. Such frameshifted clones have been shown to potentially represent biologically significant interactions (13).

[d] Examination of the full-length ORF in the GFP-LMB localization assay (see Fig. 2A). ND, not determined.

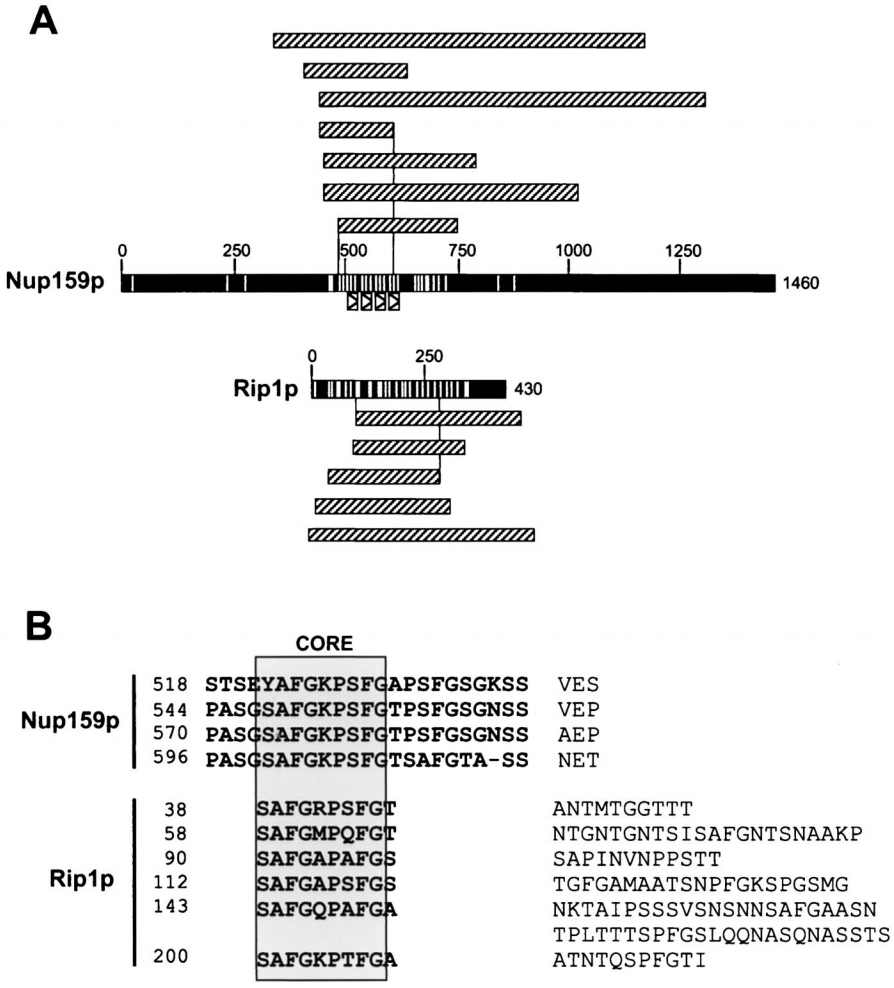

FIG. 1. Portions of Nup159p and Rip1p isolated in the Crm1p two-hybrid screen. (A) The overlapping clones of Nup159p and Rip1p found to interact with Crm1p are shown aligned with their respective full-length proteins. Minimally defined regions of interaction with Crm1p are defined by lines. FG repeats are represented as white bars within both Nup159p and Rip1p. The four 26-aa repeats found in Nup159p are shown as gray boxes below their respective sequences. Numbers indicate amino acid positions in the two proteins. (B) Nup159p and Rip1p contain similar repetitive sequences. The four 26-aa repeats found in Nup159p (adapted from reference 16) are aligned to six 10-aa similar repeats found in Rip1p. The alignment shows a core region of homology (boxed in gray) between these repeat sequences.

In Fig. 2B the LMB-mediated redistributions of GFP-Cip1p, Cip2p-GFP, and GFP-Cip3p are shown. *CIP1* corresponds to the previously uncharacterized ORF YDR499W (Table 1). In ~50% of the untreated cells, GFP-Cip1p was localized throughout the nucleus and the cytoplasm; the remaining ~50% showed nuclear accumulation of the fusion protein (Fig. 2B upper row). At 30 min after LMB addition GFP-Cip1p was entirely nuclear (Fig. 2B upper row). The redistribution is rapid, since it was clearly evident 15 min after LMB addition (data not shown). *CIP2* is the recently identified 5′ RNA-triphosphatase *CTL1* (45), isolated once in the Crm1p two-hybrid screen (ORF YMR180C; Table 1). Without LMB, Ctl1p-GFP was distributed throughout the cytoplasm and the nucleus. At 30 min after LMB addition, Ctl1p-GFP dramatically redistributed to the nucleus (Fig. 2B, middle row). Like Cip1p, the Ctl1p-GFP redistribution can be observed within 15 min of LMB addition (data not shown). The third Crm1p interacting protein (Cip3p) is the transcription factor Ace2p (YLR131C; Table 1). A single *ACE2* clone was isolated three times (A4 category candidate). GFP-Ace2p was mostly cytoplasmic and was often excluded from the nucleus (Fig. 2B, lower row). The fusion protein also appeared as bright spots, a phenomenon previously reported and attributed to Ace2p aggregation when overexpressed from the strong Gal1-promoter (5). The accumulation of GFP-Ace2p in the nucleus after LMB addition was somewhat slower than that observed for GFP-Cip1p and Cip2p-GFP. After 15 min approximately 50% and after 30 min approximately 80% of the Ace2p had accumulated in the nucleus (data not shown and Fig. 2B, lower row). GFP-Ace2p totally redistributed to the nucleus in 60 min (data not shown).

Mapping residues important for NES function. The relocalization of Cip1p, Cip2p, and Cip3p strongly suggested the presence of NESs. As the CRM1-dependent NES consensus sequence contains a minimum of three hydrophobic residues, we introduced leucine (or other hydrophobic residues) to alanine substitutions within the regions isolated in the two-hybrid screen. Substitutions of key NES residues have been shown to block NES activity (30). The substitutions were in all cases made in the context of full-length Cip proteins fused to GFP.

Five mutations were made within the Cip1p sequence spanning aa 660 to 747, corresponding to the C-terminal portion of the protein (Fig. 3A). GFP-Cip1p containing mutations L670A or L673A showed strong nuclear accumulation (100% of the cells), while the localization of the L697A, L732A, and L735A mutants resembled that of the wild-type GFP fusion protein (Fig. 3B). Thus, L670 and L673 constitute important residues

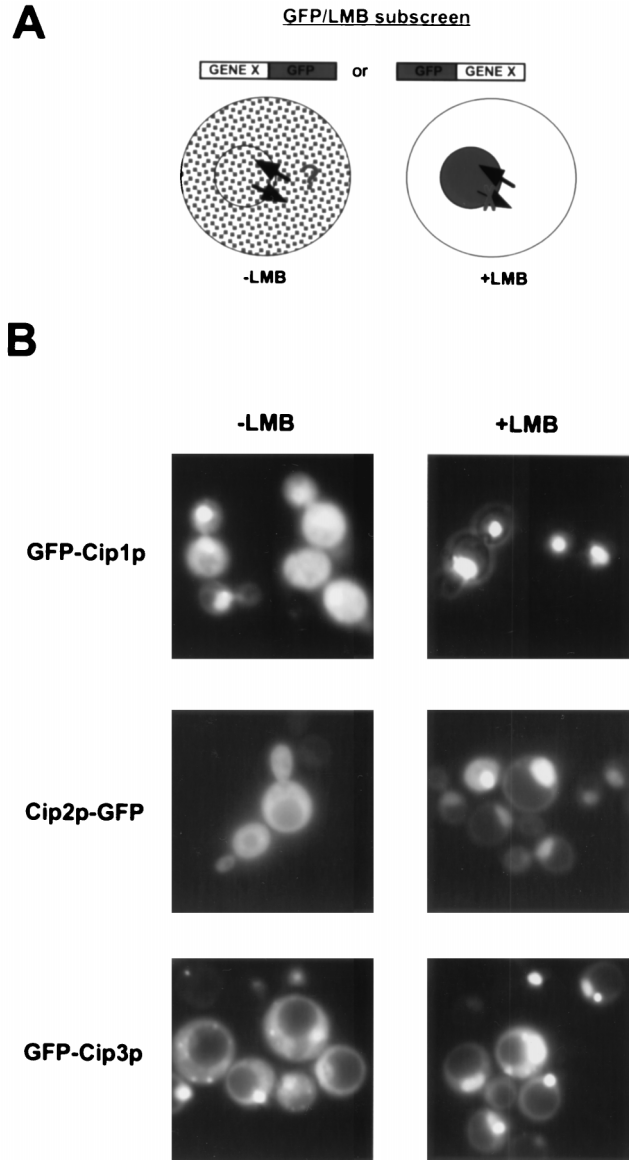

FIG. 2. Identification of three new *S. cerevisiae* proteins with nuclear export activity. (A) Schematic representation of the GFP-LMB subscreen for nuclear export activity. Entire ORFs isolated in the Crm1p two-hybrid screen were expressed as N- or C-terminal GFP fusion proteins in the LMB-sensitive strain Crm1T539C. The dark portion of the bar represents GFP. Localization of the fusion proteins was determined by fluorescent microscopy before LMB addition (−LMB) or at various times after the inhibition of Crm1p-mediated export by LMB (+LMB). LMB-induced nuclear accumulation of a given fusion protein is indicative of the presence of Crm1p-dependent nuclear export activity. (B) Localization of Cip1p (YDR499Wp), Cip2p (Ctl1p), and Cip3p (Ace2p), expressed as either C-terminal (Cip1p and Cip3p) or N-terminal (Cip2p) fusions to GFP, were determined in the absence of LMB (−LMB) or 30 min after addition of 100 ng of LMB per ml (+LMB). See the text for details.

for the NES activity of Cip1p. For Ctl1p and Ace2p, the same approach proved unable to identify critical NES residues. Figure 3C shows the 84-aa portion (aa 77 to 160) of Ctl1p, found to interact with Crm1p in the two-hybrid screen, with mutated residues indicated. The mutant fusion proteins all localized identically to wild-type Ctl1p-GFP (data not shown). For Ace2p, the following single and double mutations were made in the aa 42 to 242 portion of Ace2p isolated in the two-hybrid screen: singles (L70A, L73A, I75A, L126A, and I138A) and doubles (I145A, L146A; L157A, I158A; and I200A, L202A; Fig. 3D). None of these changes substantially shifted the subcellular localization of GFP-Ace2p to the nucleus (data not shown). The only striking changes were observed with GFP-Ace2I138Ap and GFP-Ace2L157A,I158Ap, both of which were localized throughout the cytoplasm and the nucleus and showed no aggregation (data not shown). The localization of these two mutant proteins was unresponsive to LMB, possibly because of misfolding (data not shown).

Cip1p and Ace2p physically interact with Crm1p and other constituents of the nuclear export machinery. To further characterize the Cip1p, Cip2p, and Cip3p regions isolated in the two-hybrid screen, we analyzed their interaction profiles with known factors involved in nuclear export. First, the interaction of a 19-aa region (aa 664 to 682) spanning the key leucine residues (L670 and L673) of Cip1p, the aa 42 to 242 Ace2p region and Ctl1p (aa 77 to 160) were examined as baits in the two-hybrid assay (Fig. 4A). The tested regions of Cip1p and Ace2p were sufficient to interact with the yeast Ran homologue Gsp2p and FG repeat-containing proteins, interactions characteristic of hydrophobic NESs (38, 54, 55). However, we were unable to get reliable pairwise two-hybrid interaction data with the construct containing Ctl1p (aa 77 to 160).

Next, we fused Cip1p (aa 660 to 747), Ctl1p (aa 77 to 160), and Ace2p (aa 42 to 242) to GST and examined the interactions of the fusion proteins with Crm1T539Cp protein translated in vitro in a reticulocyte lysate. In this pulldown assay, the nuclear export activity-containing regions of Cip1p and Ace2p bound Crm1T539Cp with an efficiency comparable to a GST-PKI-NES-positive control (Fig. 4B). These interactions were specific since GST protein did not associate detectably with Crm1T539Cp. Furthermore, the interactions were lost when challenged with 0.5 μM LMB, suggesting that RanGTP from the reticulocyte lysate contributes to complex formation. The reaction with GST-Ctl1p (aa 77 to 160) contained many breakdown products, both in the absence and in the presence of LMB, making it difficult to assess whether binding to Crm1T539Cp occurred (Fig. 4B). Taken together with our mutational analysis described in the previous paragraph, the results show a novel hydrophobic NES within Cip1p (Table 2). Furthermore, the interaction data strengthen the idea that Ace2p is a direct target of Crm1p.

The aa 42 to 242 fragment of Ace2p harbors both nuclear import and nuclear export activity. As we were unable to define NESs in Ctl1p and Ace2p by the site-directed mutagenesis approach outlined in Fig. 3, we performed deletion mapping of the relevant regions. To this end, the Ctl1p (aa 77 to 160) and Ace2p (aa 42 to 242) fragments were first fused to GFP, and the localization of the respective fusion proteins in the Crm1T539C strain was examined in the absence or presence of LMB. GFP-Ctl1p (aa 77 to 160) localized throughout the cells with modest nuclear accumulation (Fig. 5A, lower row). Therefore, it seems that the aa 77 to 160 region possesses some NLS activity. However, this localization was unaltered by LMB addition, indicating that the NES activity observed for full-length Ctl1p no longer functions in the context of the GFP-Ctl1p (aa 77 to 160) fusion protein. When the aa 77-160 fragment was fused to an NLS-2XGFP protein, we were also unable to detect any NES activity. The Ctl1p (aa 77 to 160) region was therefore not analyzed further.

Interestingly, LMB treatment induced nuclear accumulation of the GFP-Ace2p (aa 42 to 242) fusion protein (Fig. 5A, middle row). Thus, the aa 42 to 242 region of Ace2p harbors both NLS and NES activity. Since this region of Ace2p lies outside the originally defined C-terminal NLS, the implication

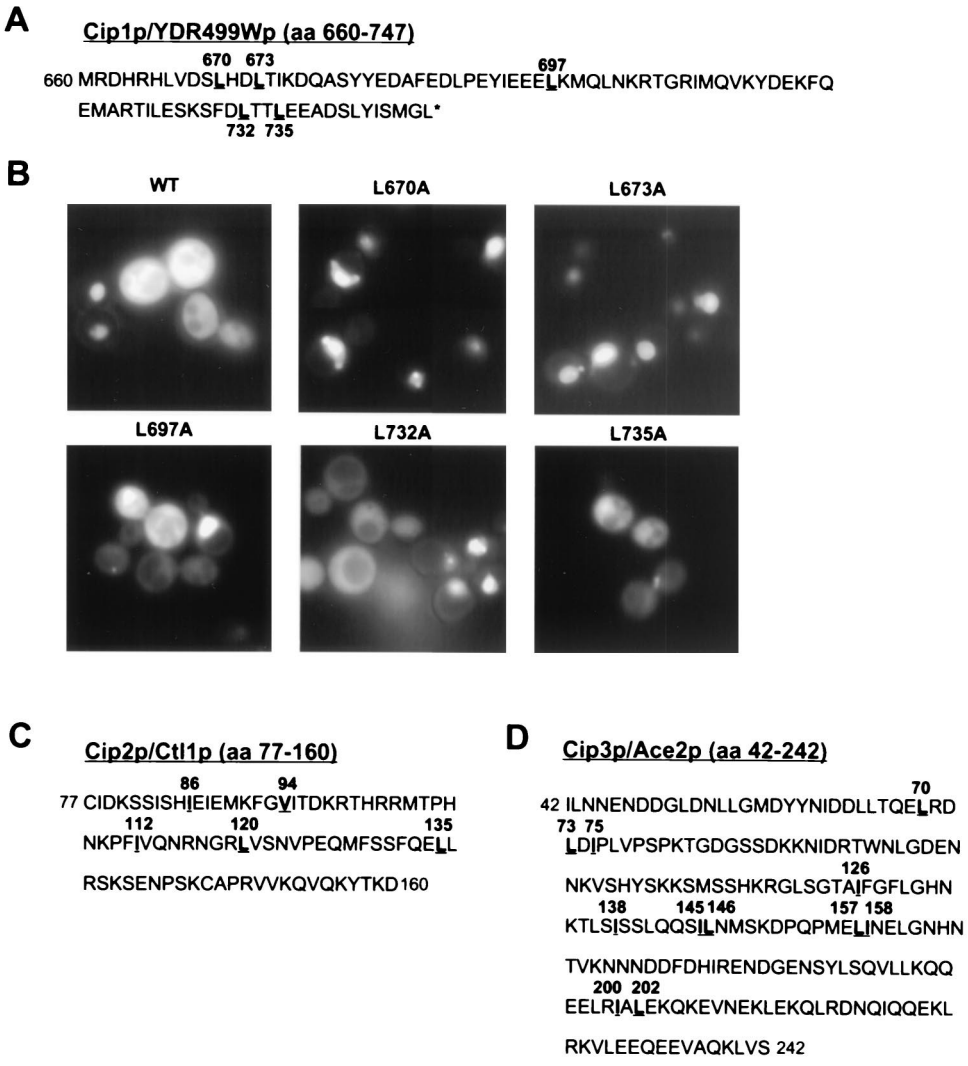

FIG. 3. Mutagenic analysis of the Crm1p-interacting sequences of Cip1p, Cip2p, and Cip3p. (A) The aa 660 to 747 portion of Cip1p isolated in the Crm1p two-hybrid system. The sequence corresponds to the C-terminal 88 aa of the protein. Underlined and bold amino acids were individually substituted to alanines in the context of full-length Cip1p fused to GFP (GFP-Cip1p). (B) GFP-Cip1p mutants were localized by fluorescent microscopy. The nature of the mutation is indicated above the pictures. Leucine-to-alanine changes of aa 670 and 673 resulted in 100% nuclear accumulation of the respective GFP fusion proteins. (C and D) Sequences of the aa 77 to 160 region of Cip2-Ctl1p and the aa 42 to 242 region of Cip3p-Ace2p isolated in the Crm1p two-hybrid system. Underlined and bold amino acids were individually or doubly substituted for alanines in the context of full-length GFP fusion proteins (see text for details).

is that Ace2p contains at least two distinct regions with NLS activity. Attempts to delineate further the N-terminal NLS activity in Ace2p, by expressing GFP-Ace2p (aa 42 to 150) and GFP-Ace2p (aa 140 to 242), were unsuccessful; neither fusion protein relocalized in response to LMB treatment (data not shown). We also made constructs expressing different subregions of the Ace2p (aa 42 to 242) fragment fused to the simian virus 40 (SV40) large T-antigen NLS and two GFP moieties. The localization of these fusion proteins was then examined in the absence or the presence of LMB. As can be seen in Fig. 5B, the NLS-Ace2p (aa 42 to 150)-2XGFP fusion protein efficiently relocalized to the nucleus in LMB-treated cells (Fig. 5B, second row). Thus, nuclear export activity is confined to this region of Ace2p. When smaller fragments of the aa 42 to 150 region were analyzed, we were able to demonstrate NES activity in a 29-aa subfragment spanning aa 122 to 150 in the Ace2p sequence (Fig. 5B, last row). The relocalization of this fusion protein in response to LMB treatment was not as dramatic as seen with the aa 42 to 150 fragment, since nuclear accumulation of the smaller fusion protein was more prominent in the absence of LMB.

Ace2p-specific genes are induced in the presence of LMB. A prediction for a transcription factor with a Crm1p-regulated NES is that target gene activation should accompany Crm1p inactivation. To confirm that Ace2p is a target of Crm1p, we examined the abundance of two Ace2p-inducible transcripts after LMB addition to the Crm1T539C strain (Fig. 6). Four different transcripts were analyzed by primer extension. A positive control was TRX2, which is activated by Yap1p and showed a robust upregulation after 30 min of LMB treatment. U2 snRNA transcription is presumably unaffected by NES containing transcription factors and constitutes a negative control. The Ace2p-regulated transcripts, CTS1 and CUP1, both showed increased abundance in cells treated with LMB compared to untreated cells. Taken together, our analysis of Ace2p shows that the protein contains a functional NES.

Ace2p can enter the nucleus independent of cell cycle. Ace2p shows extensive homology to Swi5p. Given the similarity, it was

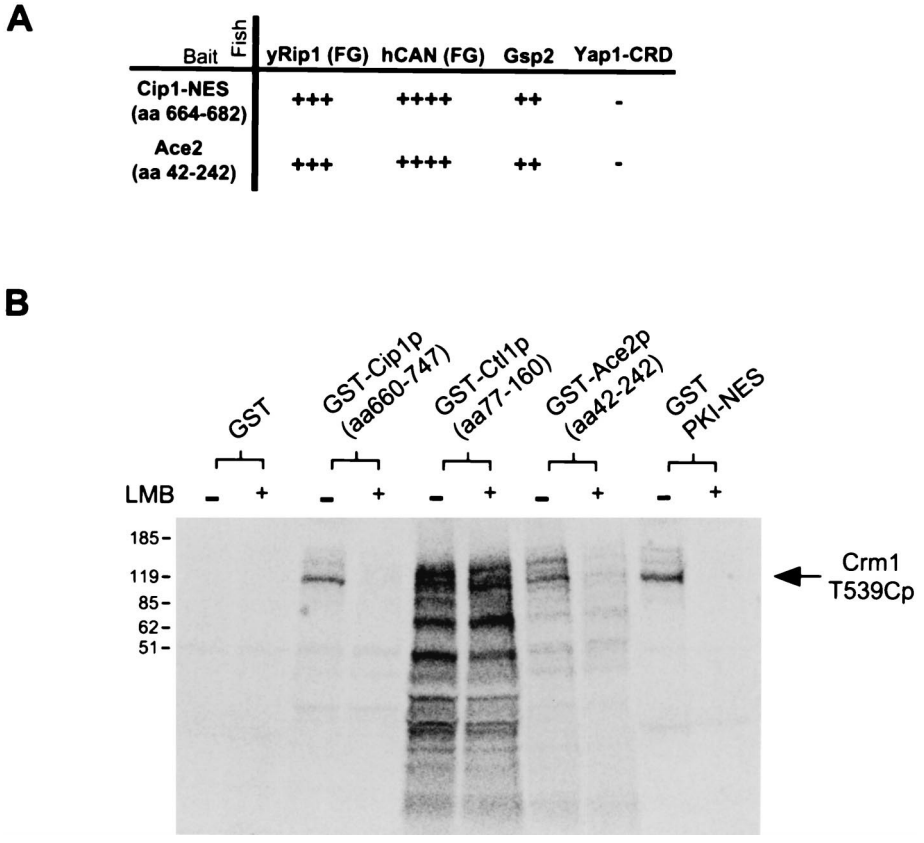

FIG. 4. In vivo and in vitro binding analysis of Cip1p, Cip2p, and Cip3p fragments. (A) A 19-aa region (aa 664 to 682) of Cip1p, as well as the aa 42 to 242 Ace2p region interacts with the FG repeats of nucleoporins and the *S. cerevisiae* Ran homologue Gsp2p. Pairwise two-hybrid interactions were tested between the Cip1p-NES and the Ace2p (aa 42 to 242) baits and the Rip1p (aa 148 to 275), hCAN (aa 1691 to 1894), and Gsp2p and Yap1p-CRD (aa 532 to 650) fish constructs. The Yap1p-CRD sequence contains the Yap1p-NES and serves as a negative control. The number of plus symbols corresponds to blue color intensity on X-Gal indicator plates. (B) The Cip1p fragment (aa 660 to 747) and the Ace2p fragment (aa 42 to 242) interact in vitro with Crm1T539Cp in an LMB-sensitive manner. In vitro translated, ^{35}S-labeled Crm1T539Cp protein was incubated at 4°C for 2 h with glutathione-Sepharose beads prebound with GST protein fused to fragments of Cip1p, Cip2p-Ctl1p, and Cip3p-Ace2p as indicated. GST and GST-PKI-NES proteins were included as negative and positive controls, respectively. ^{35}S-labeled protein retained on the beads after binding and washing was analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Binding reactions were carried out in the absence or presence of 0.5 μM LMB as indicated by the + and − signs above the autoradiogram. The migration of the Crm1T539Cp in vitro translation product is indicated by an arrow. The migration of protein size markers is indicated.

of interest to compare the localization of the two proteins in response to Crm1p inactivation by LMB. A GFP-Swi5p fusion protein showed a steady-state localization similar to that of GFP-Ace2p, with predominant cytoplasmic localization (Fig. 7A). In about 10% of the cells GFP-Swi5p was nuclear (data not shown). Cells with nuclear GFP-Swi5p were all large and unbudded, indicative of early G_1 stage of the cell cycle (data not shown). This is consistent with previous results showing that Swi5p enters the nucleus at the end of mitosis (36). In contrast to Ace2p, Swi5p localization did not change upon LMB treatment. Thus, the similarity between Ace2p and Swi5p does not include an obvious NES activity.

Ace2p localization has been suggested to be similar to that of Swi5p (5). However, apart from activating G_1-specific genes, Ace2p is also involved in maintaining basal-level expression of *CUP1*, a gene that is not regulated in a cell cycle-dependent manner (49). This suggests that Ace2p might perform a constitutive nuclear function independent of the cell cycle. To test whether Ace2p can enter the nucleus at all stages of the cell cycle, we first induced nuclear accumulation of GFP-Ace2p by LMB treatment and subsequently determined the cell cycle stage by analyzing the cell morphology. At 40 min after LMB addition, nuclear localization of GFP-Ace2p was observed in all stages of the cell cycle, i.e., cells with small as well as larger buds (Fig. 7B). Taken together with the rapidity of GFP-Ace2p nuclear accumulation upon LMB treatment (50% of the cells show nuclear GFP-Ace2p signal after 15 min of incubation with LMB [data not shown]), these observations show that Ace2p is able to enter the nucleus independent of the mitotic cycle.

Inactivation of Crm1p in S phase-arrested cells induces nuclear accumulation of Ace2p. To further investigate the relationship of Ace2p localization to the cell cycle, we arrested the Crm1T539C strain with hydroxyurea in the S phase of the cell cycle and analyzed the localization of GFP-Ace2p in response to inactivation of Crm1p by LMB. In LMB-treated S-phase cells, GFP-Ace2p accumulated in the nucleus with kinetics comparable to those observed for asynchronous cells (Fig. 8A). To rule out that the relocalization of GFP-Ace2p was due to protein overexpression, we also analyzed *CUP1* gene expression in the Crm1T539C strain not harboring the GFP-Ace2p construct. When the cells were arrested in S phase, the addition of LMB resulted in a robust increase in *CUP1* expression (Fig. 8B). This is consistent with the idea that Ace2p can enter the nucleus at a non-G_1 stage of the cell cycle. In contrast, in cells arrested in early G_1 by alpha-mating factor, LMB had much less of an effect on CUP1 mRNA levels. This is presumably because a large fraction of Ace2p is already

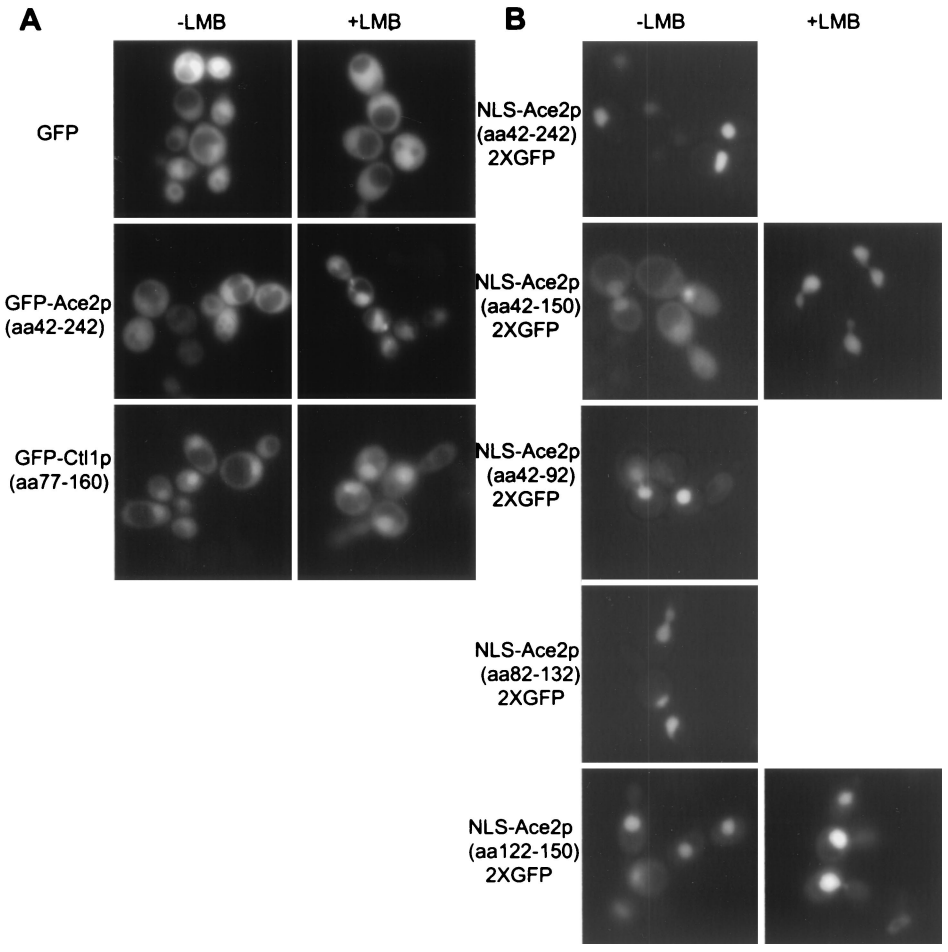

FIG. 5. An aa 122 to 150 fragment of Ace2p contains nuclear export activity. (A) Ctl1p (aa 77 to 160) and Ace2p (aa 42 to 242) fragments were fused to GFP, and the localization of the fusion proteins in the Crm1T539C strain was analyzed in the absence of LMB (−LMB) or 60 min after the addition of 100 ng of LMB per ml (+LMB). (B) Subfragments of the Ace2p (aa 42 to 242) region were inserted in between the SV40 large T-antigen NLS and two GFP moieties, and the localization of the fusion proteins was analyzed in the Crm1T539C strain (−LMB). The localization of fusion proteins that yielded detectable cytoplasmic GFP signal were further analyzed 60 min after the addition of 100 ng of LMB per ml (+LMB).

nuclear, and thus LMB has little additional effect on Ace2p-mediated *CUP1* expression. Taken together with our previous results on GFP-Ace2p localization, the data show that a pool of Ace2p is nuclear during non-G_1 stages of the cell cycle. Furthermore, it is highly suggestive that Ace2p continuously shuttles between the cytoplasm and nucleus throughout the cell cycle.

DISCUSSION

The identification of *S. cerevisiae* proteins dependent on Crm1p for their nuclear export is less advanced than in other organisms. However, the development of an LMB-sensitive *S. cerevisiae* strain has aided our attempts to identify potential NES-containing yeast proteins. This strain has two major benefits: (i) in the absence of LMB, the strain has no apparent phenotype, and (ii) LMB inhibition of Crm1p-mediated export is rapid. By using Crm1p as a bait in a two-hybrid screen and subsequently screening the positive clones using a GFP-LMB assay, we have successfully identified three *S. cerevisiae* proteins not previously reported to have nuclear export activity.

In addition to proteins with export activity, the Crm1p two-hybrid screen also identified the two nucleoporins, Rip1p and Nup159p. Because Crm1p has previously been shown to interact with a number of FG repeat containing nucleoporins in the yeast two-hybrid assay (38), it is interesting that the screen so specifically isolated Rip1p and Nup159p. A previous less-exhaustive two-hybrid screen using the NES-containing protein Rev as a bait only isolated one FG repeat-containing protein, Rip1p (55). Since the Rev-Rip1p two-hybrid interaction has been suggested to be indirect and mediated by Crm1p (38), the simplest interpretation is that Crm1p has the strongest affinity for the type of FG repeats found in Rip1p and Nup159p. The minimal Crm1p-interacting domains of Rip1p and Nup159p, defined by our two-hybrid screen, show a clear specificity for the FG repeat domains. The FG repeat domains found in Nup159p and Rip1p are closely related to each other and are unique compared to other FG repeat-containing proteins in *S. cerevisiae*. Interestingly, the FG repeats found in these yeast nucleoporins are most closely related to the mammalian nucleoporin CAN (NUP214), with which CRM1 was initially copurified (11). In fact, Nup159p is proposed to function as the yeast homologue of CAN (23). It is therefore possible that these nucleoporins play the same role in Crm1p-mediated export in different species. A recent mammalian two-hybrid screen with the human mRNA export factor TAP (Mex67p in yeast) identified two FG repeat-containing nucleoporins, CAN and hCG1 (the human homologue of yeast Rip1p [23]), sug-

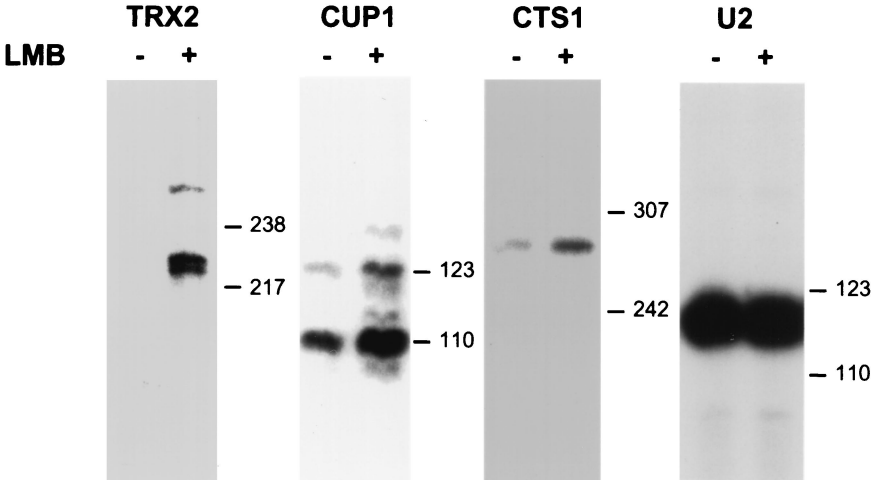

FIG. 6. LMB activates Ace2p-specific target genes. We performed primer extension analysis of RNA purified from the LMB-sensitive Crm1T539C strain in the absence of LMB (−) or 30 min after the addition of 100 ng of LMB per ml (+). RNA was isolated from log-phase cells, and 10 μg of total RNA was used for primer extension analysis. These reactions were carried out individually with primers specific for TRX2, CUP1, CTS1, and U2 RNAs as indicated above the autoradiograms. The migration of radioactive size marker fragments is indicated. When the U2 snRNA-specific primer was included as an internal control in a given reaction, similar results were obtained.

gesting that these proteins also contribute to the TAP-mediated mRNA export pathway. Inactivation of Nup159p or Rip1p in *S. cerevisiae* blocks the export of bulk poly(A)$^+$ RNA. Thus the two nucleoporins seem to be important for mRNA export, as well as the Crm1p-NES-mediated export pathway, in mammals and in yeast. It has been proposed that Crm1p is not a major mRNA export receptor in yeast (39). It is possible, however, that different export pathways converge on common NPC components such as Nup159p-CAN and Rip1p-hCG1.

Novel exported proteins in *S. cerevisiae*. Our identification and characterization of the Cip1p-NES has revealed a sequence that closely resembles many of those identified in higher eukaryotes (Table 2). This shows that at least some yeast sequences (the NES of Yap1p included) resemble hydrophobic NESs of higher organisms. *CIP1* is a previously unidentified yeast ORF, YDR499W, predicting a protein of 747 aa. The gene is essential in yeast (data not shown), and searches of the expressed sequence tag (EST) databases from various or-

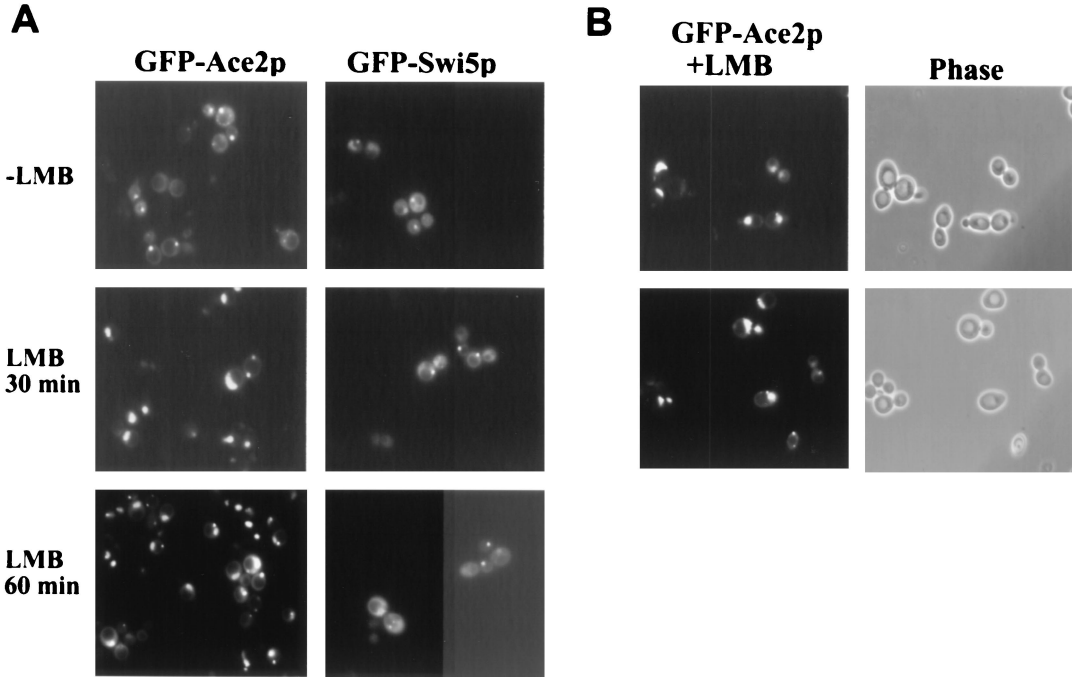

FIG. 7. Ace2p can enter the nucleus at all stages of the cell cycle. (A) Fluorescent microscopy analysis of GFP-Ace2p and GFP-Swi5p localization in the Crm1T539C strain in the absence of LMB (−) or 30 and 60 min after the addition of 100 ng of LMB per ml, respectively. GFP-Ace2p rapidly accumulates in the nucleus upon LMB treatment, whereas GFP-Swi5p does not. Note that the GFP-Swi5p fusion protein, like GFP-Ace2p, aggregates in bright fluorescent spots, the significance of which is unknown. (B) Analysis of morphology of cells containing nuclear GFP-Ace2p. The Crm1T539C strain expressing GFP-Ace2p was treated with 100 ng of LMB per ml for 40 min, and the cell morphology was analyzed in phase contrast. The two pictures represent two different fields.

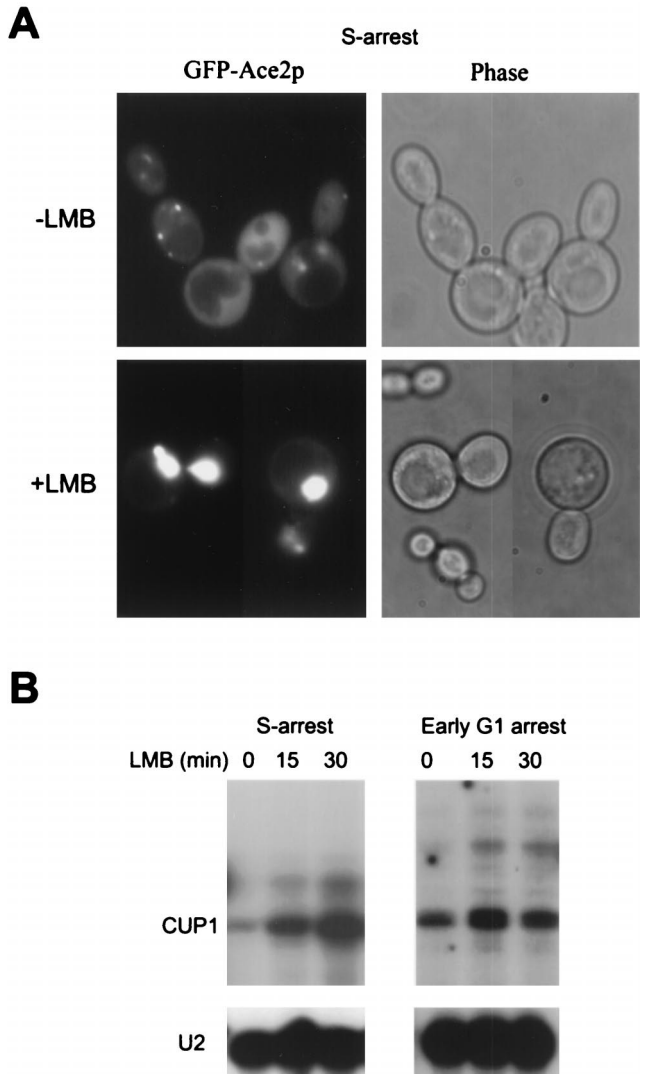

FIG. 8. GFP-Ace2p nuclear accumulation and *CUP1* gene expression can be induced in S phase-arrested cells. (A) Crm1T539C cells expressing GFP-Ace2p were arrested in S phase by a 3.5-h incubation in medium containing 100 mM hydroxyurea. After proper arrest, cells were left untreated or treated with 100 ng of LMB per ml for 60 min, and the localization of GFP-Ace2p was examined by fluorescent microscopy. (B) Crm1T539C cells were arrested in S phase as described in panel A or in early G_1 phase by a 3.5-h incubation in medium containing 2.5 μg of alpha-mating factor. After proper arrest, cells were left untreated or treated with 100 ng of LMB per ml for 15 or 30 min, respectively. Total RNA was isolated, and 5 μg was subjected to primer extension analysis with primers specific for CUP1 and U2 RNAs. Reactions were carried out individually with either CUP1 or U2 snRNA specific primers. When the two primers were included in the same primer extension reaction, similar results were obtained.

ganisms identified a single human EST (Hs d172-f) with high homology to Cip1p. Throughout the aa 629 to 683 region of Cip1p, the EST and the Cip1p protein share 95% identity. As this region spans the NES defined in Cip1p, it appears the NES is conserved in the human protein.

In contrast, site-directed mutagenesis of hydrophobic residues in the Crm1p interaction regions of Ctl1p and Ace2p, yielded no mutants that abolished NES activity. The presence of NLS activity in the particular regions of both proteins could explain our limited success in defining NES residues by this strategy. It is possible that mutations that abolish NES activity also perturb NLS activity and thus the protein's ability to shuttle. A more comprehensive analysis of the respective transport signals is required to clarify this issue. In any case, the likelihood that both proteins contain bona fide export signals is high. The rapid nuclear accumulation in response to LMB addition strongly suggests that the export block is direct. Furthermore, we have been able to define a small 29-aa subregion of Ace2p that contains NES activity when fused to an NLS-2XGFP reporter protein. This sequence in Ace2p is rich in hydrophobic residues (Fig. 3D), and further mutational analysis should help clarify whether it falls into the group of leucine-rich NESs. Whereas Cip1p and Ace2p are likely direct targets, Ctl1p might need bridging proteins to interact with Crm1p.

CTL1 is related to *CET1*, the yeast mRNA capping 5′-triphosphatase. Cet1p removes the γ-phosphate from the 5′ end of primary transcripts, the first step in the mRNA capping process. Cet1p is not related to mRNA 5′-triphosphatases found in higher eukaryotes but has been proposed to be related by sequence and metal dependency to various viral mRNA triphosphatases (4, 6, 19, 20). Although these enzymes have thus far been shown to act only in the capping of mRNA substrates, studies of *CTL1* have shown that it has no genetic or physical connection with mRNA capping (45). Whereas Ctl1p localizes throughout the nucleus and the cytoplasm (45), Cet1p is exclusively nuclear (data not shown and references 21 and 58). Ctl1p has metal-dependent RNA 5′-triphosphatase activity in vitro, but its in vivo substrate(s) has yet to be identified. Without more information, the significance of the putative Ctl1p NES remains elusive.

What is the function of the Ace2p nuclear export activity? Our evidence for Ace2p nuclear export activity includes the rapid LMB-induced nuclear accumulation, as well as the interaction of Ace2p (aa 42 to 242) with yeast Ran, FG repeat-containing nucleoporins, and Crm1p. Furthermore, treatment of the Crm1pT539C strain with LMB resulted in the activation of the Ace2p-specific transcripts CUP1 and CTS1, a finding consistent with an inhibition of Ace2p export.

Given the close similarity of Ace2p to Swi5p, it was surprising to identify an export activity in Ace2p. It has been convincingly shown that Swi5p, synthesized in the G_2 phase of the cell cycle, is cytoplasmic. Swi5p enters the nucleus in a regulated fashion at mitosis and then is rapidly degraded as cells progress through G_1 (36, 56). For such cell cycle-dependent behavior, a protein might not need an NES; indeed, our analysis of GFP-

TABLE 2. Selected NES sequences[a]

Organism and protein	Sequence
Viral	
Rev HIV-1	**LPPLERLTL**
Rex HTLV-1	**LSAQLYSSLSL**
ICP27 HSV-1	**IDLGLDLDL**
Metazoan	
PKI	**ALKLAGLDI**
p53	**RELNEALEL**
MAPKK	**QKKLEELEL**
Yeast	
Yap1p	**DIDVDGLCS**
Cip1p	**VDSLHDLTI**

[a] Prototypic NES sequences with hydrophobic residues in boldface are shown. Sequences are from Rev (9), Rex (42), ICP27 (46), PKI (60), p53 (52), MAPKK (15), Yap1p (63), and Cip1p (this study). Abbreviations: HIV-1, human immunodeficiency virus type 1; HTLV-1, human T-lymphotropic virus type 1; HSV-1, herpes simplex virus type 1; MAPKK, mitogen-activated protein kinase kinase.

Swi5p localization provided no indication of nuclear export activity.

Preliminary characterization of *ACE2* regulation showed that its cell cycle-dependent transcription and localization regulation resembled those of *SWI5* (5). Specifically, an Ace2p-LacZ fusion protein was cytoplasmic in cells arrested in early M phase and nuclear in cells arrested in early G_1 phase, a result consistent with Swi5p-like regulation. This fits well with the ability of Ace2p to specifically activate transcription of a series of G_1 genes. Swi5p nuclear entry is achieved by site-specific dephosphorylation of three key serine residues in the NLS of the protein (34). Serine phosphorylation restricts Swi5p to the cytoplasm. Two of these serine residues are conserved in the Ace2p sequence and the third is replaced by a threonine, suggesting that nuclear entry of Ace2p is regulated similarly. Indeed a mutant derivative of Ace2p, where the three residues are replaced by alanines, exhibits nuclear localization throughout the cell cycle (40). Our data, however, suggest an additional level of Ace2p regulation. The nuclear export activity in Ace2p could play a role in cell cycle-dependent protein localization: nuclear accumulation would result from specific inhibition of Ace2p export activity. This could be achieved simultaneously with the activation of nuclear import to create rapidly a large nuclear pool of Ace2p in G_1. However, we favor a different explanation, based on our data showing that Ace2p harbors N-terminal NLS activity and the fact that the protein can enter the nucleus at all stages of the cell cycle. Ace2p was first identified as a high-copy suppressor of the copper-sensitive phenotype of a deletion of *ACE1* (activator of *CUP1* expression), which encodes a DNA-binding protein that activates *CUP1* in a copper-inducible fashion (3, 53, 57). It was also shown that Ace2p is involved in basal *CUP1* transcription. We suggest that the Ace2p export activity contributes to this cell cycle-independent gene expression. A constant pool of nuclear Ace2p is probably needed to maintain basal *CUP1* transcription. Therefore, we propose that the N-terminal Ace2p NLS functions constitutively, possibly independent of the cell cycle-regulated nuclear entry in late-M–early-G_1 phase. To avoid premature activation of G_1-specific genes, however, we imagine that the nuclear pool of Ace2p must be low; therefore, constitutive nuclear entry must be countered by nuclear export. Early G_1-specific nuclear accumulation of Ace2p would still occur based on rapid and massive nuclear entry of the protein. Additionally, nuclear export of Ace2p could aid in some escape from Swi5p-like G_1-specific nuclear degradation; it would be undesirable for the cell to completely degrade a protein with a constitutive function. One prediction is that Ace2p nuclear accumulation in early G_1 would be less pronounced or more rapid than that of Swi5p. In this context, it is interesting to note that in an asynchronous culture we found that GFP-Ace2p can be found in the nucleus of cells in a given field to a much smaller extent (~0.5%) than GFP-Swi5p (~10%, data not shown). Future studies should help determine the exact function of Ace2p nuclear export.

ACKNOWLEDGMENTS

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