

## Involvement of Nucleocytoplasmic Shuttling of Yeast Nap1 in Mitotic Progression

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**Nucleosome assembly protein 1 (Nap1) is widely conserved from yeasts to humans and facilitates nucleosome formation in vitro as a histone chaperone. Nap1 is generally localized in the cytoplasm, except that subcellular localization of *Drosophila melanogaster* Nap1 is dynamically regulated between the cytoplasm and nucleus during early development. The cytoplasmic localization of Nap1 is seemingly incompatible with the proposed role of Nap1 in nucleosome formation, which should occur in the nucleus. Here, we have examined the roles of a putative nuclear export signal (NES) sequence in yeast Nap1 (yNap1). yNap1 mutants lacking the NES-like sequence were localized predominantly in the nucleus. Deletion of *NAPI* in cells harboring a single mitotic cyclin gene is known to cause mitotic delay and temperature-sensitive growth. A wild-type *NAPI* complemented these phenotypes while *nap1* mutant genes lacking the NES-like sequence or carboxy-terminal region did not. These and other results suggest that yNap1 is a nucleocytoplasmic shuttling protein and that its shuttling is important for yNap1 function during mitotic progression. This study also provides a possible explanation for Nap1's involvement in nucleosome assembly and/or remodeling in the nucleus.**

Chromatin is one of the hallmarks of eukaryotes. The eukaryotic genome DNA is complexed with chromosomal proteins to form the chromatin structure in the nucleus. Nuclear reactions such as DNA replication, transcription, DNA repair, and recombination take place on the chromatin and are regulated by the disruption and assembly of chromatin, namely chromatin remodeling. Recently, a variety of chromatin remodeling factors has been identified, including histone modification enzymes and ATP-dependent factors (reviewed in references 1, 2, 22, and 42). The histone chaperone family is one of the chromatin remodeling factors that binds to core histones and facilitates assembly and remodeling of the chromatin in an ATP-independent manner. This family includes various kinds of proteins, such as nucleoplasmin (31) and N1/N2 (28) in *Xenopus*, nucleosome assembly protein 1 (designated Nap1 in this paper) and Nap2 (13, 48), template-activating factor I $\alpha$  (TAF-I $\alpha$ ) and TAF-I $\beta$ /SET (41), chromatin assembly factor 1 (CAF-1) (6), dNLP (17), Spt6 (5), DF31 (9), CIA (40), TAF-III/B23 (45), and so on.

Nap1 was first identified from HeLa cell extracts as a protein that stimulates nucleosome formation from DNA and purified core histones (14). Nap1 directly binds to core histones and transfers them onto naked DNA (15, 36, 54). Recent studies suggest that Nap1 is involved in transcriptional regulation through chromatin remodeling (32). For example, Nap1 re-

models the chromatin-like structure of the adenovirus genome DNA and stimulates transcription as well as replication (24). Nap1 acts synergistically with ATP-dependent chromatin remodeling factors such as NURF and ACF to remodel the chromatin structure (18, 19, 23, 43). Furthermore, Nap1 and Nap2 are found to interact with p300, a histone acetyltransferase, and to stimulate transcription from p21 and E2F promoters (20, 49). Considering the roles of Nap1 in nucleosome formation and/or transcription, Nap1 should exist in the nucleus. However, it is widely accepted that Nap1 is localized in the cytoplasm but not in the nucleus (26, 35; unpublished observations). One exceptional observation is that *Drosophila melanogaster* Nap1 is present in nuclei limitedly during some stages of development (16). A possible explanation for this contradiction is that Nap1 is transiently present in nuclei and regulatedly exported from nuclei, although the static subcellular localization of Nap1 is cytoplasmic. Here, we have addressed this issue and further tried to understand the physiological meaning of nuclear localization and nucleocytoplasmic shuttling of Nap1.

In the yeast *Saccharomyces cerevisiae*, deletion of *NAPI* causes no phenotypes or very weak phenotypes, if any (26, 50). In yeasts, there are four cyclin B homologs, Clb1, Clb2, Clb3, and Clb4. Nap1 physically and genetically interacts with Clb2 (25, 26). Deletion of *NAPI* in a Clb2-dependent strain, in which *CLB1*, *CLB3*, and *CLB4* are disrupted, shows a temperature-sensitive growth phenotype and an elongated bud phenotype (25, 26). It is suggested that the elongated bud phenotype is caused by a prolonged mitotic delay because disappearance of Clb2 at the end of mitosis is delayed in the  $\Delta CLB1 \Delta CLB3 \Delta CLB4 \Delta NAPI$  strain (25). Different kinds of factors and genes are known to be involved in the elongated bud phenotype. A *cdc28-IN* mutation shows the elongated bud phenotype (52); a *clb2<sup>ts</sup>* mutant, in which *CLB1*, *CLB3*, and

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*CLB4* are disrupted, shows the elongated bud phenotype at nonpermissive temperatures (10); and overexpression of Swe1 kinase, which inactivates Cdc28 by phosphorylation, also causes the elongated bud phenotype (4, 34). These observations suggest that the compromised activity of Cdc28-Clb kinase causes the elongated bud phenotype and that a target protein(s) of this kinase is responsible for the elongated bud phenotype. Gin4 is a protein kinase that is localized in the bud neck and activated during mitosis by phosphorylation. Mutations in this gene also cause the elongated bud phenotype (3). The mitosis-specific phosphorylation of Gin4 requires Nap1 and Clb2 (3). Gin4 phosphorylation is downstream of Clb2 because an ectopic expression of destruction box-deleted Clb2 causes phosphorylation of Gin4 in interphases (3). It is interesting that Gin4 physically and genetically interacts with Nap1 (3). Thus, Nap1 may play an important role during mitotic progression from Cdc28-Clb regulation to cytokinesis, although an exact *in vivo* function(s) of yeast Nap1 (yNap1) remains unclear. Identification of interactors of Nap1 further emphasizes this point. Nap1 interactors identified by biochemical methods and two-hybrid screening (8) includes Clb2 (26), Nbp1 (50), Gin4 (3), CK2 (33), Hta1, Kcc4, Yrb1, Jip1 (53), Yap6, and Kap95 (21). Note that Yrb1 and Kap95 are closely related to the nuclear import mechanism (30). Recently, Mossammarparast et al. reported that Kap114 is involved in the nuclear import of Nap1 (38).

In this study, we show that yNap1 is a nucleocytoplasmic shuttling protein. A region of yNap1 involved in histone binding was found to be important for nuclear import of yNap1, and nuclear export of yNap1 was dependent on a nuclear export signal (NES)-like sequence. To elucidate the role of the Nap1 nucleocytoplasmic shuttling, we used a *yNAP1*-disrupted strain in complementation assays and showed that nucleocytoplasmic shuttling, but not cytoplasmic localization of yNap1, is important for mitotic progression. Our findings solve the contradiction in Nap1 localization and show the possibility that Nap1 acts as a histone chaperone *in vivo*. In addition, these data provide a cue to its physiological function during mitotic progression.

#### MATERIALS AND METHODS

**Strains and culture conditions.** Except where noted, all yeast strains were grown at 25°C in yeast extract-peptone-dextrose (YPD) medium. For protein expression induced by galactose, single colonies were picked up and first inoculated into synthetic defined (SD) medium containing 2% glucose. Cells were grown at 25°C until the optical density at 600 nm ( $OD_{600}$ ) reached 0.8. Then cells were collected by centrifugation at 800 × g and washed twice with phosphate-buffered saline (PBS). The collected cells were grown in synthetic galactose (SG) medium containing 2% galactose for 6 to 8 h, allowing the induced expression of proteins.

**Vector constructions.** To construct expression vectors of yNap1 derivatives in yeast cells, promoter and terminator fragments of the *yNAP1* gene were amplified by PCR with primers 5'-GTGGCGGCCCGGGCACTCCCTTCTTTCT TCC-3' and 5'-GGAGGATCCACTAGTTCTAGAGATCTTGGCCTTTGCTC TTGGTCC-3' for the promoter and 5'-ATCTCTAGAACTAGTGGATCCTC CCAACGCACCTTCGCAAGAGTG-3' and 5'-CCCCTCGAGTAACTCGTCC AACTTGAAGGCC-3' for the terminator, with the yeast genomic DNA as a template. Overlap extension was performed by PCR with primers 5'-GTGGCG GCCCGGGCACTCCCTTCTTTCTCC-3' and 5'-CCCCTCGAGTAACT CGTCCAACCTTGAAGGCC-3', with promoter and terminator PCR products as templates. PCR products were digested with *NotI* and *XhoI* and cloned into *NotI*- and *XhoI*-digested pRS316 plasmid, generating pRS316-yNap1pt, which contains a unique *BamHI* site between the promoter and the terminator

fragments. pBluescript-Flag was constructed from *BamHI*- and *XhoI*-digested pBluescript SK(+) (Stratagene) and a DNA fragment made by annealing two synthetic oligonucleotides, 5'-GATCCGCCGCCACCATGGACTACAAGGAT GACGACGACAAGCATATGC-3' and 5'-TGCAGCATATGCTTGTCTGCTCG TCATCCTTGTAGTCCATGGTGGCGCG-3'. *yNAP1* cDNA fragments were amplified by PCR with primers 5'-AGCGCAAGCATATGTCAGACCCCTATC AGAACGAAAC-3' and 5'-GCGGGATCCTTATGACTGCTTGCATTCAGG AG-3'. The amplified DNA fragment was phosphorylated with T4 polynucleotide kinase (Toyobo), blunted with Klenow fragment, and digested with *NdeI*. Then the fragment was cloned into *NdeI*- and *EcoRV*-digested pBluescript-Flag (pBS-Flag-yNap1). For yNap1ΔNES, DNA fragments corresponding to the N- and C-terminal segments were prepared by PCR with primers 5'-AGCGCAAG CATATGTCAGACCCCTATCAGAACGAAAC-3' and 5'-CAACTTCGAATA GCTCGCTTTGACCCACACATACCCGCTGT-3' and primers 5'-CAAAGC GAGCTATTTCGAAGTTG-3' and 5'-GCGGGATCCTTATGACTGCTTGCATTCAGGAG-3', respectively, with the yNap1 cDNA as a template. The two segments were ligated by overlap extension PCR with primers 5'-AGCGCAAG CATATGTCAGACCCCTATCAGAACGAAAC-3' and 5'-GCGGGATCCTTATGACTGCTTGCATTCAGGAG-3'. Amplified DNA fragments were phosphorylated with T4 polynucleotide kinase, blunted with Klenow fragment, and digested with *NdeI*. The fragment was cloned into *NdeI*- and *EcoRV*-digested pBS-Flag and designated pBS-Flag-yNap1ΔNES. For yNap1 (L99A L102A), DNA fragments corresponding to the N- and C-terminal segments were prepared by PCR with primers 5'-AGCGCAAGCATATGTCAGACCCCTATCA GAACGAAAC-3' and 5'-CGCTTTGTGCGTCTTAGCGTCCAGCAGC-3' and primers 5'-GCTGAGCGCTAAGACTGCACAAAGCGAGC-3' and 5'-GCGGGATCCTTATGACTGCTTGCATTCAGGAG-3', respectively, with the yNap1 cDNA as a template. These two segments were ligated by overlap extension PCR with the primers 5'-AGCGCAAGCATATGTCAGACCCCTATCAGA ACGAAAC-3' and 5'-GCGGGATCCTTATGACTGCTTGCATTCAGGAG-3'. Amplified DNA fragments were phosphorylated with T4 polynucleotide kinase, blunted with Klenow fragment, and digested with *NdeI*. The fragment was cloned into *NdeI*- and *EcoRV*-digested pBS-Flag and designated pBS-Flag-yNap1 (L99A L102A). Flag-yNap1 wild-type (WT) and mutant fragments, except for ΔC1 mutants, were excised from pBS-Flag-yNap1 derivatives by digestion with *BamHI* and cloned into the *BamHI* site of pRS316-yNap1pt or pYES2. Flag-yNap1ΔC1 and yNap1ΔNESΔC1 were excised from pBS-Flag-yNap1 or pBS-Flag-yNap1ΔNES by digestion with *BamHI* and *XhoI* and ligated with the plasmid pYES2, which was digested with *BamHI* and *XhoI*. To construct yNap1 expression vectors for HeLa cells, Flag-yNap1 fragments excised from pBS-Flag-yNap1 plasmids with *BamHI* were cloned into *BamHI*-digested pcDNA3 (Invitrogen). To construct Flag-yNap1ΔC1 and Flag-yNap1ΔNESΔC1 expression vectors, plasmids pBS-Flag-yNap1 and pBS-Flag-yNap1ΔNES were digested with *BamHI* and *XhoI*, blunted with Klenow, and cloned into the *EcoRV* site of pcDNA3. To construct enhance green fluorescent protein (EGFP)-Nap1 expression vectors, Nap1 coding sequences were cut out with *NdeI* and *BamHI* from the pBS-Flag-yNap1 plasmids and cloned into the *BglII* site of pEGFP-C1 (Clontech) by blunt-end ligation. To construct the glutathione S-transferase (GST)-fused recombinant yNap1 protein expression vector, yNap1 cDNA fragments digested with *NdeI* and *BamHI* and then blunted with Klenow fragment were cloned into pGEX-5X-3 (Amersham Biosciences), which had been digested with *Sall* and filled with Klenow fragment. To construct pYES2-Clb2-EGFP, two DNA fragments were made by PCR as follows. The Clb2 fragment was obtained by PCR with oligonucleotides 5'-CGTAGCGAATTCGCCACCATGTCCA ACCAATAGAAAACACA-3' and 5'-TGGTGGCGACCGGTGGATCTTCA TGCAAGTTCATTATATCATAGCC-3' as primers and with CLB2 cDNA as a template. The EGFP fragment was obtained by PCR with oligonucleotides 5'-GATCCACCGTCCGCCACCATGG-3' and 5'-CGGATCCTCGAGTTACT TGTACAGTCTCGTCCATGCC-3' as primers and with pEGFP-N1 (Clontech) as a template. Then overlap extension PCR was performed with oligonucleotides 5'-CGTAGCGAATTCGCCACCATGTCCAACCCAATAGAAAACACA A-3' and 5'-CGGATCCTCGAGTTACTTGTACAGTCTCGTCCATGCC-3' as primers and with the two DNA fragments as templates. The overlap extension products were gel purified, digested with *EcoRI* and *BamHI*, and cloned into *EcoRI*- and *BamHI*-digested pYES2 to generate pYES2-Clb2-EGFP. To construct yCP, a low-copy-number plasmid carrying the *NAP1* gene, the coding region of *NAP1* was cloned between *SmaI* and *SphI* sites in a *TRP1/CEN4*-based yeast plasmid, YCp19, so that the region between the *SmaI* and *SphI* sites in *URA3* was replaced by *NAP1*. To construct yEP, a high-copy-number plasmid carrying the *NAP1* gene, the coding region of *NAP1* was cloned between the *SmaI* and *SphI* sites in a *TRP1, URA3, 2*-based plasmid, YEp24.

**Preparation of recombinant proteins.** To prepare recombinant GST-yNap1, *Escherichia coli* BL21(DE3) cells were transformed with pGEX-5X-3 derivatives

and grown in Lurai-Bertani medium containing 100  $\mu$ g of ampicillin/ml. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added at a concentration of 1 mM when the OD<sub>600</sub> reached 0.3, and cells were further incubated for 3 h. The cells were harvested by centrifugation and washed with PBS. Recombinant proteins were purified by using glutathione Sepharose beads according to the manufacturer's instructions (Amersham Biosciences).

**Examination of morphology and temperature sensitivity.** Single colonies of DK168 and its transformants were grown at 25°C in YPD for 12 h. Cells were plated onto slide glasses, covered with coverslips, and subjected to examination under a microscope (Axioscope; Zeiss). To examine the temperature sensitivity of each strain, single colonies were inoculated into 2 ml of YPD medium and incubated at 25°C for 24 h. Cell concentrations were adjusted to ODs of 0.1, 0.01, and 0.001 with YPD, and 2  $\mu$ l of each dilution was spotted onto SD plates lacking His, Trp, Leu, and Ura and incubated at either 25 or 37°C for 1 or 2 days. To examine the growth rate of yNap1-overexpressing strains, a single colony of each strain was streaked onto SD and SG plates lacking His, Trp, Leu, and Ura and incubated at 25°C for 2 or 3 days.

**Construction of NAPI-deleted strains.** The *NAP1* gene was disrupted by insertion of *LEU2*. To this end, a *LEU2* gene sandwiched with upstream and downstream flanking regions of a *nap1* gene was amplified by PCR from the genomic DNA of DK168, whose *NAP1* is disrupted by *LEU2*. The  $\Delta nap1::LEU2$  DNA was transformed into PSY1785 and PSY1784 strains carrying WT KAP114 and disrupted KAP114 alleles, respectively (37). The disruption of the *NAP1* gene was confirmed by PCR.

**Western blotting analysis of yeast cell lysates.** Exponentially growing yeast cells were harvested by centrifugation and washed twice with PBS. Cells were suspended in a cell lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 145 mM  $\beta$ -mercaptoethanol, and 0.25% sodium dodecyl sulfate (SDS). Glass beads were added, and cells were lysed by vigorously shaking and boiling at 98°C for 5 min. The lysates were cleared by centrifugation at 15,000  $\times$  g for 5 min, and their protein concentrations were determined by using the protein assay kit (Bio-Rad). Aliquots (equivalent to 10  $\mu$ g of protein) were subjected to SDS-7.5% polyacrylamide gel electrophoresis (PAGE) followed by Western blotting with the antibodies indicated in each figure legend.

**Immunoprecipitation assay.** Yeast cell extracts were prepared from exponentially growing cells cultured in SG (without Ura) for 6 to 8 h. The cell cultures were harvested by centrifugation and frozen in liquid nitrogen. The cells were sonicated in lysis buffer (50 mM HEPES-KOH [pH 7.6], 50 mM KCl, 0.5 mM EGTA, 1 mM MgCl<sub>2</sub>, 10% glycerol, 2  $\mu$ g of aprotinin/ml, 2  $\mu$ g of leupeptin/ml, 2  $\mu$ g of pepstatin A/ml, 1 mM phenylmethylsulfonyl fluoride) in the presence of glass beads. The cell lysates were obtained from sonicated lysates by centrifugation. The protein concentration was determined by the Bradford method. The soluble yeast cell extracts (200  $\mu$ g of protein) and anti-Flag antibody-conjugated agarose beads (Sigma) were mixed and incubated at 4°C for 30 min. Then the beads were washed four times with the lysis buffer, and bound proteins were separated by SDS-7.5% PAGE followed by immunoblotting with anti-Flag or anti-Clb2 (Santa Cruz) antibodies.

**GST pull-down assays.** To prepare resins bound by GST or GST fusion proteins, glutathione-Sepharose beads (Amersham Bioscience) were mixed with *E. coli* lysates containing GST or GST fusion proteins, washed three times with a binding buffer containing 500 mM NaCl, 50 mM Tris (pH 7.9), 1 mM EDTA, and 1% Triton X-100, and washed three times with a buffer containing 50 mM KCl, 50 mM HEPES-KOH (pH 7.6), 1 mM EGTA, 1 mM MgCl<sub>2</sub>, and 0.01% Tween 20. To prepare [<sup>35</sup>S]methionine-labeled yNap1 proteins, in vitro transcription-translation reactions were performed according to the manufacturer's instructions (Invitrogen). As a template for transcription with T7 RNA polymerase, pYES2-Clb2-EGFP was digested with *Nco*I, which removes the EGFP coding region downstream of Clb2. In vitro-translated Clb2 was incubated with the resins bound by GST or GST-Nap1 at 4°C for 1 h. Beads were washed 3 times with binding buffer and eluted with an SDS sample buffer. Bound materials were separated by SDS-10% PAGE and visualized by Coomassie brilliant blue (CBB) staining and autoradiography.

**Supercoiling assay.** Supercoiling assays were performed essentially as described previously (11). Recombinant yNap1 and its derivatives were prepared as GST fusion proteins.

**Mammalian cell culture and transfection experiments.** HeLa cells were grown in minimal essential medium supplemented with 10% fetal calf serum. For the subcellular localization study, HeLa cells were split and grown on coverslips placed in 6-cm-diameter tissue culture dishes for 16 h before transfection. Cells in each dish were transfected with 10  $\mu$ g of mammalian expression vectors by the standard calcium phosphate method (27). At 6 h posttransfection, the medium was changed and cells were further incubated for 18 h. Cells were washed twice with PBS and fixed for 10 min at room temperature with 4% formaldehyde that

was freshly prepared from paraformaldehyde. Coverslips were washed with 0.5% Triton X-100 in PBS for 10 min and washed three times with PBS. To visualize Flag-tagged yNap1 proteins, fixed cells were serially incubated with 1% nonfat dried milk in PBS for 30 min, with 10  $\mu$ g of anti-Flag M2 antibody (Sigma)/ml in 1% nonfat dry milk in PBS for 1 h, with rhodamine-conjugated anti-mouse immunoglobulin G (Cappel) for 1 h, and with 1  $\mu$ g of 4',6'-diamino-2-phenylindole dihydrochloride (DAPI)/ml in PBS for 10 min. The cells were then observed under a fluorescence microscopy (Axioscope). To measure the fluorescent intensity in the nucleus and cytoplasm, EGFP-Nap1, EGFP-Nap1 $\Delta$ NES, EGFP-Nap1 $\Delta$ C1, and EGFP-Nap1 $\Delta$ NES $\Delta$ C1 were expressed in HeLa cells by conventional calcium phosphate transfection and fixed with 4% paraformaldehyde for 10 min and DNA was stained with DAPI. Transfected cells were analyzed by the laser scanning cell cytometer LSC2 (Olympus).

**Indirect immunostaining of yeast cells.** Indirect immunostaining of yeast cells with anti-Flag antibody was performed as described previously (51). DNA was visualized by DAPI staining.

## RESULTS

**Identification of NES in the yNAP1 sequence.** Nap1 has been identified as a protein that facilitates formation of nucleosomes from DNA and histones in vitro. Therefore, Nap1 should be present in the nucleus where nucleosome formation occurs. However, it is reported that the subcellular localization of Nap1 in mammalian and yeast cells is restricted to the cytoplasm (26, 35). To get insight into a physiological function(s) of Nap1 as a histone chaperone, we initiated this study by carefully reexamining the subcellular localization of Nap1. In a previous study (16), a putative NES-like sequence was pointed out in *Drosophila* Nap1, leading to a hypothesis that Nap1 may be imported into and exported from the nucleus. To study this point further, we looked for NES-like sequences in Nap1 homologs of other species. We found NES-like sequences in Nap1's in humans, nematodes, and yeasts (Fig. 1A). To examine whether the putative NES is functionally active, we constructed expression vectors for Flag-tagged yNap1 $\Delta$ NES mutant protein, which lacks the region between amino acid positions 88 to 102 encompassing the NES-like sequence, and two kinds of amino acid substitution mutant proteins in the NES-like sequence, Flag-yNap1 (L88A V92A) and Flag-yNap1 (L99A L102A) (Fig. 1). We tested the subcellular localization of these yNap1 mutant proteins in mammalian (Fig. 2) and yeast (see Fig. 4) cells. Flag-tagged proteins were visualized by using anti-Flag antibody combined with a fluorescein-conjugated secondary antibody, whereas nuclei were visualized by staining DNA with DAPI. WT Flag-yNap1 is predominantly localized in the cytoplasm (Fig. 2a and e). In contrast, Flag-yNap1 $\Delta$ NES and Flag-yNap1 (L99A L102A) are predominantly localized in the nucleus (Fig. 2b, c, d, f, g, and h). We could not see expression of Flag-yNap1 (L88A V92A) for unknown reasons (data not shown). These results show that the NES-like sequence is functional and responsible for the export of yNap1 to the cytoplasm. Therefore, it is quite possible that the cytoplasmic localization of yNap1 is not static and yNap1 is dynamically shuttled between the nucleus and the cytoplasm.

**Subcellular localization of yNap1 that loses the histone-binding activity.** An interesting question is raised as to how Nap1 is imported into the nucleus. Several lines of the evidence suggest that histones could be involved in the nuclear import of Nap1. First, Nap1 interacts with H2A/H2B dimers in the cytoplasm (7, 16). Second, the N-terminal regions of histones act as nuclear localization signals (NLS) (39). Third,



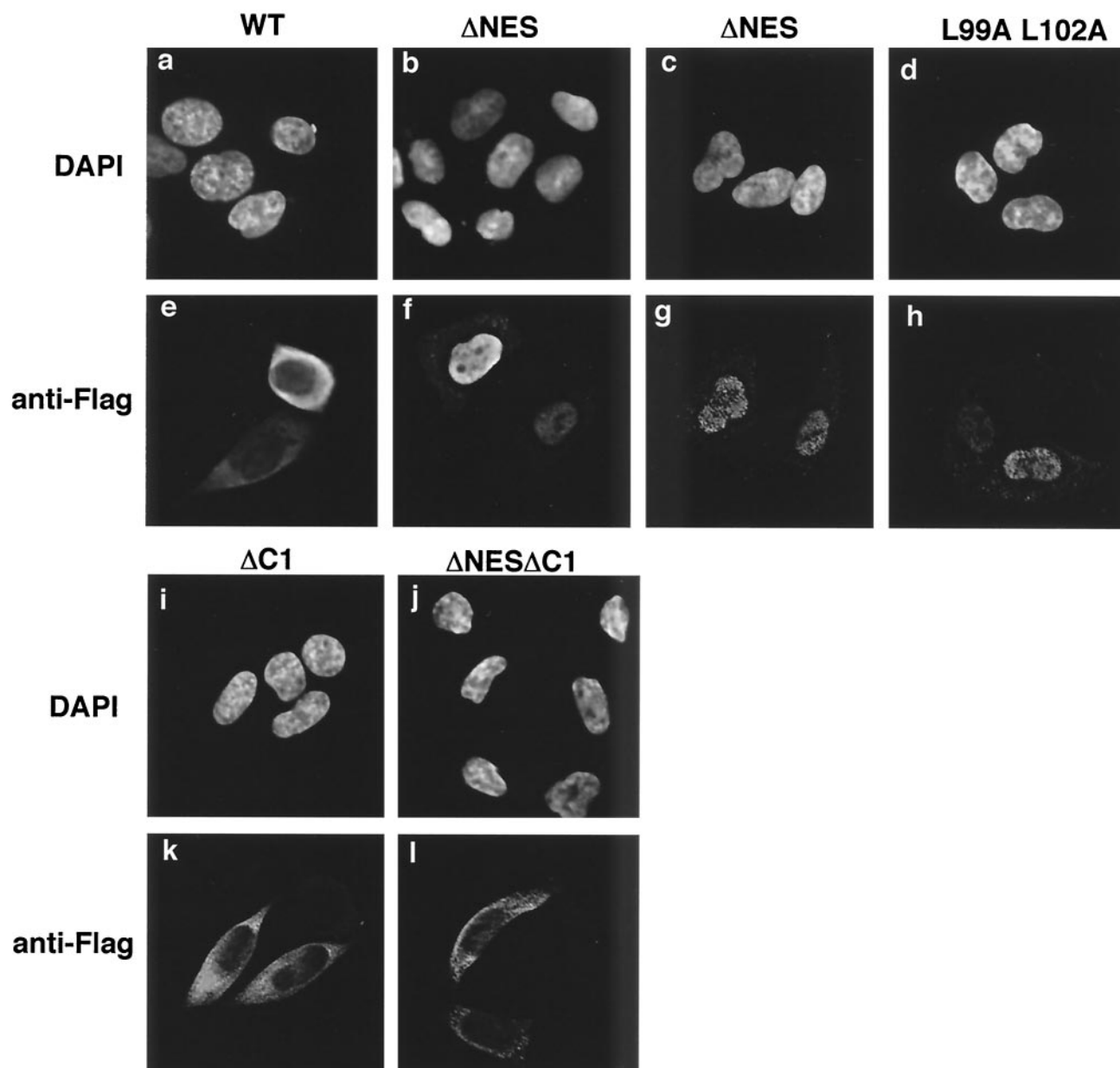


FIG. 2. Subcellular localization of Flag-yNap1 derivatives in mammalian cells. HeLa cells grown on coverslips were transfected with expression plasmids for Flag-yNap1 (a and e), Flag-yNap1 $\Delta$ NES (b, c, f, and g), Flag-yNap1 (L99A L102A) (d and h), Flag-yNap1 $\Delta$ C1 (i and k), or Flag-yNap1 $\Delta$ NES $\Delta$ C1 (j and l). For cells expressing Flag-yNap1 $\Delta$ NES, two independent optical fields are shown. Cells were fixed, stained with DAPI (a to d, i, and j) or anti-Flag antibody (e to h, k, and l), and then examined under a fluorescence microscope and analyzed by a deconvolution system (Zeiss).

Thus, it is concluded that WT yNap1 is dominantly localized in the nucleus and that the C-terminal H2A/H2B binding region of yNap1 is important for the nuclear localization of yNap1. EGFP-yNap1 $\Delta$ NES is a nuclear dominant in 89.2% of total cells expressing EGFP-yNap1 $\Delta$ NES. This confirms that the NES-like sequence is involved in exclusion of yNap1 from the nucleus. Among cells expressing EGFP-yNap1 $\Delta$ NES $\Delta$ C1, we could not see the cell that is dominantly stained in the nucleus (data not shown). Again, the C-terminal region is likely to be essential for the nuclear localization of yNap1.

**Nucleocytoplasmic shuttling of yNap1 is involved in mitotic progression.** We tried to examine the physiological role of

nucleocytoplasmic transport of yNap1 by genetic complementation assays with the DK168 strain ( $\Delta$ NAP1  $\Delta$ CLB1  $\Delta$ CLB3  $\Delta$ CLB4). DK168 shows a temperature-sensitive growth and an elongated bud phenotype below the permissive temperature (25, 26). Before carrying out complementation assays, we first examined the subcellular localization of Flag-yNap1 mutant proteins in yeast DK168 cells (Fig. 4A). Flag-yNap1 is dominantly localized in the cytoplasm, whereas Flag-yNap1 $\Delta$ NES and Flag-yNap1 (L99A L102A) are localized dominantly in the nucleus (Fig. 4A). Therefore, it is confirmed that the mechanism of nuclear import and export of yNap1 in yeast cells are similar to that in mammalian cells.

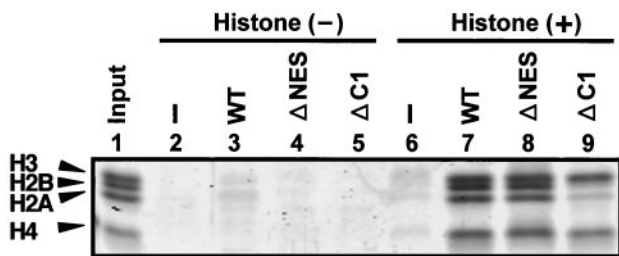


FIG. 3. Interactions between yNap1 derivatives and core histones. GST pull-down assays were performed with purified core histones and GST-tagged recombinant yNap1 proteins. Lysates prepared from *E. coli* cells expressing GST (lanes 2 and 6), WT GST-yNap1 (lanes 3 and 7), GST-yNap1ΔNES (lanes 4 and 8), and GST-yNap1ΔC1 (lanes 5 and 9) were incubated with (lanes 6 to 9) or without (lanes 2 to 5) 2 μg of core histones purified from HeLa cells as described previously (44) and then mixed with glutathione-Sepharose beads. Beads were washed with a buffer containing 150 mM NaCl and 0.5% NP-40 (lanes 2 to 9). Core histone (lane 1, 0.4 μg) and the bound proteins (lanes 2 to 9) eluted by an SDS-PAGE loading buffer were separated by SDS-15% PAGE and visualized by CYPRO Orange staining. The positions of each core histone component are indicated. The amounts of the bound histones were measured by National Institutes of Health imaging.

It is suggested that the elongated bud phenotype of DK168 is caused by the prolonged mitotic delay followed by the inability of Gin4 kinase phosphorylation (3). Next, we performed complementation experiments with various yNap1 derivatives by using the DK168 strain. WT Flag-yNap1 complements the elongated bud phenotype (Fig. 5A, b) and the temperature-sensitive (*ts*) phenotype (Fig. 5B). In sharp contrast, Flag-yNap1ΔNES and Flag-yNap1 (L99A L102A) cannot complement the elongated bud phenotype (Fig. 5A, c and d) and the *ts* phenotype (Fig. 5B). The artificial mutations sometimes affect protein stability so that it is possible that yNap1ΔNES and yNap1 (L99A L102A) could not complement the phenotypes due to its instability and low abundance. Western blot analyses of lysates with anti-Flag antibody have revealed that Flag-yNap1, Flag-yNap1ΔNES, and Flag-yNap1 (L99A L102A) driven by the native promoter are equally expressed (Fig. 4B, lanes 2 to 4). Therefore, cytoplasmic localization and/or nucleocytoplasmic shuttling of yNap1 is required for the putative mitotic function of yNap1. To distinguish these possibilities, we performed further complementation experiments with Flag-yNap1ΔC1 and Flag-yNap1ΔNESΔC1, which are predominantly localized in the cytoplasm (Fig. 2k and l). Flag-yNap1ΔC1 and Flag-yNap1ΔNESΔC1 do not complement the elongated bud phenotype (Fig. 5A, e and f). As these mutant proteins are localized in the cytoplasm and the C-terminal region is important for the nuclear import of Nap1, it is likely that nucleocytoplasmic shuttling, but not cytoplasmic localization, of yNap1 is important for the mitotic progression.

**Overexpressed NES-deficient yNap1 mutants inhibit normal growth in the Clb2-dependent strain.** Nucleocytoplasmic shuttling could be divided into two steps, that is, nuclear import and export. To elucidate the molecular mechanisms of the role of Nap1, it is necessary to clarify whether either one of the two steps or both steps are required for the mitotic function of Nap1. As NES-deficient Nap1's do not complement the mitotic function (Fig. 5), it is likely that the nuclear export of Nap1 is

essential for its function. However, it is possible that the loss of the NES function reduces the level of nuclear import of Nap1, possibly due to the decrease of recycling of Nap1 for repeated nuclear import processes by the loss of its nuclear export. To test this, we performed overexpression experiments of NES-deficient yNap1's. Since a part of yNap1ΔNES is localized in the cytoplasm, it is possible that overexpression of yNap1ΔNES may result in the increase of the cytoplasmic portion of yNap1ΔNES. Thus, the increased amount of NES-deficient yNap1 may overcome the reduced nuclear import. Overexpression of yNap1 and its derivatives was performed by using a galactose-inducible multicopy plasmid, pYES2 (Fig. 6A). The expression level of each galactose-inducible derivative was examined in comparison with that derived from a single-copy plasmid containing the native promoter by Western blot analysis with anti-Flag antibody (Fig. 4B, lanes 5 to 10, and data not shown). It is shown that the expression level in the galactose-inducible derivatives tested here was at least five times or more than that derived from the native promoter. Under these conditions, the growth rate of each strain was observed. Overexpression of yNap1ΔNES and yNap1 (L99A L102A) could not complement the *ts* and elongated bud phenotypes (data not shown). To be more precise, the slight growth inhibition by overexpression of yNap1ΔNES or yNap1 (L99A L102A) was observed even at the permissive temperature (Fig. 6A). yNap1ΔNES appears to inhibit cell growth more potently than yNap1 (L99A L102A). Possibly, yNap1 (L99A L102A), but not yNap1ΔNES, may retain a weak NES function, although they both show similar subcellular localizations (Fig. 2 and 4) and both are unable to complement the WT function (Fig. 5). These results show that the increased nuclear import of NES-deficient Nap1 is still insufficient for mitotic function of Nap1. Furthermore, the nuclear accumulations of NES-deficient Nap1's have a *trans* dominant-negative effect for mitotic progression. Therefore, it is strongly suggested that the nuclear export of Nap1 is essential for the mitotic function of Nap1.

**Genetic interaction between NAP1 and KAP114.** We further examined the effect of the balance between nuclear import and export on the Nap1 function. As Flag-yNap1ΔC1 did not complement the mitotic function of Nap1 and the C-terminal re-

TABLE 1. Quantitative analysis of cells expressing nuclear and cytoplasmic Nap1 proteins<sup>a</sup>

Protein	% of cells in nucleus	% of cells in cytoplasm	Total cell no.
WT yNap1	10.2	89.1	356
yNap1ΔC	0	100	212
yNap1ΔNES	89.2	10	120

<sup>a</sup> EGFP-yNap1 and its derivatives were transiently expressed in HeLa cells. Cells were fixed with 4% paraformaldehyde, stained with DAPI, and observed under a laser scanning cytometer (LSC2; Olympus). The integral intensity value of fluorescence in the nucleus and that in the nuclear periphery area were measured, and the former value was plotted versus the latter value. Cells were sorted into nucleus- or cytoplasm-staining dominant populations by checking each area of each cell on the graph. The number of nucleus-staining dominant cells and that of cytoplasm-staining dominant cells are indicated as percentages of the total scanned cell number. This table does not include the results of the experiment with yNap1ΔNESΔC1, since the scanned cell number was not sufficient to correctly evaluate the result. However, as described in the text, we could not detect nucleus-staining dominant cells among cells expressing yNap1ΔNESΔC1 as far as we searched in the sample.

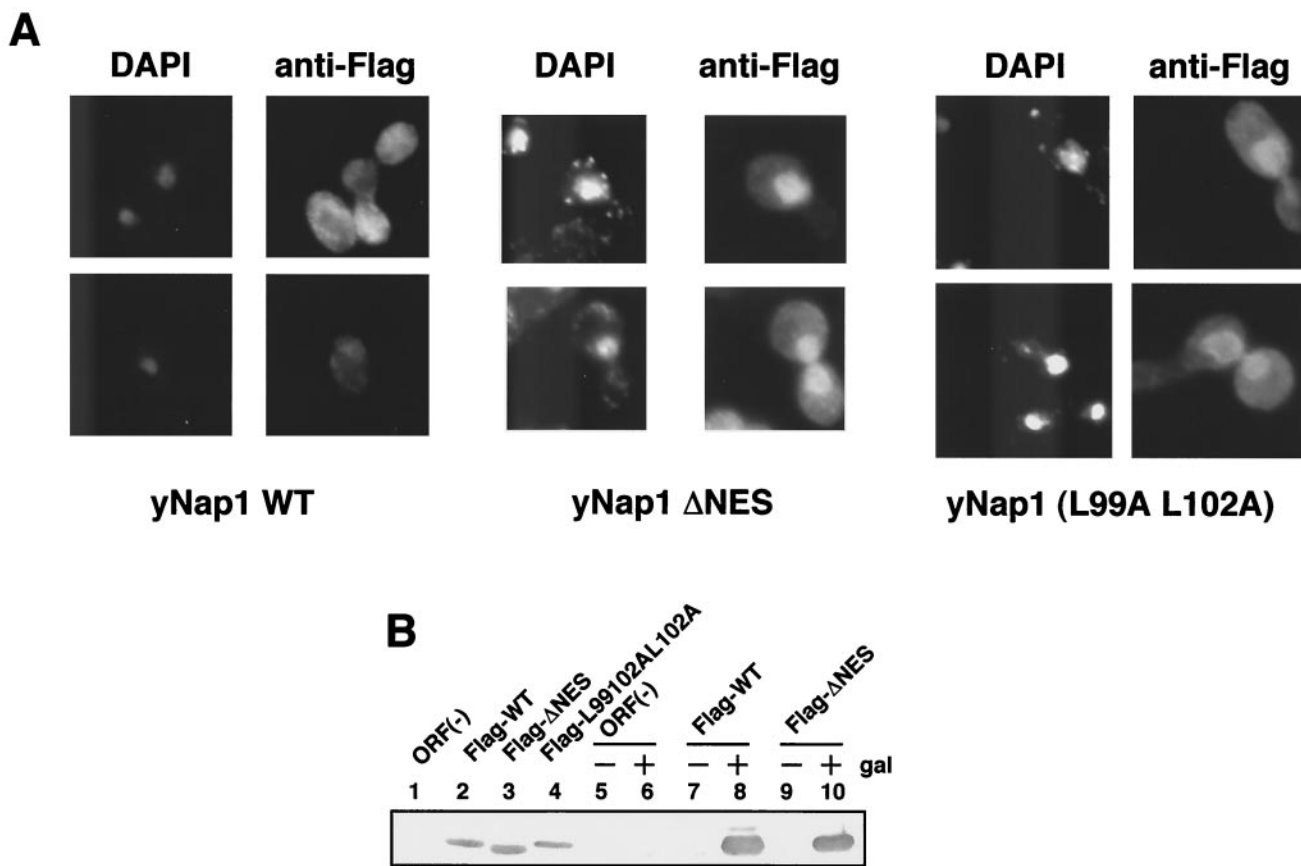


FIG. 4. Subcellular localization of yNap1 derivatives in yeast cells. (A) Exponentially growing cells expressing WT Flag-yNap1, Flag-yNap1 $\Delta$ NES, or Flag-yNap1 (L99A L102A) were fixed, and DNA and Flag-tagged proteins were stained with DAPI and anti-Flag antibody, respectively. (B) Amounts of Flag fusion proteins in transformed cells. DK168 cells were transformed with pRS316 (lane 1), pRS316-Flag-yNap1 (lane 2), pRS316-Flag-yNap1 $\Delta$ NES (lane 3), or pRS316-Flag-yNap1 (L99A L102A) (lane 4). Exponentially growing transformants were lysed, and lysates were subjected to Western blot analysis with anti-Flag antibody. DK168 cells were separately transformed with pYES2 (lanes 5 and 6), pYES2-Flag-yNap1 (lanes 7 and 8), or pYES2-Flag-yNap1 $\Delta$ NES (lanes 9 and 10). Lysates prepared from exponentially growing transformants in either SD (lanes 5, 7, and 9) or SG (lanes 6, 8, and 10) were subjected to Western blot analysis.

gion was shown essential for nuclear import of Nap1, it is possible that not only nuclear export but also nuclear import is important for the Nap1 function. For instance, it is possible that the loss of nuclear import causes reduced nuclear export, since nuclear import is the primary step for nuclear export. To know the effect of nuclear import of Nap1 on the Nap1 function, we examined a genetic interaction between *NAP1* and *KAP114*, which is involved in the nuclear import of Nap1 (38) and other proteins, including TATA-binding protein and histone H2A/2B (37, 46). It has been reported that disruption of either *NAP1* or *KAP114* does not show any cell growth defect (25, 26, 37, 46). The *NAP1* and *KAP114* double-gene-disrupted strain PSY1784 $\Delta$ nap1 shows slower growth than the *KAP114*-positive strain PSY1785 $\Delta$ nap1 (Fig. 6B). The decreased growth rate caused by *NAP1* gene disruption was partially complemented by the *NAP1* gene with both low- and high-copy-number plasmids. These results indicate the genetic interaction between these two genes. Disruption of *KAP114* shows the reduced level of nuclear import of some proteins, such as histone H2A/2B and TATA-binding protein (37, 46), so that the genetic interaction between *NAP1* and *KAP114* suggested

that Nap1 has some functions in nuclear import which is important for the cell growth.

**Interaction of NES-deficient yNap1 proteins with Clb2.** Clb2s play key roles in mitotic progression (10). It has been noted that the physical and genetic interactions between Clb2 and yNap1 have been indicated (25, 26). As *clb2<sup>ts</sup>* mutant shows the elongated bud phenotype at nonpermissive temperatures, it is likely that Nap1 regulates some Clb2 function through a direct interaction. Thus, we examined the Clb2-binding ability of yNap1 mutant proteins by GST pull-down assays. Clb2 was prepared by in vitro transcription-translation reactions in the presence of [<sup>35</sup>S]methionine. GST-fused WT yNap1, but not GST alone, interacts with Clb2 in a dose-dependent manner (Fig. 7A, lanes 2 to 7), which is in good agreement with the previous result (3). yNap1 $\Delta$ NES and yNap1 (L99A L102A) also interact with Clb2 as efficiently as WT yNap1 (Fig. 7, lanes 8 to 13). Therefore, the mutation in the NES sequence does not affect the interaction ability of Nap1 to Clb2.

Next, we examined the in vivo Clb2 interacting abilities of WT yNap1, yNap1 $\Delta$ NES, and yNap1 (L99A L102A) by coimmunoprecipitation assays (Fig. 7C). Flag-tagged WT yNap1,

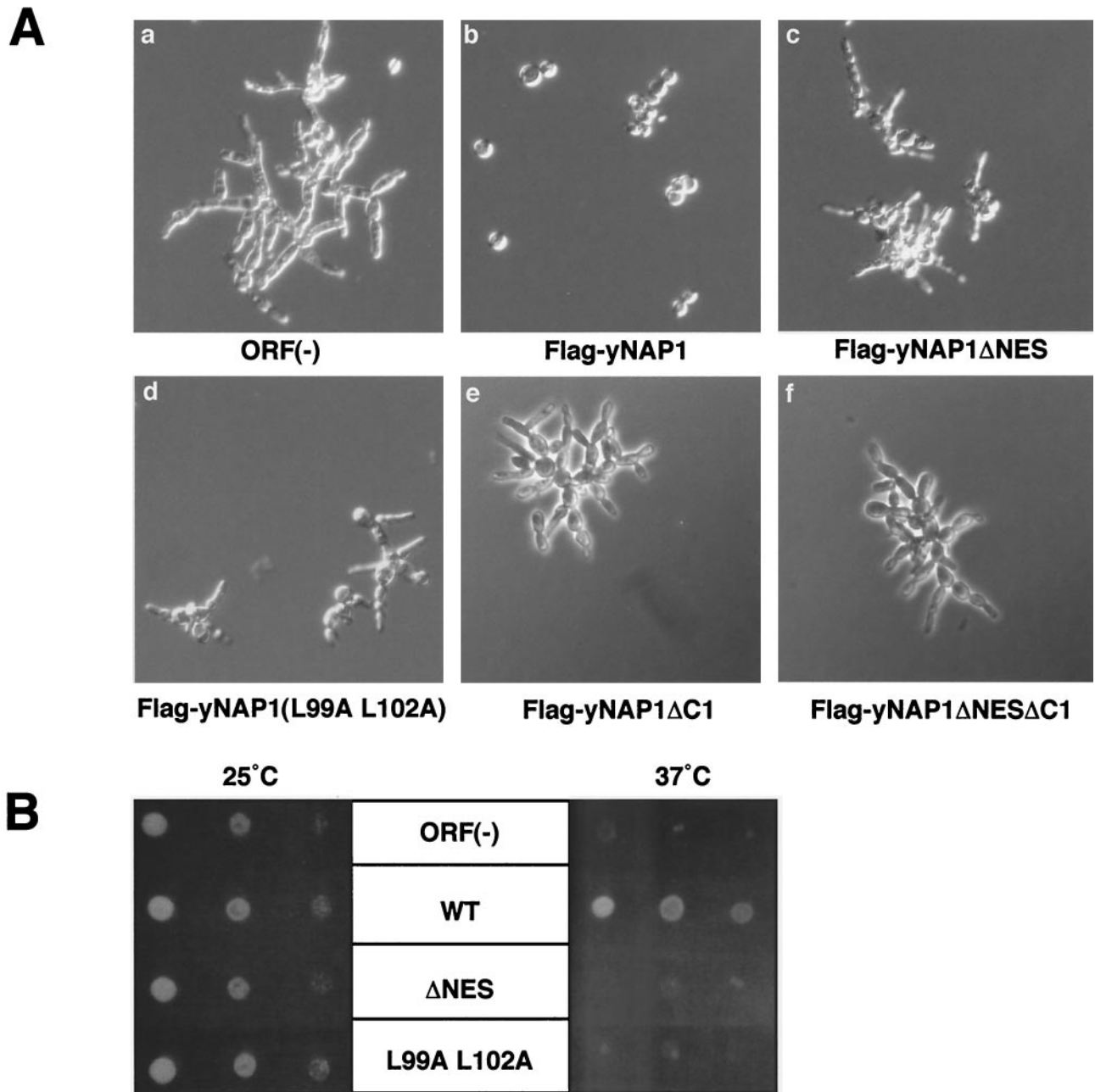


FIG. 5. Complementation of a *NAP1* disruptant with *yNAP1* derivatives. The morphological change (A) and *ts* phenotype (B) of a *NAP1*-disrupted strain, DK168, were examined when DK168 was transformed with an empty vector [ORF(-)] (a) or expression vectors for Flag-*yNap1* WT (WT) (b), Flag-*yNap1*ΔNES (ΔNES) (c), Flag-*yNap1*(L99A L102A) (L99A L102A) (d), Flag-*yNap1*ΔC1 (e), or Flag-*yNap1*ΔNESΔC1 (f). (A) The morphologies of exponentially growing transformants were observed under a phase microscope. (B) The *ts* phenotypes during growth were examined by incubation of serially diluted cell suspension spots at permissive (25°C) and nonpermissive (37°C) temperatures.

*yNap1*ΔNES, and *yNap1* (L99A L102A) proteins were expressed in DK168 strains by using the *Gall* promoter. Flag-tagged WT *yNap1*, *yNap1*ΔNES, and *yNap1* (L99A L102A) proteins were precipitated by anti-Flag antibody (Fig. 7, upper panel). Clb2 was coprecipitated with WT Flag-*yNap1*, Flag-*yNap1*ΔNES and Flag-*yNap1* (L99A L102A) (Fig. 7, lower panel). Therefore, the interaction between Nap1 and Clb2 is not dependent on either the NES-like sequence or the subcellular localization.

**Nucleosome assembly activity of *yNap1* mutant proteins.** A question is raised whether the mitotic function of *yNap1* is correlated with its nucleosome assembly activity. We next examined the nucleosome assembly activity of NES-mutated *yNap1* proteins (Fig. 8B) by using purified recombinant GST-*yNap1* proteins (Fig. 8A). In the supercoiling assay, a protein containing the nucleosome assembly activity facilitates nucleosome formation with core histones and a relaxed circular plasmid DNA in the presence of topoisomerase I. Removal of



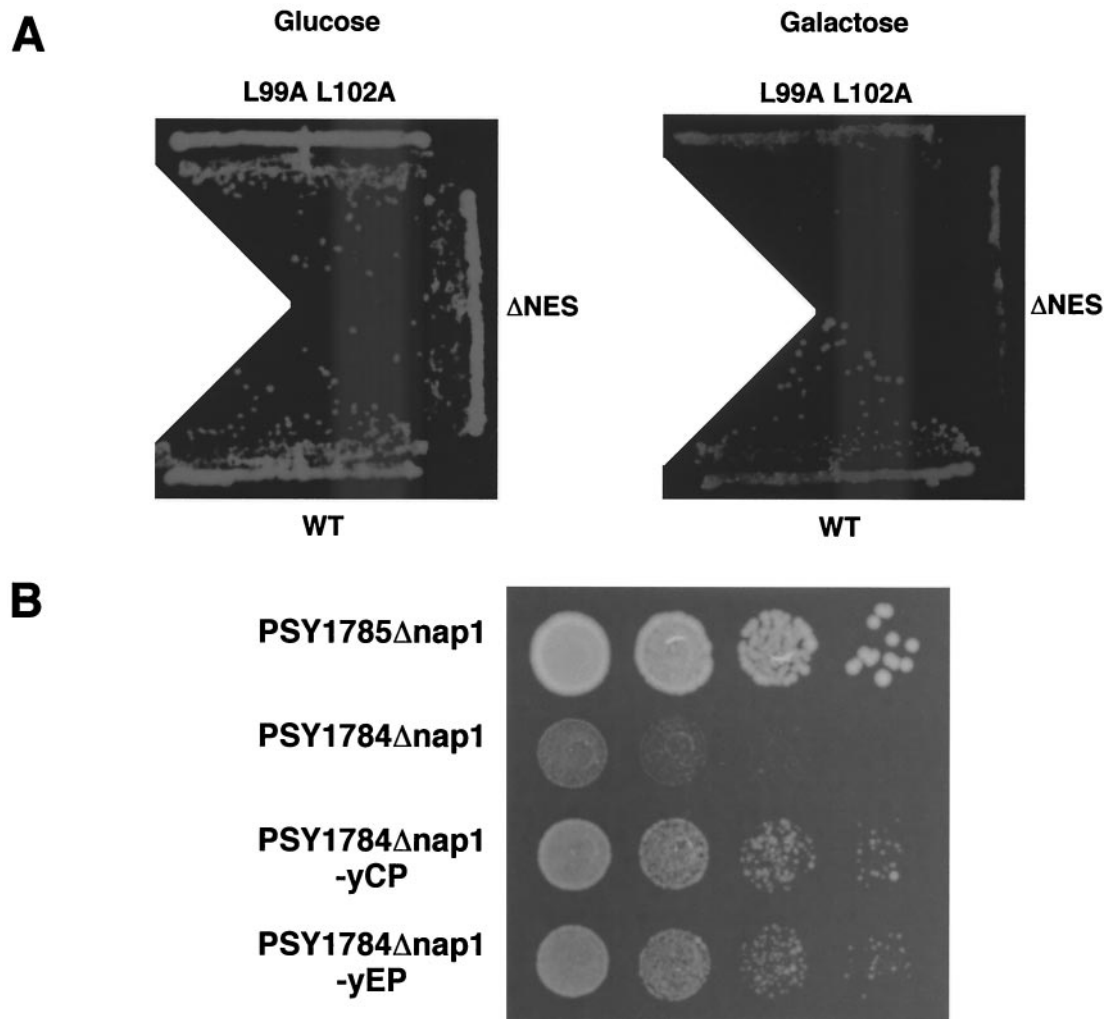


FIG. 6. Genetic analyses of *NAPI*. (A) Effect of overexpression of NES-deficient yNap1 in a Clb2-dependent strain. DK168 cells were transformed with pYES2-Flag-yNap1 (WT), pYES2-Flag-yNap1ΔNES ( $\Delta$ NES), or pYES2-Flag-yNap1 (L99A L102A) (L99A L102A). Single colonies on an SD (without Ura) plate were isolated, and well-grown cultures were streaked onto SD (without Ura) and SG (without Ura) plates and incubated at 25°C for 2 and 3 days, respectively. (B) Genetic interaction between *NAPI* and *KAP114*. Yeasts (PSY1785 $\Delta$ nap1 = *KAP114 nap1::LEU2*; PSY1784 $\Delta$ nap1 = *kap114::HIS3 nap1::LEU2*; PSY1784 $\Delta$ nap1-yCP = *kap114::HIS3 nap1::LEU2* carrying a low copy number of *NAPI*; PSY1784 $\Delta$ nap1-yEP = *kap114::HIS3 nap1::LEU2* carrying a high copy number of *NAPI*) fully grown in liquid media were diluted to OD<sub>600</sub>s of 0.1 with distilled water. Then 1/10 serially diluted cultures were prepared from the 0.1-OD<sub>600</sub> cell suspensions in distilled water, and 5  $\mu$ l of each dilution was grown on YPD agar plates for 2 days at 30°C.

proteins after the reaction gives a supercoiled DNA. A relaxed DNA (Fig. 8B, lane 9) generated by treatment of a supercoiled DNA with *E. coli* topoisomerase I (Fig. 8B, lanes 1 and 2) was mixed with core histones that had been preincubated with yNap1 proteins. The supercoiled DNA was formed by GST-yNap1 in a dose-dependent manner (Fig. 8B, lanes 3 and 4). GST-yNap1 (L99A L102A) has the same level of nucleosome assembly activity as GST-yNap1 (Fig. 8B, lanes 3, 4, 7, and 8). Therefore, the substitution mutations in the NES region of yNap1 do not affect the nucleosome assembly activity in vitro. GST-yNap1 $\Delta$ NES has less, but significant, activity than WT GST-yNap1 (Fig. 8B, lanes 3, 4, 5, and 6). Densitometric scanning revealed that the activity of GST-yNap1 $\Delta$ NES is approximately 50% of that of WT GST-yNap1 (Fig. 8B, lanes 4 and 5). The low nucleosome assembly activity of yNap1 $\Delta$ NES could be due to its low histone-binding ability, as shown in

Fig. 2. We could not find any strict correlation between the mitotic complementation ability and the nucleosome assembly activity.

## DISCUSSION

Our analyses described in this paper started with a question of whether Nap1 is stably localized in the cytoplasm because the nucleosome assembly activity should be a nuclear function. By examining the subcellular localization and biochemical activities of mutant Nap1 proteins, we could propose a novel regulatory role of Nap1 in mitotic progression through its nucleocytoplasmic shuttling. We have shown that yNap1 is localized in the nucleus when the NES-like sequence is deleted or mutated (Fig. 2 and 4A). The histone binding was found to be important for nuclear import of yNap1 (Fig. 2 and 3). Since

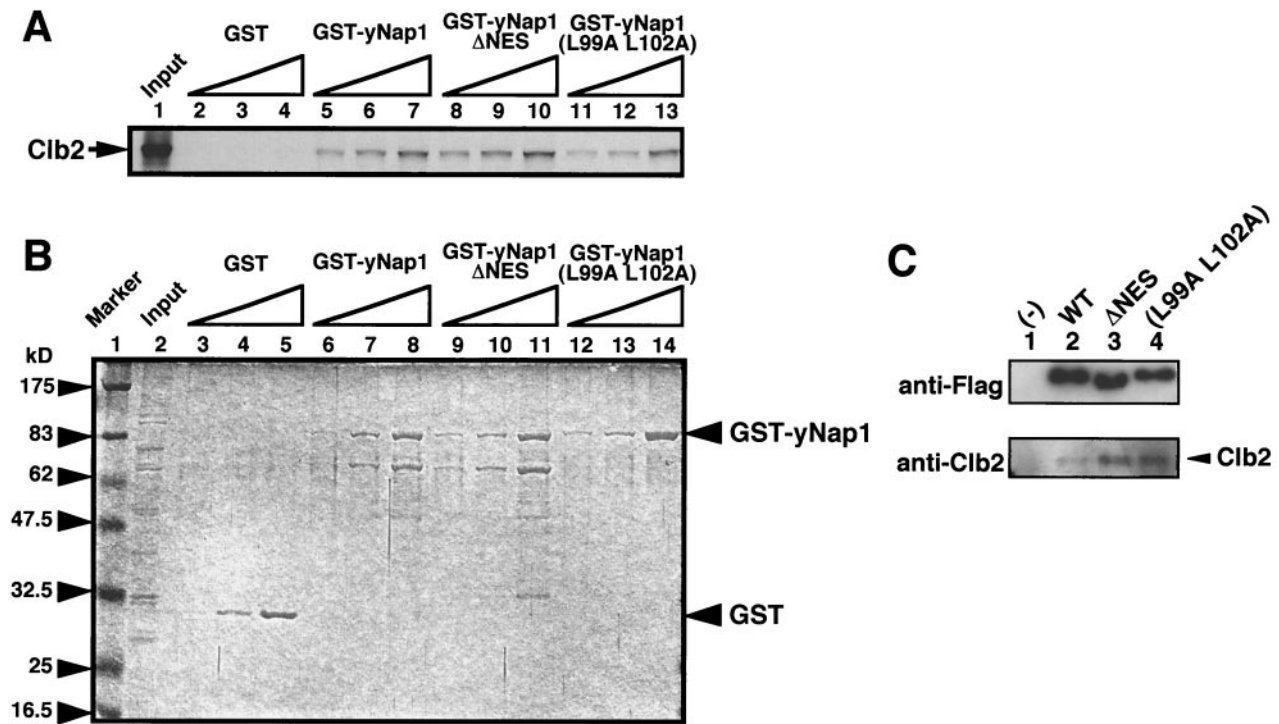


FIG. 7. Interaction between yNap1 and Clb2. (A) Fifty nanograms (lanes 2, 5, and 8), 150 ng (lanes 3, 6, and 9), or 500 ng (lanes 4, 7, and 10) of GST (lanes 2 to 4), GST-yNap1 (lanes 5 to 7), GST-yNap1 $\Delta$ NES (lanes 8 to 10), or GST-yNap1 (L99A L102A) (lanes 11 to 13) was mixed with glutathione beads and incubated with in vitro-translated [ $^{35}$ S]methionine-labeled Clb2 (lane 1, input). Bound proteins were separated by SDS-10% PAGE and visualized by autoradiography. (B) The amount of loaded GST proteins used in panel A is shown by CBB R-250 staining. (C) Yeast cell lysates prepared from DK168::pYES2 (lane 1), DK168::pYES-Flag-yNap1 (lane 2), DK168::pYES-Flag-yNap1 $\Delta$ NES (lane 3), or DK168::pYES-Flag-yNap1 (L99A L102A) (lane 4) were precipitated with anti-Flag antibody-conjugated agarose beads, and bound proteins were separated by SDS-7.5% PAGE and stained with anti-Flag and anti-Clb2 antibodies.

NES-deficient Nap1's had no complementing activity for mitotic function of Nap1 (Fig. 5), it is suggested that nucleocytoplasmic shuttling of Nap1 is required for its function. Overexpression of NES-deficient Nap1's did not complement the mitotic function of Nap1 (Fig. 6). Therefore, it is concluded that nuclear import of Nap1 is insufficient and the nuclear export is essential for Nap1 function.

**Nucleocytoplasmic shuttling of Nap1.** As yNap1 is predominantly localized in the cytoplasm (26) (Fig. 4A) and Gin4, which is one of the Nap1 interactors which plays a critical role in mitosis, is localized in the bud neck, it is reasonably assumed that Nap1 acts as part of the mitotic function in the cytoplasm. However, our results suggest that nucleocytoplasmic shuttling, but not stable cytoplasmic localization, of Nap1 is required for the mitotic function of Nap1. First, C-terminal deletion mutants (Flag-yNap1 $\Delta$ C1 and Flag-yNap1 $\Delta$ NES $\Delta$ C1), which are not effectively imported into the nucleus, thereby staying in the cytoplasm, have no complementing activity (Fig. 5A). Second, overexpression of NES mutant proteins is still insufficient to complement the DK168 phenotypes (Fig. 6A). Ectopically expressed NES-deficient yNap1's are predominantly localized in the nucleus, but a significant level of cytoplasmic staining is also observed (Fig. 4A). Therefore, the cytoplasmic amount of the mutant yNap1 could be increased, as the total amount of mutant yNap1 proteins is increased by its overexpression. These observations strongly suggest that the constitutive cyto-

plasmic existence of Nap1 is not enough and that nucleocytoplasmic shuttling is required for the mitotic function of Nap1.

**Nuclear import of Nap1.** As discussed above, the nuclear export of Nap1 is important for the mitotic function of Nap1. However, we could not rule out the possibility that nuclear import of Nap1 is also critical because nuclear import is the primary step for nuclear export. We addressed this point by using *KAP114* mutants. Kap114 physically interacts with Nap1 and is involved in the nuclear import of TATA-binding protein (37, 46) and histone H2A/2B (38). Kap114 and Nap1 make a complex with H2A/2B (38). The nuclear import of Nap1 is shown to be dependent on its histone-binding domain (Fig. 2). Therefore, it is likely that Nap1 assists the Kap114 function in nuclear import of some proteins as a nuclear import partner. Indeed, a double gene disruption (*kap114* and *nap1*) resulted in a decreased growth rate phenotype (Fig. 6B), although a single gene disruption of either one of these two genes did not give any growth defect. This synthetic phenotype between two genes suggests that Nap1 may be involved in nuclear import of some nuclear proteins.

**Nuclear export of Nap1.** Experimental results strongly suggest that the nuclear export of Nap1 is essential for its mitotic function. Overexpression of NES-deficient Nap1s caused the cell growth inhibition (Fig. 6A). Such growth inhibition was not observed when they were expressed from the authentic y*NAP1* promoter on a single-copy plasmid (Fig. 5B). A yNap1 itself

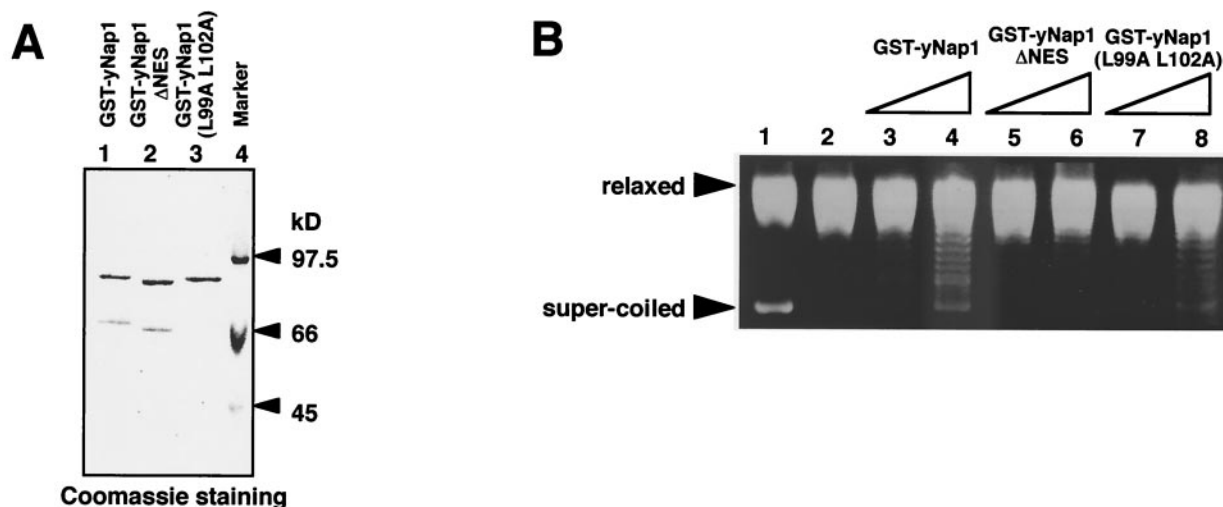


FIG. 8. Nucleosome assembly activities of yNap1 mutants. (A) Purified GST-fused yNap1 proteins used for nucleosome assembly assays. Four hundred nanograms of each protein (lane 1, GST-yNap1; lane 2, GST-yNap1 $\Delta$ NES; lane 3, GST-yNap1 [L99A L102A]) was separated by SDS-7.5% PAGE and stained with CBB R-250. (B) Supercoiling assays. Nucleosome assembly reactions (lanes 2 to 8) were carried out with relaxed circular DNA and purified core histones in the absence (lane 2) or presence of GST-yNap1 (lanes 3 and 4), GST-yNap1 $\Delta$ NES (lanes 5 and 6), or GST-yNap1 (L99A, L102A) (lanes 7 and 8). After the reaction, DNA in each reaction mixture was deproteinized, separated by 1% agarose gel electrophoresis, and visualized by staining with Cyber gold (Molecular Probes). A mixture of fully supercoiled DNA and relaxed DNA is also shown in lane 1.

may be inhibitory when stably localized in the nucleus. On this line, it is noted that NAP1 has the chromatin disruption activity (24) as well as the nucleosome assembly activity (36, 54). The cell cycle is likely to be inhibited at the mitotic phase because nuclei of these cells were localized at the bud neck (data not shown). Such inhibition was not observed in NAP1-positive strains, a WT YPH499 strain, and a DK166 strain ( $\Delta$ *CLB1*  $\Delta$ *CLB3*  $\Delta$ *CLB4*) (data not shown). Under the same conditions, yNap1-NLS, which is a fusion yNap1 protein with simian virus 40 NLS at the C terminus of yNap1 and predominantly localized in nucleus, could complement the DK168 phenotypes and did not have any inhibitory effect on the cell growth (data not shown). Thus, the inhibitory effect is suppressed by endogenous Nap1. These observations suggest that nuclear yNap1 does not have a mitotic cell arrest ability, but an export-deficient Nap1 that accumulates in the nucleus provides the inhibitory effect on cell growth.

An alternative explanation for the growth inhibition would be that the yNap1 mutants in the nucleus may anchor a yNap1 interactor(s) that exerts its normal function transiently in the nucleus and is needed to be exported to the cytoplasm. The target of Nap1 during its nuclear export is an open question. Several lines of evidence suggest that Clb2 is one of the candidates. First, Clb2 is also a nucleocytoplasmic shuttling protein and is dominantly localized in the nucleus, in particular during the G<sub>2</sub>/M phase (12). Second, an NES mutation of Clb2 causes an elongated bud phenotype (12). Therefore, we examined the difference of Clb2 localization in the absence and presence of *NAP1*. We confirmed that Nap1 and NES-mutated Nap1s bind to Clb2 (Fig. 7). We tried to detect the difference of Clb2-EGFP localization in *NAP1*-positive and -negative strains. However, the difference is not so significant between two strains.

Although systematic analyses are needed, we would like to

propose the term shuttling chaperone for Nap1. The shuttling chaperone may modulate nuclear import and/or export of proteins rather than itself. A variety of Nap1-interacting proteins are being identified, and their intracellular localization patterns are closely related to the regulation of mitotic progression. It is possible that these interacting proteins are also targets of the Nap1 shuttling chaperone activity. Thus, it is interesting and necessary to examine the involvement of Nap1 in the subcellular localization of these interacting proteins and to ask whether their aberrant localization eliminates the proper mitotic progression.

**Regulation of Nap1 localization.** We have discussed the function of Nap1 associated with its nucleocytoplasmic shuttling. It is interesting how the shuttling is regulated. It has long been thought that Nap1 is localized in the cytoplasm in a variety of species. Since the NES-like sequence in yNap1 is well conserved among species, the conclusion from our analyses with yeast Nap1 is probably applicable for other species. It is reported that the fly Nap1 is temporally localized in the nucleus at some stages during development (17). We have shown that EGFP-Nap1 is dominantly detected in cytoplasm, whereas in 10.1% of cells expressing EGFP-yNap1, the signal is dominantly detected in nucleus (Table 1). These observations point out that Nap1 could be localized in the nucleus under a certain condition(s). It is possible that the rate of nuclear import and export is varied depending on cell conditions. Based on the histone-binding activity and nucleosome assembly activity of Nap1, it is assumed that Nap1 functions as a chaperone for histones during nuclear import and in the cytoplasm as well as at the deposition to DNA. Thus, the rate of the nuclear import of Nap1 might be increased at the S phase, since histones are most actively synthesized at the S phase. The modification may also be involved in the regulation of nuclear transport and nuclear function, if any, of Nap1. It is reported that a cytoplas-

mic fraction of Nap1, but not the nuclear fraction, is possibly phosphorylated by CK2 and that the histone-binding activity is stimulated by phosphorylation of Nap1 by CK2 (48).

The balance of nuclear import and export of Nap1 may be important for the nuclear function of Nap1. Nap1 could be involved in not only de novo assembly of nucleosomes but also structural change of chromatin, although we do not know an exact function of Nap1 in the nucleus. Nap1 family proteins are shown to be involved in transcription regulation (20, 24, 49). Our preliminary experiments with one-hybrid assays indicated that Nap1 stimulates the transcription from chromatin under a certain condition (data not shown). To consider the nuclear function of Nap1, it is worthwhile to note that the nuclear localizations of NES-mutated yNap1s show speckled patterns in the nuclei (Fig. 2g, h, and j). Determination of the nuclear domain may support understanding of the nuclear function of Nap1.

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