

nup1 Mutants Exhibit Pleiotropic Defects in Nuclear Pore Complex Function

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Abstract. The *NUPI* gene of *Saccharomyces cerevisiae* encodes one member of a family of nuclear pore complex proteins (nucleoporins) conserved from yeast to vertebrates. We have used mutational analysis to investigate the function of Nup1p. Deletion of either the amino- or carboxy-terminal domain confers a lethal phenotype, but partial truncations at either end affect growth to varying extents. Amino-terminal truncation causes mislocalization and degradation of the mutant protein, suggesting that this domain is required for targeting Nup1p to the nuclear pore complex. Carboxy-terminal mutants are stable but do not have wild-type function, and confer a temperature sensitive phenotype. Both import of nuclear proteins and export of poly(A) RNA are defective at the nonpermiss-

sive temperature. In addition, *nup1* mutant cells become multinucleate at all temperatures, a phenotype suggestive of a defect in nuclear migration. Tubulin staining revealed that the mitotic spindle appears to be oriented randomly with respect to the bud, in spite of the presence of apparently normal cytoplasmic microtubules connecting one spindle pole body to the bud tip. EM analysis showed that the nuclear envelope forms long projections extending into the cytoplasm, which appear to have detached from the bulk of the nucleus. Our results suggest that Nup1p may be required to retain the structural integrity between the nuclear envelope and an underlying nuclear scaffold, and that this connection is required to allow reorientation of the nucleus in response to cytoskeletal forces.

THE nuclear envelope serves as a selective barrier to the flow of macromolecules between the nucleus and cytoplasm (for review see Forbes, 1992; Silver, 1991; Davis, 1992). Transport of protein and RNA occurs exclusively through the nuclear pore complex (NPC)¹, an ~112 megadalton structure that has been estimated to contain over 100 different polypeptides (Reichert et al., 1990). The NPC spans the double membrane of the nuclear envelope and consists of two eightfold symmetrical rings, each of which has spokes that project into a central channel (Unwin and Milligan, 1982; Hinshaw et al., 1992; Akey and Radermacher, 1993). Recent studies using both scanning and transmission EM suggest that the NPC has a much more extended structure than previously appreciated (Ris, 1991; Jarnik and Aebi, 1991; Goldberg and Allen, 1992). On the cytoplasmic side, short filaments have been observed emanating from each of the eight ring subunits. A basket-like structure, referred to

as a "fishtrap," extends from the nuclear rings some 50–100 nm into the nucleus. Goldbert and Allen (1992) have also reported the presence of a filamentous lattice on the nucleoplasmic side of the nuclear envelope that connects the distal rings of each basket. These results suggest that some constituents of the NPC are linked to the nucleoskeleton, and provide support for the idea that the NPC may play a role in organizing nuclear structure that is distinct from (although perhaps interdependent with) its function in nucleocytoplasmic transport.

Few of the constituents of the NPC are known. Much recent work has focused on a family of proteins present in both vertebrate and yeast NPCs (Davis and Blobel, 1986, 1987; Snow et al., 1987; Aris and Blobel, 1989). These proteins have amino acid similarity within a domain containing degenerate repeats, and the mammalian proteins, at least, are modified by the addition of O-linked *N*-acetylglucosamine (Davis and Blobel, 1987; Holt et al., 1987; Hanover et al., 1987). Yeast genes *NUPI*, *NUP2*, and *NSPI*, and vertebrate genes p62, pom121 and nup153, form one subfamily (Davis and Fink, 1990; Nehrbass et al., 1990; Starr et al., 1990; Carmo-Fonseca et al., 1991; Cordes et al., 1991; Sukegawa and Blobel, 1992; Loeb et al., 1993; Hallberg et al., 1993). Each has a domain consisting of degenerate XFXFG repeats that is recognized by mAbs 306 and 414. The carboxy-

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1. *Abbreviations used in this paper:* 5FOA, 5-fluoroorotic acid; HA, hemagglutinin; NPC, nuclear pore complex; SPB, spindle pole body.

terminal domain of *NUP1* and *nup153*, and the amino-terminal domain of *NSP1* are also distantly related to one another, and to the GLFG repeat domain that characterizes the other subset: *NUP49*, *NUP100*, and *NUP116* (Wente et al., 1992; Wimmer et al., 1992).

The exact function of these proteins remains unclear. In vertebrate systems, the translocation step of protein import can be blocked either by mAbs against the O-linked NPC glycoproteins (Dabauvalle et al., 1988) or by WGA (Dabauvalle et al., 1988; Finlay et al., 1987; Yoneda et al., 1987; Adam and Gerace, 1991). RNA export is also inhibited (Featherstone et al., 1988). Nuclei reconstituted from egg extracts that have been depleted of all or a subset of WGA-binding proteins are incapable of docking import substrates at the NPC (Finlay and Forbes, 1990). Furthermore, incubation of cytosolic extract with WGA-binding proteins renders the extract incapable of supporting nuclear import, presumably due to depletion of a factor required for docking nuclear proteins at the NPC (Sterne-Marr et al., 1992). Thus, docking and translocation may represent two separate functions carried out by one or more of the NPC glycoproteins.

Genetic analysis of the yeast nucleoporins has provided insights into the functional and physical interactions between these proteins, and has helped to identify new proteins with which they interact. Viable *nsp1* and *nup1* mutants are lethal when combined with a disruption of *NUP2*, which encodes a nucleoporin that is normally nonessential (Loeb et al., 1993). *nsp1* mutations are also synthetically lethal with deletions of *NUP116* and *NUP49* (Wimmer et al., 1992). Nsp1p, Nup49p, and Nup54p can be coimmunoprecipitated (Grandi et al., 1993), suggesting that the synthetic phenotype results from destabilization of a physical complex. On the other hand, Nup1p and Nup2p form distinct complexes which contain common components, and thus may function on partially redundant pathways (Belanger et al., 1994). Two novel nucleoporins have been identified by virtue of their association with these complexes; Nic96 is part of the Nsp1p complex (Grandi et al., 1993), and Srp1p associates with both Nup1p and Nup2p (Belanger et al., 1994).

The role of individual proteins in NPC function can now be investigated by phenotypic analysis of yeast strains containing mutations or deletions in each of the known nucleoporins. For example, Wente and Blobel (1993) have shown that deletion of *NUP116* confers a temperature sensitive phenotype, and results in fusion of the outer nuclear membrane above the NPC at the nonpermissive temperature. This "sealing" of the nuclear envelope, which prevents movement of mRNA out of the nucleus (and presumably also inhibits protein import), may be caused by loosening of the membrane-NPC interface (pore wall). Thus Nup116p may play a role in tethering the peripheral components of the NPC to the nuclear envelope membrane.

Here we present phenotypic analysis of strains carrying mutations in the yeast nucleoporin, Nup1p. We find that *nup1* mutants are defective for protein import and mRNA export, and exhibit structural alterations of the nuclear envelope very different from those of Δ *NUP116*. NPCs themselves appear normal, but the nuclear envelope forms long finger-like projections that extend out from the nuclear mass. This apparent detachment of the envelope from the underlying nuclear structure may explain why *nup1* mutants are also defective for nuclear migration.

Materials and Methods

Reagents

Enzymes for molecular biology were purchased from Boehringer Mannheim Corp. (Indianapolis, IN), Pharmacia LKB Biotechnology (Piscataway, NJ) and New England Biolabs (Beverly, MA). Lyticase was obtained from Enzogenetics (Corvallis, OR). 5-fluoroorotic acid (5FOA) was purchased through the Genetics Society of America consortium. The anti-tubulin mAb YOL1/34 was obtained from Harlan Bioproducts (Indianapolis, IN). All fluorescently labeled secondary antibodies were obtained from Jackson ImmunoResearch Labs. (Westgrove, PA). Digoxigenin-labeled dUTP and anti- β -galactosidase antibody were purchased from Boehringer Mannheim. mAb 12CA5 was obtained from the Harvard Cell Culture Facility (Cambridge, MA).

Strains and Microbial Techniques

The plasmids and yeast strains used are listed in Tables I and II. Yeast cell culture and genetic manipulations were performed essentially according to Sherman et al. (1986). Yeast shuttle plasmids and linear fragments were introduced into yeast by lithium acetate transformation (Ito et al., 1983). Selection against *Ura*⁺ strains was accomplished by culture on solid synthetic media containing 1 mg/ml 5FOA (Boeke et al., 1984).

Construction of Truncated *nup1* Alleles

Truncations from either the amino or carboxy terminus of Nup1p were generated by a modification of the ExoIII protocol of Henikoff (1984). For amino-terminal truncations, restriction sites for SphI and StyI were first introduced after the third codon, using the single stranded mutagenesis technique of Kunkel et al. (1987). Strain RZ1032 (*dur⁻ung⁻*) was used to isolate single-stranded DNA from pB2289, and second strand synthesis was performed as described, using an oligonucleotide of sequence 5'-AGTCATTCATGCTTCAGCATGCCGCGATTACCTAGGGAACACTTCTCTGTGAT as a primer. A nested series of deletions in the resulting plasmid (pLDB23) was then made using ExoIII to digest from the StyI site, and the plasmid religated. Junctions were sequenced to identify those that contained in-frame fusions with downstream sequences (pB2291, pLDB27, and pLDB33; see Table I). Carboxy-terminal truncations were made in a similar manner, using an oligonucleotide of sequence 5'-TGGCGAACAGAAAGATTCCAAGGGTACGCTTGCATGCAAGAATGAGGCATC to introduce StyI and SphI sites eight codons upstream of the stop codon, generating plasmid pLDB24. The resulting truncations are listed in Table I (pB2300 and pLDB35).

Preparation of Cell Extracts

Yeast strains were grown to mid log phase, and cells were spheroplasted as described by Kalinich and Douglas (1989). Spheroplasts were lysed by resuspension in 10 ml/g ice cold 20 mM Hepes-HCl, pH 7.5; 5 mM MgCl₂ and protease inhibitors (see Kalinich and Douglas, 1989), followed by vortexing and incubation on ice for 5 min. Lysates were centrifuged at 9,000 rpm in an SA600 rotor for 45 min at 4°C. The supernatant, representing soluble cellular components, was decanted. The pellet was resuspended in 10 ml/g ice cold 20 mM Hepes-HCl, pH 7.4; 5 mM MgCl₂; 1 M NaCl and protease inhibitors, incubated on ice 10 min, and centrifuged at 9,000 rpm for 45 min. The supernatant was decanted.

Protein Import Assay

Yeast strains carrying pFBI-33C (Moreland et al., 1987) were grown at room temperature in synthetic complete medium lacking uracil, and containing 0.1% glucose. The cells were grown to early log phase, and half of each culture was shifted to 36°C for 2 h before transfer into fresh medium containing 2% galactose. After addition of galactose, cultures were continued at the same temperature for 1.5 h, and then fixed in fresh 4% formaldehyde. Fixation was performed at room temperature for 1 h, followed by overnight incubation on ice. Cells were treated for immunofluorescence essentially as described (Davis and Fink, 1990), using Lyticase to dissolve the cell wall, and including treatment with methanol. Samples were viewed with a Nikon Optiphot-2 microscope with a 100 \times CF Fluor objective, and photographed using Tmax 400 film.

Table I. Plasmids Used in This Study

Plasmid	Relevant markers	Comments	Source
pB2337	<i>nup1-2::LEU2</i>	Complete disruption of <i>NUP1</i> in pGEM7	Loeb et al., 1993
pLDB125	<i>nup1-3::HIS3</i>	Complete disruption of <i>NUP1</i> (SnaBI-HpaI) with <i>HIS3</i> in pLD1*	This study
pB2487	<i>CEN URA3 NUP1</i>	<i>NUP1</i> wild-type inserted into pRS316†	Loeb et al., 1993
pLDB13	<i>CEN HIS3 NUP1</i>	<i>NUP1</i> wild-type in pRS313	This study
pLDB18	<i>CEN HIS3 NUP1^{HA}</i>	HA tagged <i>NUP1</i> (EcoRV-EcoR1) fragment from pLD1-3* cloned into pRS313	This study
pB2289	<i>CEN TRP1 NUP1</i>	<i>NUP1</i> wild-type inserted into pRS314	Loeb et al., 1993
pLDB23	<i>CEN TRP1 NUP1</i>	Amino-terminal SphI and StyI sites created in pB2289 by site-directed mutagenesis	This study
pLDB24	<i>CEN TRP1 NUP1</i>	Carboxy-terminal SphI and StyI sites created in pB2289 by site-directed mutagenesis	This study
pB2291	<i>CEN TRP1 nup1-8</i>	Exo III deletion from pLDB23: Δaa 4-141	This study
pLDB33	<i>CEN TRP1 nup1-15</i>	Exo III deletion from pLDB23: Δaa 4-191	This study
pLDB27	<i>CEN TRP1 nup1-9</i>	Exo III deletion from pLDB23: Δaa 4-212	This study
pB2300	<i>CEN TRP1 nup1-21</i>	Exo III deletion from pLDB24: Δaa 1041-1076	This study
pLDB35	<i>CEN TRP1 nup1-22</i>	Exo III deletion from pLDB24: Δaa 989-1076	This study
pLDB107	<i>CEN TRP1 NUP1^{HA}</i>	<i>NUP1</i> fragment (Xho-Bam) from LDB18 into pRS314	This study
pLDB56	<i>CEN TRP1 nup1-8^{HA}</i>	pB2291 with HA tag inserted at amino acid 460	This study
pLDB97	<i>CEN TRP1 nup1-9^{HA}</i>	pLDB27 with HA tag inserted at amino acid 460	This study
pLDB96	<i>CEN TRP1 nup1-15^{HA}</i>	pLDB33 with HA tag inserted at amino acid 460	This study
pLDB104	<i>CEN TRP1 nup1-21^{HA}</i>	pLDB34 with HA tag inserted at amino acid 460	This study
LDB123	<i>NUP1^{HA} LEU2</i>	<i>NUP1^{HA}</i> (BamHI-XhoI) fragment from LDB18 into pRS305	This study
LDB139	<i>nup1-8^{HA} LEU2</i>	<i>nup1-8^{HA}</i> (BamHI-XhoI) fragment in pRS305	This study
LDB140	<i>nup1-15^{HA} LEU2</i>	<i>nup1-15^{HA}</i> (BamHI-XhoI) fragment in pRS305	This study
LDB141	<i>nup1-21^{HA} LEU2</i>	<i>nup1-21^{HA}</i> (BamHI-XhoI) fragment in pRS305	This study
pLDB19	<i>CEN HIS3 nup1-106</i>	Nonsense mutation, amino acid 73 of <i>NUP1</i> in pLDB18	This study
pLDB69	<i>CEN HIS3 nup1-3410^{HA}</i>	Frameshift at amino acid 1032 of <i>NUP1</i> in pLDB18	This study
pLDB71	<i>CEN HIS3 nup1-3518^{HA}</i>	Frameshift at amino acid 1032 of <i>NUP1</i> in pLDB18	This study
pLDB99	<i>CEN TRP1 nup1-106</i>	<i>nup1-106</i> fragment (Bam-Xho) from LDB19 into pRS314	This study
pFB1-33C	<i>2μ URA3 GAL10::H2B::LacZ</i>	H2B NLS fused in frame to β-galactosidase	Moreland et al., 1987
pLDB94	<i>CEN HIS3 RNA1</i>	<i>RNA1</i> fragment (Xba-SacI) from pRU35‡ into pRS313	This study

* Davis and Fink, 1990.

† All pRS vectors were obtained from P. Hieter (Sikorski and Hieter, 1989).

‡ Traglia et al., 1989.

Table II. Yeast Strains Used in This Study

Strain	Genotype	Source
L4242	<i>MATa ura3-52 leu2^{Δ1} his3^{Δ200}</i>	Fink collection
L2612	<i>MATα ura3-52 leu2-3,-112 trp1-289 can1 cyh2</i>	Fink collection
9933-13A	<i>MATa ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200}</i>	Fink collection
9933-20C	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200}</i>	Fink collection
LDY80	<i>MATa/MATα ura3-52/ura3-52 leu2^{Δ1}/leu2-3,-112 his3^{Δ200}/HIS3 TRP1/trp1-289 CAN1/can1 CYH2/cyh2 NUP1/nup1-2::LEU2</i>	L4242 X L2612 diploid disrupted with pB2337
LDY176	<i>his3^{Δ200} leu2 ura3-52 trp1^{Δ289} nup1-2::LEU2</i> [pB2487]	segregant from LDY80 [pB2487]
LDY386	<i>MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,112 his3^{Δ200}/his3^{Δ200} trp1^{Δ1}/trp1^{Δ1} NUP1/nup1-3::HIS3</i>	9933-13A X 9933-20C diploid disrupted with pLDB125
LYD392	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200} nup1-3::HIS3</i> [pB2487]	segregant from LDY386[pB2487]
LDY415	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200} nup1-3::HIS3 nup1-21::LEU2</i>	LDY392 with pLDB141 integrated at <i>leu2</i>
LDY416	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200} nup1-3::HIS3 nup1-8::LEU2</i>	LDY392 with pLDB139 integrated at <i>leu2</i>
LDY417	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200} nup1-3::HIS3 nup1-15::LEU2</i>	LDY392 with pLDB140 integrated at <i>leu2</i>
LDY418	<i>MATα ura3-52 leu2-3,-112 trp1^{Δ1} his3^{Δ200} nup1-3::HIS3 NUP1::LEU2</i>	LDY392 with pLDB123 integrated at <i>leu2</i>
F95	<i>MATa adel ade2 gall1 his7 lys2 rna1-1 tyr1 ura1</i>	Fink collection
LDY351	<i>MATα trp1-289 his3^{Δ200} nup1-2::LEU2 trp1 ade2 rna1-1</i> [pB2487]	segregant from 169-10a*X 177-55a‡
168-1A	<i>MATa ura3-52 leu2 mtr1-1</i>	segregant from L4242x T127§
BJ5457	<i>MATα ura3-52 trp1 lys2-801 leu2Δ1 his3Δ100 pep4::HIS3 prb1Δ1.6R can1</i>	ATCC

* Belanger et al., 1994.

† Segregant from a cross between F95 and L2612.

‡ Kadowaki et al., 1992.

In Situ Hybridization

Yeast strains were grown to early log phase at 25°C. Half of each culture was then shifted to 36°C for 3.5 h before fixation in 4% freshly prepared formaldehyde. Fixation was performed at 25°C for 1 h, and continued on ice overnight. Cells were spheroplasted and adhered to coverslips essentially as described (Davis and Fink, 1990), through the methanol fixation step. The remaining steps were modified according to Forrester et al. (1992) from the protocol described by Amberg et al. (1992). Coverslips were rinsed briefly in buffer B (0.1 M KPO₄, pH 7.0; 1.2 M sorbitol), and then incubated for 10 min at -20°C in 70% ethanol. After rinsing briefly in 2× SSC, coverslips were inverted onto a 20- μ l drop of hybridization solution placed on a strip of parafilm. To make 20- μ l hybridization solution, 50 ng digoxigenin-labeled oligo dT-50, 10 μ g calf thymus DNA and 10 μ g tRNA were mixed in formamide (30% final) and heated to 70°C for 10 min. An equal volume of a solution containing 4× SSC, 0.4% BSA, 40 mM vanadyl ribonucleoside complex and 20% dextran sulfate was then added. Coverslips were incubated in a humid chamber at 36°C overnight. They were then washed three times for 10 min each in 2× SSC at 36°C, and the washes repeated at 25°C. Coverslips were next incubated in solution B containing 0.1% Triton X-100 for 5 min at 25°C, after which they were rinsed three times with solution B. Subsequent steps (starting with blocking) were performed essentially as described previously (Davis and Fink, 1990). Samples were viewed with a Nikon Optiphot-2 microscope with a 100× CF Fluor objective, and photographed using T-max 400 film.

Results

Isolation of *nup1* Mutants

To identify the domains of Nuplp that are required for function, we made truncations from either end of the *NUPI* gene

in pB2289 (see Materials and Methods). The resulting plasmids were transformed into a Δ *NUPI* strain (LDY176) harboring pB2487 (*NUPI CEN URA3*). Transformants were plated on 5FOA, which allows growth only of those cells that have lost pB2487 (Fig. 1 A). Under these conditions, amino-terminal deletion mutants *nup1-8* (Δ 4-142) and *nup1-15* (Δ 4-191) conferred only a slight growth defect, whereas *nup1-9* (Δ 4-212) was incapable of supporting growth. Similar results were obtained by truncation of the carboxy-terminal domain. In this case removal of the last 35 amino acids (*nup1-21*) caused very slow growth. A more extensive truncation, *nup1-22* (Δ 989-1076) was inviable.

To test whether the truncations affect the stability of Nuplp, as well as to investigate the subcellular localization of the mutant proteins, we performed crude fractionation on strains carrying each of the mutants. We previously showed that wild-type Nuplp fractionates with the nucleus, and is solubilized by extraction with 1 M NaCl (Davis and Fink, 1990). To test whether the mutant proteins behave in the same manner, we tagged each of them with the nine amino acid hemagglutinin (HA) epitope as previously described (Davis and Fink, 1990), and, after confirming that insertion of the tag conferred no additional phenotype, transformed these constructs into yeast strain BJ5457. Transformants were fractionated into soluble and particulate fractions by low speed centrifugation of spheroplast lysates. The particulate fraction was then extracted with buffer containing 1 M

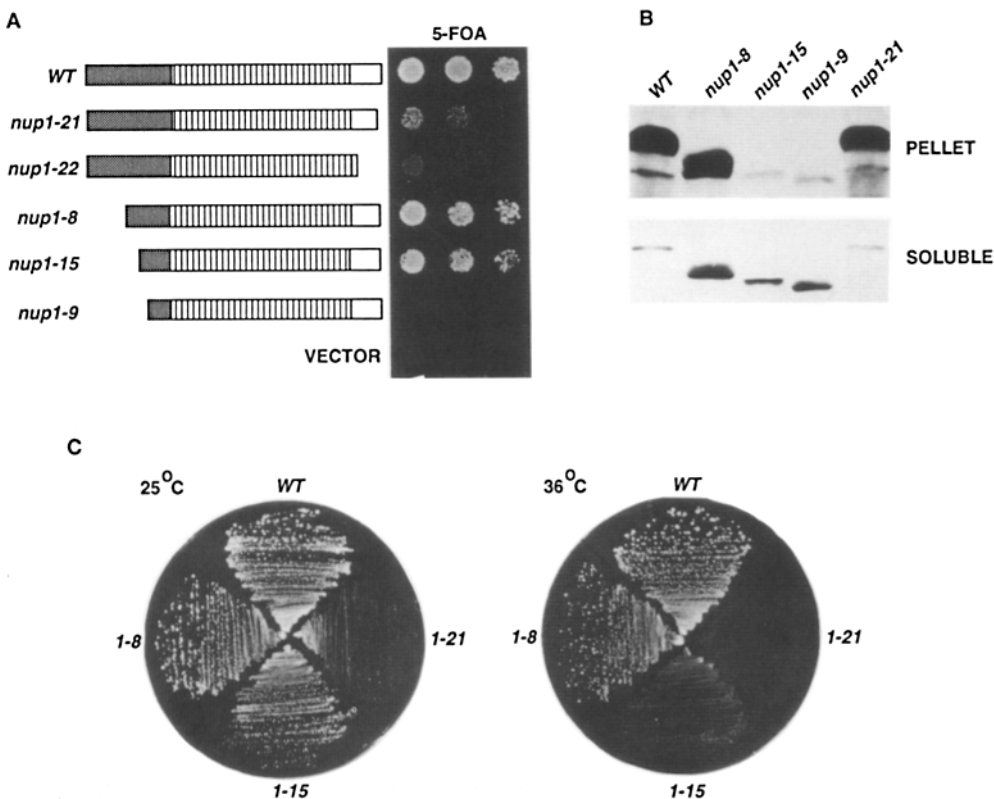


Figure 1. Characterization of Nuplp truncations. (A) A schematic showing the extent of each truncation is shown at the left. *nup1-8* (pB2291), *nup1-15* (pLDB33), and *nup1-9* (pLDB27) have progressively more severe truncations from the amino terminus (stippled), whereas *nup1-21* (pB-2300) and *nup1-22* (pLDB35) are truncated from the carboxy terminus (white). The central repetitive domain is delineated by the vertical stripes. Strain LDY176 was transformed with *CEN TRPI* plasmids bearing each construct, as well as wild-type *NUPI* (pB2289) and vector alone (pRS314) as controls. The ability of each mutant to support growth in the absence of wild-type *NUPI* is shown at the right. Serial dilutions (left to right) of each transformant were plated on media containing 5FOA to cure them of the wild-type *NUPI*-bearing *CEN URA3* plasmid. (B) BJ5457 cells harboring an HA-tagged

copy of the indicated mutants were fractionated as described in Materials and Methods. The soluble fraction, shown in the lower panel, consists of proteins released into a low speed supernatant after spheroplast lysis. The pellet fraction represents material released from the resulting pellet by 1 M salt extraction. Fractions were subject to SDS-PAGE and blotted to nitrocellulose. Blots were probed with mAb 12CA5, directed against the HA epitope, to visualize Nuplp. (C) Growth of isogenic wild-type (LDY418), *nup1-8* (LYD416), *nup1-15* (LDY417), and *nup1-21* (LDY415) integrants at 25°C (left) and 36°C (right).

NaCl. Western blot analysis of these two fractions (Fig. 1 B) showed that *nup1-21* is expressed at levels comparable to wild-type, thus the slow growth of strains carrying carboxy-terminal truncations is not due to instability of the mutant protein. Nup1-21p was also quantitatively recovered in the 1 M salt extract, as is wild-type Nuplp. Although crude cofractionation is not sufficient to determine NPC localization, these data are consistent with the idea that Nup1-21p is correctly localized. Therefore, the slow growth of the *nup1-21* mutant strain may reflect a functional role for the carboxyl-terminal domain within the NPC.

The amino-terminal truncations behaved quite differently. Expression of *nup1-8* was close to the wild-type level, but a significant amount of the protein was recovered in the soluble fraction. Association of Nup1-15p and Nup1-9p with the pellet fraction was further reduced, and in both cases the total amount of protein recovered was much less than that of wild-type. These results suggest that the amino-terminal domain of Nuplp is required for tight association with the NPC, and that the unassembled protein is degraded in the cytoplasm. Because the amount of Nup1-15p and Nup1-9p protein remaining in the particulate fraction was approximately the same even though only the *nup1-15* mutant is viable, the amino-terminal domain may have an additional function which is not provided by Nup1-9p. Alternatively, Nup1-15p may not compete well with wild-type Nuplp for assembly into the NPC, but may be able to assemble more readily than Nup1-9p in the absence of wild-type Nuplp.

Strains harboring *nup1-8* and *nup1-15* showed similar growth rates, in spite of the fact that the amount of Nuplp present in the particulate fraction is very different in the two mutants. If the level of correctly localized Nup1-15p were just above the threshold required for growth, then lowering the copy number would be expected to affect the viability of cells carrying *nup1-15* far more than those with *nup1-8*. To test the effect of copy number on each of the mutants, we integrated them at the *LEU2* locus of a *nup1-3::HIS3* strain (LDY392) and plated the transformants on 5FOA to cure them of the wild-type *NUPI* plasmid (Fig. 1 C). Under these conditions, the *nup1-15* mutant grew more slowly than *nup1-8* at room temperature, and was incapable of growth at 36°C. Strains containing *nup1-21* in single copy also grew more slowly than those harboring it on a plasmid, and were temperature sensitive.

We also attempted to isolate conditional missense mutations by random mutagenesis. Mutations were introduced by hydroxylamine or PCR mutagenesis of pLDB13 or pLDB18 (*NUPI CEN HIS3*) and the resultant library was transformed into LDY176. Transformants were screened for those that conferred a temperature sensitive phenotype when replica plated to 5FOA. Two classes of mutations were recovered. One group, exemplified by *nup1-106*, contain nonsense mutations within the amino-terminal domain. Growth of these mutants was dependent upon the presence of a nonsense suppressor (ψ element), because they were incapable of growth on medium containing guanidine hydrochloride, which rids cells of ψ (Tuite et al., 1981). Given the results we obtained with the truncations, we presume that read through translation generates enough Nuplp to support growth at 25°C, but not at 36°C. The other class of conditional mutants, *nup1-3410* and *nup1-3518*, are frameshifts at the carboxy terminus. The two mutations frameshift at the same place (12 amino

acids upstream of *nup1-21*), but into different reading frames. The behavior of these mutants upon cell fractionation was identical to that of *nup1-21* (data not shown). We failed to obtain any missense mutations that conferred a conditional phenotype. We have also used site-directed mutagenesis (including "alanine scanning") to introduce multiple point mutations throughout amino- and carboxy-terminal domains of *NUPI* (Bogerd, A. M., and L. I. Davis, unpublished), but in no case was growth affected. It thus appears that the Nuplp protein is quite tolerant to small changes in its primary sequence.

The above results suggest that the essential function of Nuplp is mediated through its carboxy-terminal domain, and that the loss of this domain from the NPC through mislocalization, underexpression or truncation of Nuplp leads to conditional growth.

nup1 Mutants Are Defective for Nuclear Migration

To determine whether *nup1* mutants showed gross defects in nuclear morphology, we stained the mutants with DAPI to visualize nuclear DNA (Fig. 2). While an isogenic wild-type strain showed normal nuclear morphology (Fig. 2 A), the nuclei of *nup1* mutant cells were abnormally shaped, often appearing fragmented and elongated (Fig. 2 B), and many cells appeared to have two or more nuclei (Fig. 2 C). These changes in nuclear morphology were apparent to varying extents in all of the mutant strains, at all temperatures tested (data not shown). The presence of multinucleate cells suggested that migration of the daughter nucleus into the bud is defective. This phenotype is characteristic of mutants in which cytoplasmic microtubule function is preferentially affected, because these structures are required for orienting the nucleus at the bud neck (Jacobs et al., 1988; Sullivan and Huffaker, 1992).

Tubulin staining of the mutant cells showed that many contained multiple spindles (Fig. 3, C-E), as expected if nuclear division occurs in the absence of normal migration. Furthermore, although both spindle and cytoplasmic microtubules were present, their morphology was altered. This was most easily visualized in cells that still contained only a single nucleus. In wild-type cells (Fig. 3 A), one spindle pole body (SPB) is normally oriented towards the bud, and a single cytoplasmic microtubule bundle emanating from it is tethered at the bud tip. In *nup1* mutants, the microtubules connecting the SPB and the bud tip are still present, but the SPB from which they arose appears to be randomly oriented with respect to the bud neck. Cells in which the SPB is oriented away from the bud thus have one unusually long cytoplasmic microtubule bundle that stretches from the SPB into the bud (arrows, Fig. 3, C and D). Thus, it appears that microtubule organization per se is not defective in *nup1* mutants but the nucleus is unable to respond by rotating and correctly orienting itself at the bud neck.

As with many other mutations that have profound effects on nuclear migration, this defect is not lethal to the cell population. It may be that as the elongating spindle shifts randomly within the mother it will often encounter the bud neck, through which it can then lengthen unimpeded. Once this has occurred, the chances that it will recede back into the mother are probably low. In any given population then, enough cells may correctly segregate their nuclei to allow

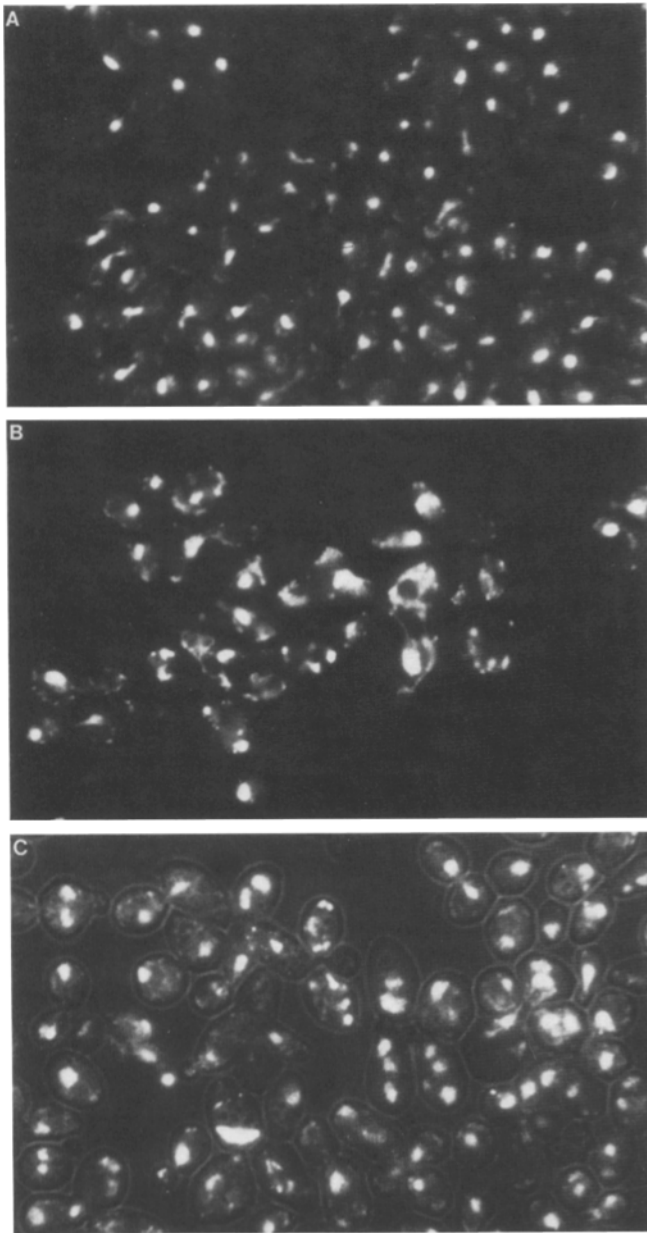


Figure 2. DAPI staining of *nup1* mutants. Mutant strain LDY415 (*B* and *C*) and isogenic wild-type strain LDY418 (*A*) were grown to early log phase at 25°C, and then fixed in 50% ethanol. Fixed cells were stained with DAPI to visualize DNA and viewed using fluorescence with (*C*) or without (*A* and *B*) concomitant phase contrast illumination.

survival even in the absence of directed nuclear migration. The slow growth of *nup1* mutant colonies at the permissive temperature may therefore reflect the small number of viable cells produced at each division, rather than a lengthening in the division cycle of all cells.

To investigate further the structural alterations in the *nup1* mutants, we examined cells by thin section EM (Fig. 4). Consistent with the irregular DAPI staining, we observed that the shape of the nucleus was altered in *nup1* mutant cells (Fig. 4, *B–E*) as compared to wild-type (Fig. 4 *A*). Long projections of nuclear envelope emanating from the bulk of the nucleus were apparent, and these often extended around

other cellular organelles. We also observed layers of double membranes containing NPCs located close to the nuclear envelope (Fig. 5). While these are somewhat reminiscent of annulate lamellae seen in higher eukaryotes, they may simply be nuclear envelope projections emanating from some other region of the nucleus. In spite of the profound changes in nuclear shape, the nuclear envelope appeared to be intact, and we found no evidence of obvious changes in number or morphology of NPCs (Fig. 5, *arrows*).

nup1-106 Is Defective for Protein Import

Because there is considerable evidence that one or more of the nucleoporins are essential components of the protein import machinery in mammalian cells, we examined protein import in the temperature sensitive *nup1* mutants. Strains harboring either *nup1-106* (pLDB19) or wild-type *NUPI* (pLDB13) were transformed with plasmid pFBI-33C, which contains the nuclear localization sequence of the yeast histone H2B gene, fused in frame to *LacZ* (Moreland et al., 1987). This gene fusion is under control of the *GAL10* promoter, allowing expression to be induced by shifting from glucose to galactose. Cells were induced on galactose for 1.5 h, and the ability of the newly synthesized fusion protein to enter the nucleus was assayed by immunofluorescence with anti- β -galactosidase antibody. At 25°C, the mutant cells efficiently localized β -galactosidase to the nucleus (Fig. 6 *A*), and the pattern was indistinguishable from that seen in an isogenic wild-type strain (not shown, the pattern was identical to that observed at 36°C, Fig. 6 *C*). We then shifted cells to 36°C for various times before induction. Mutant cells induced at the time of shift looked identical to wild-type, and those shifted for more than 4 h before induction lost detectable β -galactosidase expression, presumably due to inhibition of transcription or mRNA export (data not shown). However, when cells were shifted to 36°C for 2 h before induction, the *nup1-106* mutant showed staining of β -galactosidase throughout the cell (Fig. 6 *E*), whereas the wild-type strain showed normal accumulation in the nucleus (Fig. 6 *C*). The pattern we observed in the mutant is identical to that seen in wild-type cells when an inactivating mutation is introduced into the nuclear localization sequence (data not shown, see Moreland et al., 1987). We conclude that depletion of Nup1p interferes with transport through the NPC. All of the temperature sensitive *nup1* mutants, but none of the others, showed an import defect at 36°C similar to that observed with *nup1-106* (data not shown).

nup1-106 Mutants Show Retention of poly(A) RNA

Several yeast mutants have been identified that are defective for export of messenger and other RNAs (Wente and Blobel, 1993; Amberg et al., 1992; Shiohawa and Pogo, 1974; Kadowaki et al., 1992). In situ localization of poly(A) RNA in these mutants shows a characteristic pattern of bright nuclear staining, in contrast to normal cells which stain weakly throughout the cell. The altered localization presumably reflects the fact that poly(A) RNA is more stable within the nucleus and thus accumulates to high levels if transport is blocked. To determine whether Nup1p function is also required for movement of mRNA out of the nucleus, in situ hybridization was performed to localize poly(A) RNA before and after temperature shift. At 25°C, both wild-type (Fig. 7

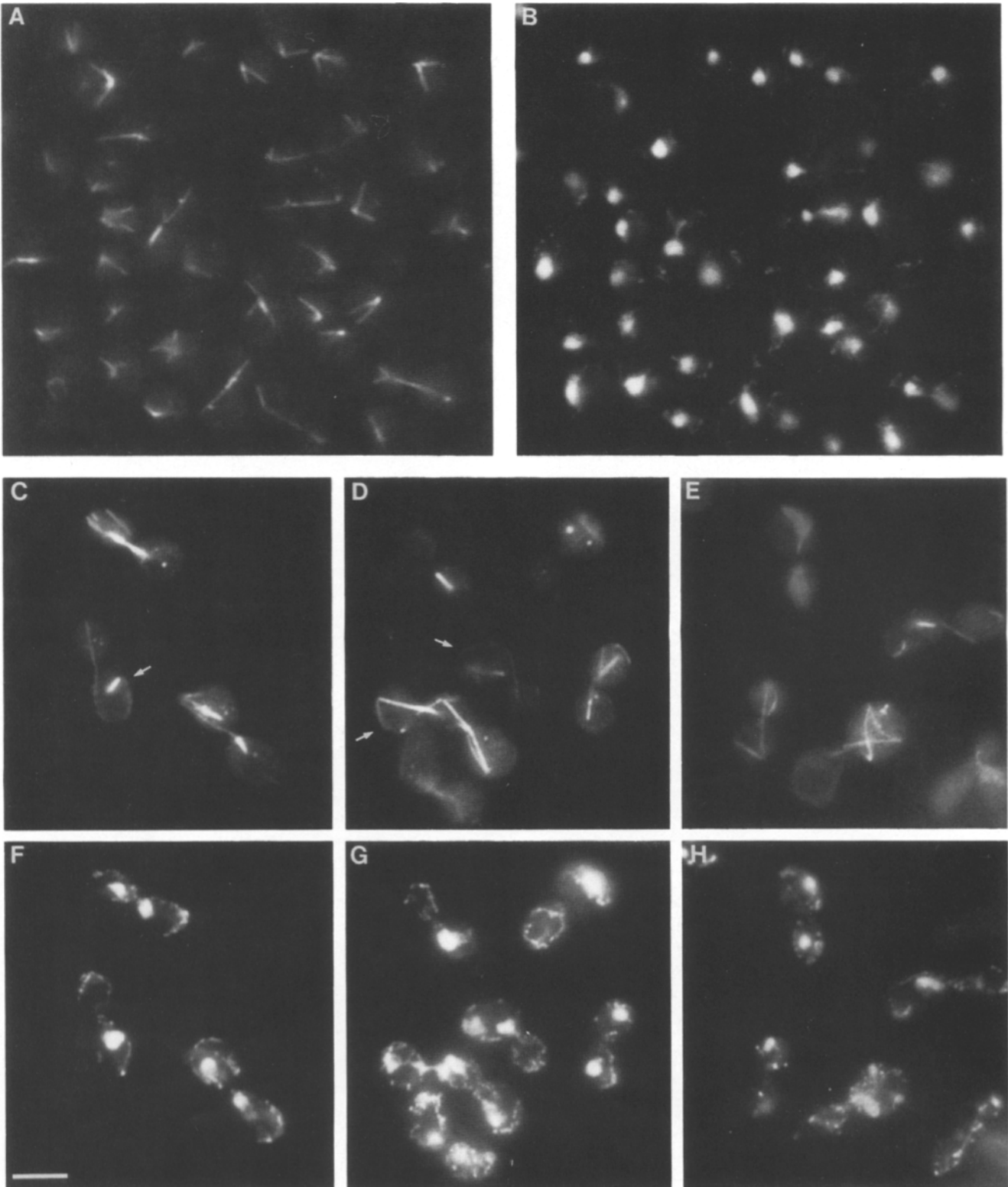


Figure 3. Tubulin staining of *nup1* mutants. Mutant strain LDY415 (C–H) and isogenic wild-type strain LDY418 (A and B) were grown to early log phase at 25°C, and processed for immunofluorescence as described (Kilmartin and Adams, 1984), using the anti-tubulin mAb, YOL1/34. Tubulin staining is shown in A, C, D, and E; DAPI staining in B, F, G, and H. Arrows point to cytoplasmic microtubules emanating from misoriented spindles.

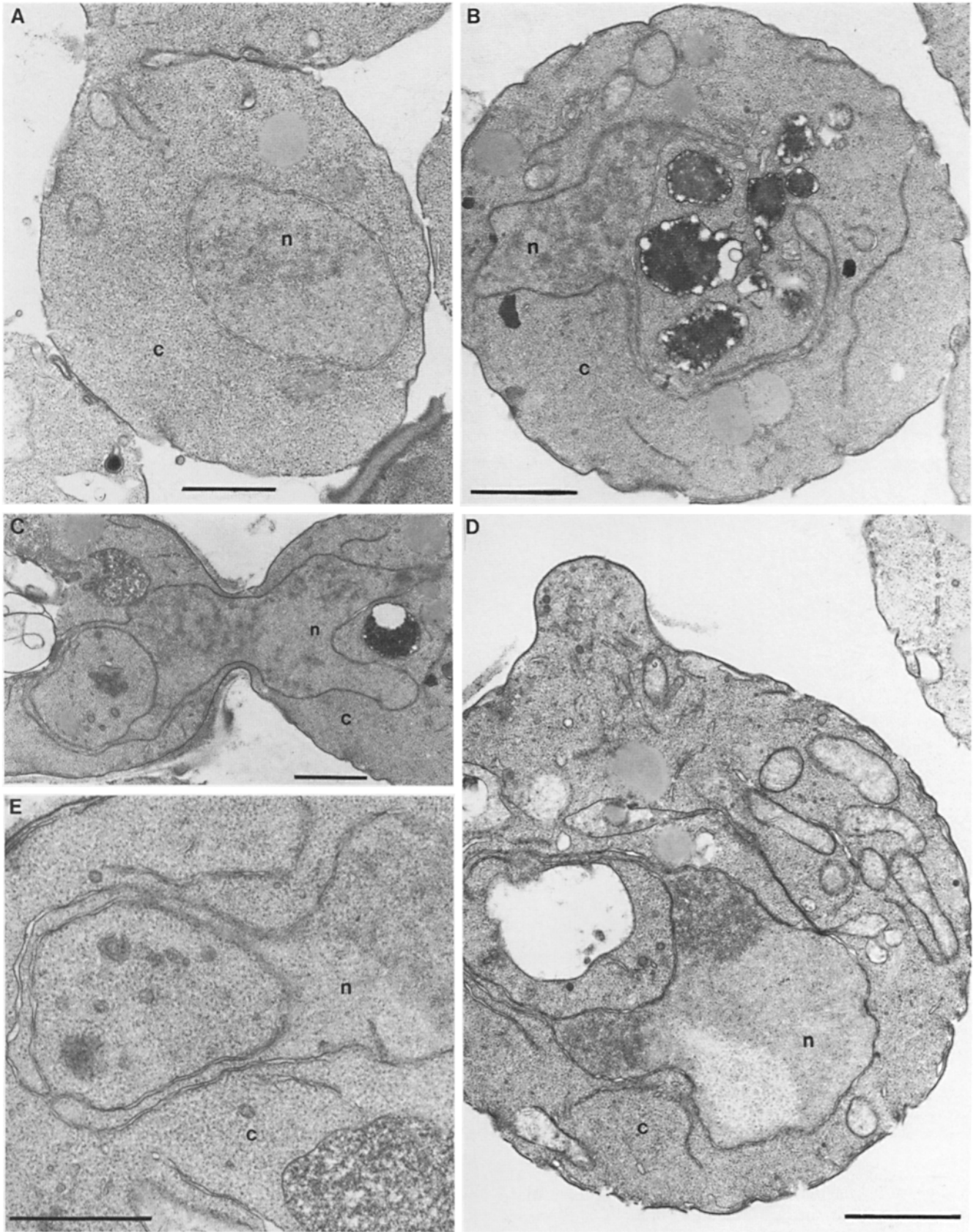


Figure 4. Nuclear structure in *nup1* mutants. Strain LDY176 carrying either pLDB19 (*nup-106*, *b-e*) or pLDB18 (*NUPI*, *A*), and cured of pB2587, was grown to early log phase at 25°C, and then processed for thin section EM, using the standard procedure for vegetatively growing cells described by Byers and Goetsch (1991). Nuclei (*n*) and cytoplasm (*c*) are denoted. Bar equals 1 μ m.

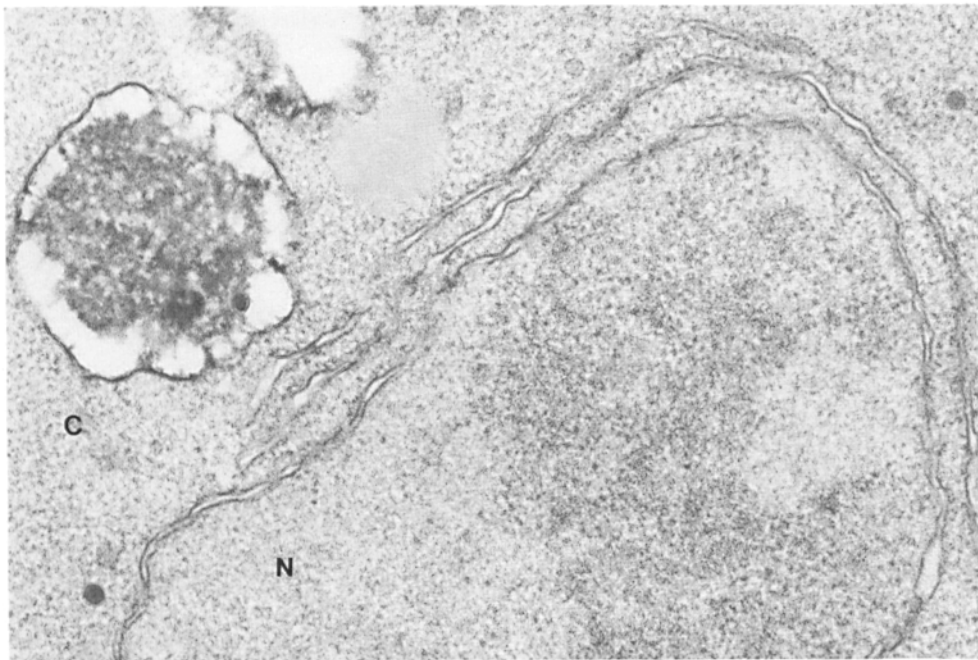
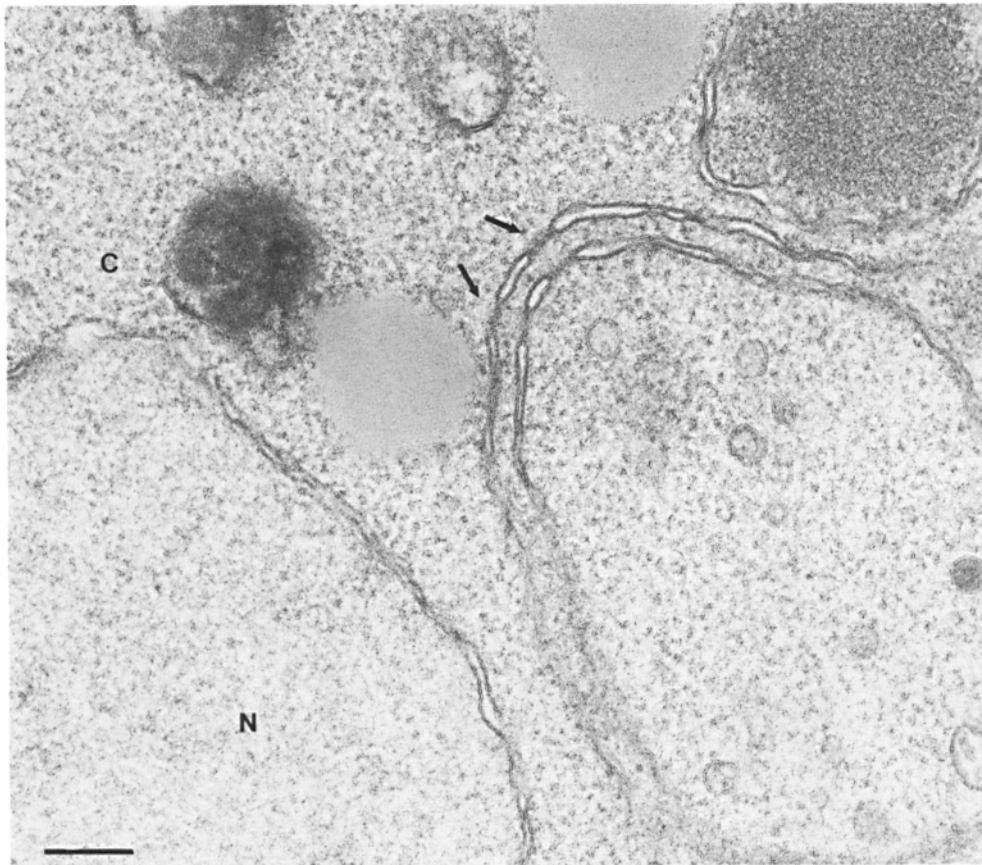


Figure 5. Nuclear envelope structure in *nup1* mutants. Strain LDY176 carrying pLDB19 (*nup1-106*), and cured of pB2587, was processed as described in the legend to Fig. 4. Arrows point to NPCs. Nucleus (*n*) and cytoplasm (*c*) are denoted. Bar equals 0.2 μ m.

A) and *nup1-106* mutant (Fig. 7 *B*) strains exhibited very faint staining throughout the cell, and this was also true of wild-type cells after 3.5 h at 36°C (Fig. 7 *C*). However, upon shifting the *nup1-106* mutant to 36°C, we observed significant nuclear staining in \sim 20% of the cells examined (Fig. 7 *D*), suggesting that *nup1* mutants are also defective for export of mRNA at the nonpermissive temperature. The

nuclear staining pattern looked similar to that seen in other mutants such as Δ *NUP116* and *mal-1* (Wente and Blobel, 1993; Amberg et al., 1992), but was present at a lower frequency. It may be that Nuplp depletion slows but does not stop transport. Alternatively, the window of time in which the localization phenotype is observable may simply be much shorter. The *nup1-106* strain required a longer shift

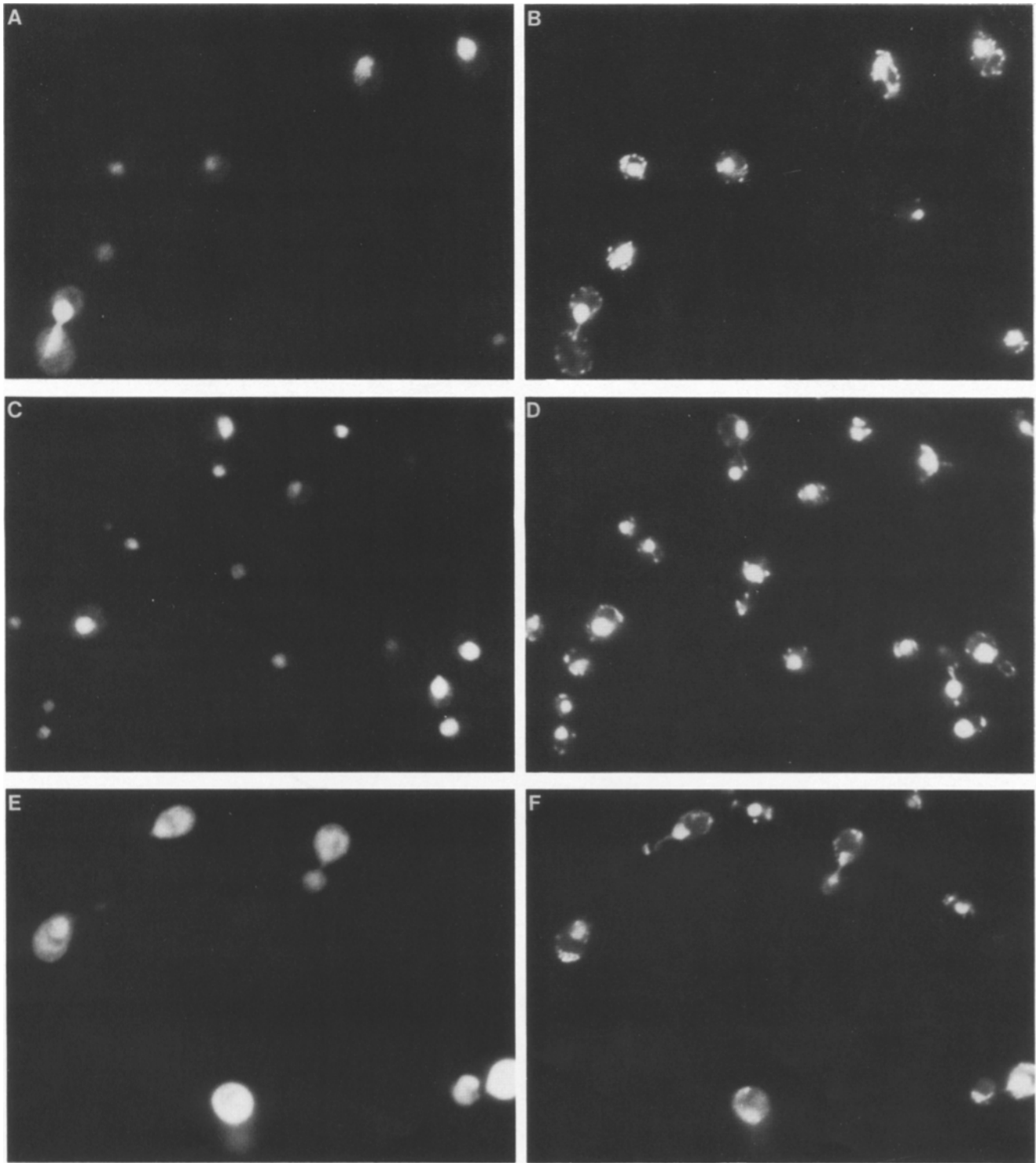


Figure 6. Nuclear protein import in *nup1* mutants. Strain LDY176 carrying either pLDB19 (*nup1-106*, *a*, *b*, *e*, and *f*) or pLDB18 (*NUPI*, *C* and *D*), and cured of pB2587, were grown to early log phase at 25°C and H2B-β-galactosidase synthesis was induced after 0 (*A* and *B*) or 2 h (*c-f*) shift to 36°C. Cells were processed for immunofluorescence as described in Materials and Methods, and stained with anti-β-galactosidase antibody (*A*, *C*, and *E*) and DAPI (*B*, *D*, and *F*).

than does the *rna1-1* strain, and staining of *nup1-106* decreased after ~4 h at the nonpermissive temperature, suggesting that mRNA transcription ceases (probably because nuclear import has been inhibited). Thus, the number of cells that are still synthesizing mRNA but can no longer export it is likely to be fairly low.

***nup1* and *rna1-1* Mutants Show Synthetic Lethality**

We next wanted to establish whether there is a functional relationship between Nup1p and other proteins thought to be involved in nuclear transport. We crossed a *nup1* strain to several mutants thought to be involved in some aspect of nuclear

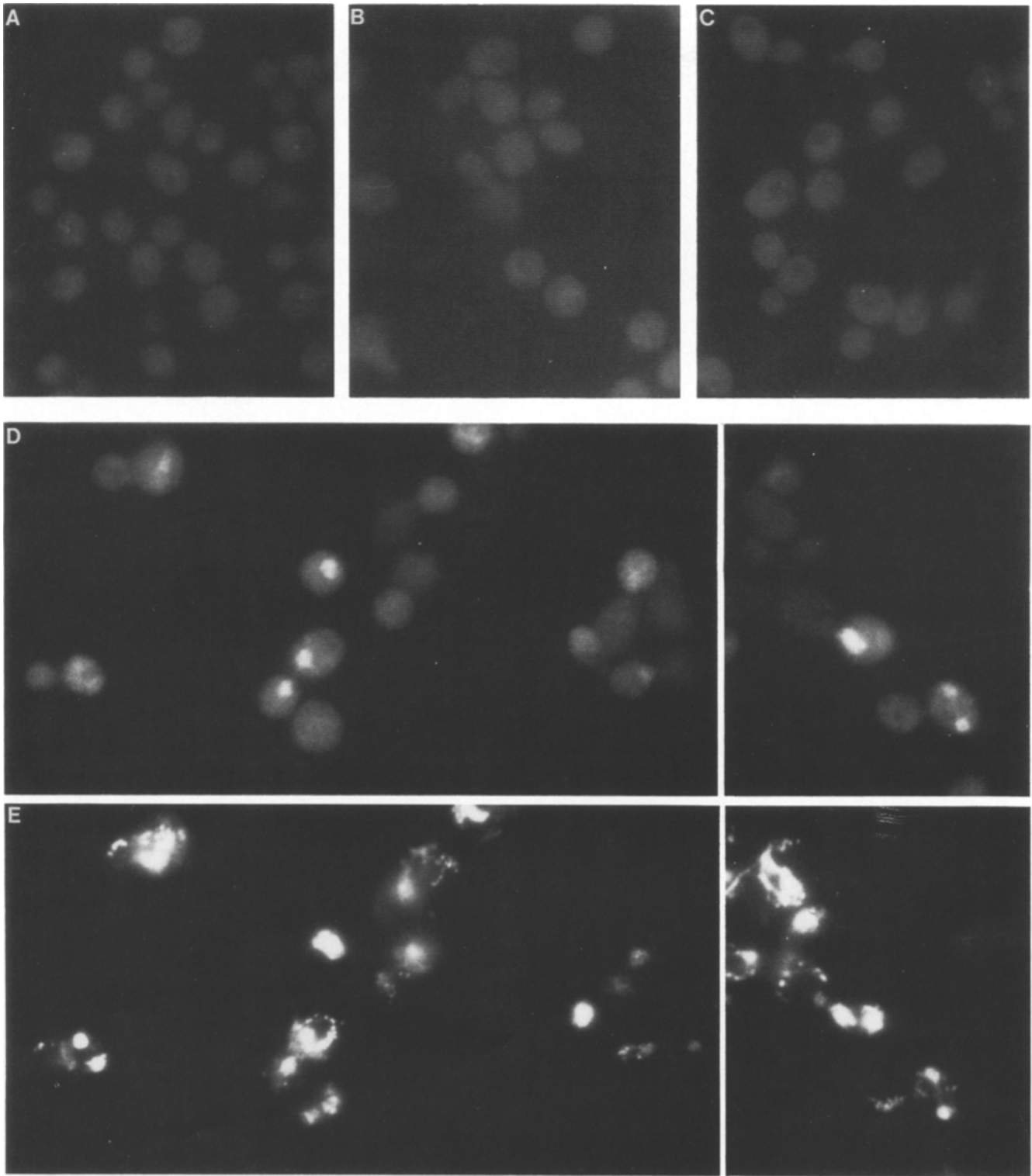


Figure 7. Poly(A) RNA localization in *nup1* mutants. Strain LDY176 carrying either pLDB19 (*nup1-106*, *B*, *D*, and *E*) or pLDB18 (*NUPI*, *A* and *C*), and cured of pB2587, were grown to early log phase at 25°C. Cells were then shifted to 36°C (*C*, *D*, and *E*) or left at 25°C (*A* and *B*) for 3.5 h, and then processed for in situ hybridization with oligo dT as described in Materials and Methods. Oligo dT (*A–D*); DAPI (*E*).

import or export. These include *rna1-1* and *mtr1/prp1*, both of which accumulate mRNA in the nucleus at the nonpermissive temperature (Shiokawa and Pogo, 1974; Amberg et al., 1992; Kadowaki et al., 1992) and Δ *NSRI*, a deletion of the gene encoding a nuclear localization sequence-binding pro-

tein that has been localized to the nucleolus (Lee et al., 1991). Double mutants between *nup1* and *mtr1* or *nsr1* were recovered at the expected frequency, and no synthetic phenotype was observed (data not shown). However, we never recovered double mutants when the *nup1* mutant was crossed

to *mal-1*. To confirm that the double mutant was inviable, we constructed a strain (LDY351) carrying *mal-1* and a *nup1-2::LEU2* deletion covered by pB2487 (*NUPI CEN URA3*). This strain was then cotransformed with either pLDB99 (*nup1-106 TRP1*), pLDB33 (*nup1-15 TRP1*) or pB2300 (*nup1-21 TRP1*) plasmids, and either pLDB94 (*RNAI HIS3*) or pRS313 (*HIS3*). Growth of transformants in the absence of *NUPI* was assayed by plating on 5FOA (Fig. 8). Both *nup1-106* and *nup1-21* were completely inviable in combination with *mal-1*, and the *nup1-15 mal-1* double mutant grew much more slowly than either single mutant. Thus, mutations that either lower the level of Nuplp or alter the carboxy-terminal domain are incapable of growth in an *mal-1* background.

Discussion

We have used deletion analysis to identify two distinct functional domains of Nuplp. Progressive deletion into the amino-terminal domain causes proportionately less protein to be recoverable in the particulate fraction, and the growth phenotype to become progressively worse, suggesting that the amino-terminal domain targets Nuplp to the NPC. Carboxy-terminal truncated proteins are stable and are found in the particulate fraction, but confer very slow growth. Our results contrast with those obtained from similar studies of the related nucleoporin Nsplp. The carboxy-terminal domain of Nsplp is sufficient for function (Nehrbass et al., 1990), and can direct a reporter protein to which it is fused to the NPC (Hurt, 1990). We note that the carboxy-terminal domain of Nuplp shares some similarity with the

amino-terminal domain of Nsplp; both consist of degenerate FGxNN repeats and are almost devoid of charged residues. The amino-terminal domain of Nuplp and carboxyl-terminal domain of Nsplp share no detectable similarity, except that they are both highly charged. The apparent difference in the requirements for various domains of these two proteins for viability may be genuine, or may be the result of strain variation. For example, strains that have different complements of nonessential nucleoporins exhibit more or less stringent requirements for intact Nuplp or Nsplp (Wimmer et al., 1992; Loeb et al., 1993). In this regard, we note that some yeast strains can grow slowly in the complete absence of *NUPI*, and that a single locus appears to determine whether or not *NUPI* is essential (Belanger et al., 1994). Therefore, the phenotypes described below may require mutation in another gene in addition to *NUPI*, perhaps a partially redundant nucleoporin. We note, however, that this second mutation has no discernable phenotype on its own, as evidenced by the wild-type behavior of the isogenic *NUPI* strain. Therefore, the phenotypic changes that we describe below can be attributed to mutation in *NUPI*.

Temperature sensitive *nup1* mutants exhibit defects in nuclear import and mRNA export. The effects of mRNA and protein transport may be distinct. Alternatively, the primary defect could involve a single one of these processes, which in turn affects the other. For example, inhibition of nuclear import could prevent the inward movement of a protein required for RNA processing or export. The converse, that defects in RNA export indirectly affect protein import, is less easy to visualize. Moreover, *mal-1* mutant strains import

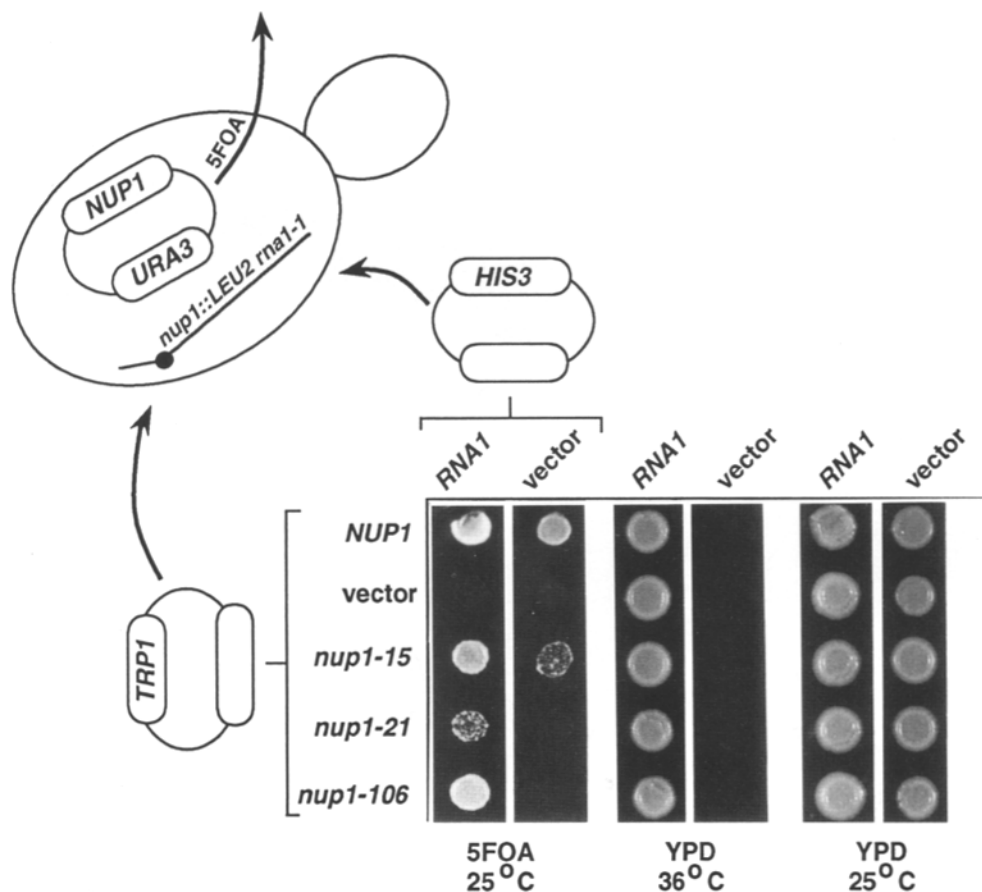


Figure 8. Synthetic lethality between *nup1* and *mal-1*. Starting strain LDY351 (top left) was transformed with a *CEN TRP1* vector containing the indicated *nup1* allele: *NUPI* (pB2289); vector (pRS314); *nup1-15* (pLDB33); *nup1-21* (pB2300); *nup1-106* (pLDB99), as well as *CEN HIS3* vector alone (pRS313) or containing the wild-type *RNAI* gene (pLDB94), as indicated. As expected, all transformants grow on YPD at the permissive temperature for *mal-1* (25°C, right panel), but only those transformed with pLDB94 grow at 36°C (middle panel). To test the viability of *nup1 mal-1* double mutants, the wild-type *NUPI* plasmid was evicted by plating cells on 5FOA (left panel). The first column therefore shows the growth rates of each single *nup1* mutant while the second column shows the growth rates of the corresponding *nup1 mal-1* double mutants.

H2B::lacZ fusions normally under conditions in which mRNA export is defective (Bogerd, A. M., and L. I. Davis, unpublished). A direct effect of *NUPI* mutation on RNA transport is suggested by the fact that *nup1* mutants are synthetically lethal with *rna1-1*. No interaction was observed with *mtr1/prp1*, which exhibits nuclear mRNA retention at levels similar to those observed in *rna1-1* mutants (Kadowaki et al., 1992; Amberg et al., 1992, 1993; Forrester et al., 1992). Thus, simply inhibiting mRNA export is not sufficient to cause synthetic lethality. The synthetic lethality observed between *rna1-1* and *nup1* is therefore specific. This interaction is particularly interesting in light of the fact that, although it affects a variety of RNA processing steps within the nucleus, Rnalp is largely cytoplasmic (Hopper et al., 1990). A plausible model is that Rnalp interacts transiently with components of the NPC to facilitate RNA export. Immunofluorescence localization of the *S. pombe* Rnalp homologue (Melchior et al., 1993) shows a concentration of Rnalp in a punctate pattern at the nuclear envelope, in addition to diffuse cytoplasmic staining, thus supporting the idea that Rnalp may transiently associate with the NPC.

Nuplp depletion also leads to a defect in nuclear migration during mitosis. A similar phenotype is observed when cytoplasmic microtubules are lost, either by mutation (Sullivan and Huffaker, 1992) or after nocodazole treatment (Jacobs et al., 1988). Deletion of the gene encoding a microtubule-associated protein, Biklp, has a similar phenotype (Berlin et al., 1990). These experiments suggest that cytoplasmic microtubules play an important role in positioning the dividing nucleus such that it segregates correctly between mother and bud. The apparently random orientation of the spindle in *nup1* mutants, in spite of the presence of properly connected cytoplasmic microtubules, suggests that the latter are no longer able to transmit the information required to orient the spindle correctly. Using immunofluorescence, we have previously shown that in wild-type cells the nuclear envelope enters the bud as a long projection at a point in time when the spindle and DNA remain entirely within the mother cell (Davis and Fink, 1990). This observation suggested that nuclear migration is not a passive result of spindle elongation into the bud, but may be a separate event that involves nuclear envelope movement along cytoplasmic microtubules. If so, then the *nup1* phenotype could be explained if attachment between the nuclear envelope and the cytoplasmic microtubules occurs normally, but the structural integrity between the envelope and underlying nuclear structures is weakened, thus preventing the nucleus itself from properly orienting or migrating in response. According to this model, the long nuclear envelope cisternae observed by EM analysis could result from extension of the nuclear envelope along cytoplasmic microtubules without concomitant nuclear movement.

An alternative explanation for the aberrant morphology of the nucleus is that it represents uncontrolled proliferation of nuclear envelope. We think this is unlikely because we would expect such an event to generate a structure more reminiscent of "karmellae" (Wright et al., 1990), which consist of closely packed layers of ER membrane wound around the nucleus. Moreover, it is difficult to imagine how depletion of an NPC protein (as opposed to overexpression) would induce uncontrolled synthesis of nuclear envelope. Furthermore, if alteration in the stoichiometry of NPC components leads to such a phenotype, we might expect this to be true for all NPC proteins. In fact, neither Nsplp depletion nor *NUPI16* dele-

tion produces a similar result (Mutvei et al., 1992; Went and Blobel, 1993).

A model in which Nuplp function is required to provide integrity between nucleoskeletal elements and the nuclear envelope is supported by our observation that Nuplp and Nup2p form complexes with Srplp (Belanger et al., 1994), a protein that has distant homology to the plakoglobin/armadillo/ β -catenin protein family (Yano et al., 1994). The members of this family are components of desmosomal and adherens junctions, and are thought to link cytoskeletal elements to transmembrane junctional components (for review see Kemler, 1993). The phenotype of *nup1* mutants is consistent with a similar role for Srplp/Nuplp. While the localization of Nuplp within the NPC is not known, we note that the majority of the WGA-binding proteins of the vertebrate NPC are localized at the distal ring of the nuclear fishtrap (Pante and Aebi, 1993), which appears to be connected to a filamentous lattice of unknown composition (Goldberg and Allen, 1992). It is tempting to suggest that Nuplp and Srplp provide this connection, although the extent to which these structural elements are conserved in yeast is not yet clear.

It is interesting to contrast the nuclear envelope morphology in *nup1* mutants to the striking defects observed in Δ *NUPI16* strains (Wente and Blobel, 1993). In the latter, a membrane "cap" forms over the cytoplasmic side of the NPC upon temperature shift, presumably resulting from fusion of the outer nuclear envelope over the NPC. This peculiar structural defect suggests that Nup116p normally provides a rivet at the interface between the "pore wall" and the cytoplasmic rings, and that deletion causes this connection to loosen, allowing the membrane on the cytoplasmic side of the pore wall to bulge outward and over the NPC. In contrast, the NPC itself appears to remain firmly associated with the underlying nucleus. The different but related phenotypes in *nup1* and *nup116* mutants suggests that perhaps each of these proteins provides a distinct structural connection for the NPC.

The results presented here suggest that *nup1* mutants have defects both in nuclear envelope structure and in nuclear transport. This could reflect numerous functions for this protein, but we think it more likely that the two phenotypes are linked. It may be that structural changes deform the NPC, and compromise the function of the transport machinery indirectly. Alternatively, association of the NPC with an underlying nuclear structure may be directly required for nuclear transport to occur.

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