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GLE2, a *Saccharomyces cerevisiae* Homologue of the *Schizosaccharomyces pombe* Export Factor *RAE1*, Is Required for Nuclear Pore Complex Structure and Function

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To identify and characterize novel factors required for nuclear transport, a genetic screen was conducted in the yeast *Saccharomyces cerevisiae*. Mutations that were lethal in combination with a null allele of the gene encoding the nucleoporin Nup100p were isolated using a colony-sectoring assay. Three complementation groups of *gle* (for GLFG lethal) mutants were identified. In this report, the characterization of *GLE2* is detailed. *GLE2* encodes a 40.5-kDa polypeptide with striking similarity to that of *Schizosaccharomyces pombe RAE1*. In indirect immunofluorescence and nuclear pore complex fractionation experiments, Gle2p was associated with nuclear pore complexes. Mutated alleles of *GLE2* displayed blockage of polyadenylated RNA export; however, nuclear protein import was not apparently diminished. Immunofluorescence and thin-section electron microscopic analysis revealed that the nuclear pore complex and nuclear envelope structure was grossly perturbed in *gle2* mutants. Because the clusters of herniated pore complexes appeared subsequent to the export block, the structural perturbations were likely indirect consequences of the export phenotype. Interestingly, a two-hybrid interaction was detected between Gle2p and Srp1p, the nuclear localization signal receptor, as well as Rip1p, a nuclear export signal-interacting protein. We propose that Gle2p has a novel role in mediating nuclear transport.

INTRODUCTION

For macromolecules too large to diffuse freely through the nuclear pore, transport across the nuclear envelope in either direction is an extremely selective process (Forbes, 1992; Gerace, 1995; Gorlich and Mattaj, 1996). Efforts by many groups have focused on elucidating the mechanism of protein import as well as the molecular composition of the nuclear pore complexes (NPCs[1]; Rout and Wente, 1994; Davis, 1995; Melchior and Gerace, 1995; Simos and Hurt, 1995). As with protein import, the export of proteins and ribonucleoprotein (RNP) particles through the NPCs is a facilitated and signal-dependent process (Izaurralde and Mattaj, 1995). However, the cellular factors required for export are less well defined.

The mechanism of nuclear import can be divided into at least two steps: nuclear localization signal (NLS)-dependent binding at the pore complex followed by nucleotide-dependent transport through the pore (Newmeyer and Forbes, 1988; Richardson *et al.*,

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[1] Abbreviations used: 5-FOA, 5-fluoroorotic acid; GLFG, glycine-leucine-phenylalanine-glycine; $Gal4_{BD}$, DNA-binding region of Gal4p; $Gal4_{AD}$, activation region of Gal4p; GST, glutathione S-transferase; $LexA_{BD}$, DNA-binding region of LexA; MBP, maltose-binding protein; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; ORF, open reading frame; poly(A)$^+$, poly-adenylated; Sec$^-$, nonsectoring; Sec$^+$, sectoring; TS$^-$, temperature sensitive; TS$^+$, nontemperature sensitive; Δ, null.

1988). Each of these steps is mediated by multiple proteins. A cytosolic receptor is required for recognition of NLSs, distinct cytosolic components facilitate docking at the NPC, and additional soluble factors carry out the subsequent post-docking energy-dependent translocation step (reviewed in Moore and Blobel, 1994; Powers and Forbes, 1994; Adam, 1995; Melchior and Gerace, 1995; Gorlich and Mattaj, 1996).

Recently, amino acid sequences that are necessary and sufficient for mediating the export of proteins from the nucleus have been characterized in mammalian cells. The nuclear export signals (NESs) for protein kinase inhibitor (Wen *et al.*, 1995), HIV-1 Rev protein (Fischer *et al.*, 1995), TFIIIA (Fridell *et al.*, 1996), and hnRNP protein A1 (Michael *et al.*, 1995) bear no similarity to NLSs (Dingwall and Laskey, 1991). Both the hnRNP A1 protein and the Rev protein specifically bind RNA and shuttle back and forth between the nucleus and cytoplasm (Daly *et al.*, 1989; Malim *et al.*, 1989; Zapp and Green, 1989; Pinol-Roma and Dreyfuss, 1992; Meyer and Malim, 1994). Thus, for protein-bound RNA molecules, the mechanism of RNA export may in essence be that of protein export.

If the mechanism of nuclear export is analogous to import, export would be a sequential process that begins with recognition of the NES and then targeting to the NPC for translocation to the cytoplasm. The yeast Rip1p (Stutz *et al.*, 1995) and the mammalian Rab/hRIP1 (Bogerd *et al.*, 1995; Fritz *et al.*, 1995) are candidates for a NES receptor that were isolated in independent two-hybrid screens. These proteins contain multiple FG amino acid repeats typical of NPC proteins (nucleoporins; Rout and Wente, 1994). Because *RIP1* is not an essential gene and its deletion is not reported to affect endogenous RNA export (Stutz *et al.*, 1995), other factors are probably involved in NES recognition and nuclear export. Export defects have been characterized with mutations in Gsp1p (the yeast homologue of a small Ras-like GTP-binding protein required for nuclear import, Ran/TC4; Belhumeur *et al.*, 1993; Kadowaki *et al.*, 1993; Melchior *et al.*, 1993; Moore and Blobel, 1993; Schlenstedt *et al.*, 1995) and in Prp20p (the yeast homologue of RCC1, a guanine nucleotide exchange protein for Ran/TC4; Aebi *et al.*, 1990; Bischoff and Ponstingl, 1991a,b; Amberg *et al.*, 1993; Kadowaki *et al.*, 1993; Cheng *et al.*, 1995). This has implied a requirement for GTP hydrolysis in RNA export.

Quantitative in vitro assays for nuclear export have not yet been developed, and thus genetic approaches are proving to be particularly useful in identifying potential export factors. Collections of temperature-sensitive mutant yeast strains have been directly screened for blocks in polyadenylated poly(A)$^+$ RNA export (Amberg *et al.*, 1992; Kadowaki *et al.*, 1992, 1994). Interestingly, numerous *rat* mutants isolated by Cole and coworkers encode for nucleoporins (*rat2/nup120*, *rat3/nup133*, *rat7/nup159*, and *rat9/nup85*; Gorsch *et al.*, 1995; Heath *et al.*, 1995; Li *et al.*, 1995; Goldstein *et al.*, 1996) that may play a direct role in export.

We have focused our efforts on studying a subset of five yeast nucleoporins that all possess an amino terminal region of tetrapeptide GLFG repeats separated by uncharged spacer sequences: this includes Nup49p, Nup57p, Nup100p, Nup116p, and Nup145p (Wente *et al.*, 1992; Wimmer *et al.*, 1992; Fabre *et al.*, 1994; Wente and Blobel, 1994; Grandi *et al.*, 1995). Nup116p, Nup100p, and, to a lesser extent, Nup145p are also related by sequence similarities that extend outside their GLFG regions (Wente and Blobel, 1994; Fabre *et al.*, 1994). Mutational analysis of the GLFG nucleoporins has revealed that they are required for the functional integrity of the NPC (Schlenstedt *et al.*, 1993; Wente and Blobel, 1993; Doye *et al.*, 1994; Fabre *et al.*, 1994; Wente and Blobel, 1994). The essential function of the GLFG region of Nup116p may be due to its interactions with Kap95p (Iovine *et al.*, 1995), an import factor required in the NPC-docking step (Adam and Adam, 1994; Chi *et al.*, 1995; Gorlich *et al.*, 1995; Radu *et al.*, 1995a).

To reveal the transport roles of yeast nucleoporins, several investigators have used genetic screens for new mutations which are lethal in combination with a mutation in a given known nucleoporin gene (Doye and Hurt, 1995). Such "synthetic lethal" screens with *nsp1*, *nup49*, *nup1*, and *pom152* have isolated mutated alleles of at least 10 other NPC-associated proteins required for NPC assembly and/or transport (Wimmer *et al.*, 1992; Belanger *et al.*, 1994; Aitchison *et al.*, 1995b; Doye and Hurt, 1995). In this study, a synthetic lethal genetic screen was conducted with the *nup100* null (Δ) mutation. Three different *gle* (for GLFG lethal) complementation groups were identified. *GLE1* encodes the first essential cellular RNA export mediator with a NES (Murphy and Wente, 1996). In this report, we have characterized the second complementation group from the *nup100*Δ screen and identified another potential nuclear export factor, Gle2p.

MATERIALS AND METHODS

Synthetic Lethal Screen to Identify gle1, gle2, and gle3

The strains used in this study are listed in Table 1. All DNA manipulations were conducted essentially as described by Sambrook *et al.* (1989). Yeast strains were grown in either YP (1% yeast extract, 2% bactopeptone) or synthetic minimal medium plus appropriate amino acids supplemented with 2% glucose (SD). Yeast transformations were completed using the lithium acetate method (Ito *et al.*, 1983) and general manipulations of yeast cells were conducted as described by Sherman *et al.* (1986). *Escherichia coli* strain DH5α was used as the bacterial host for all plasmids and was cultured in SOB medium and transformed using standard methods (Sambrook *et al.*, 1989).

A large part of the 2881-bp *NUP100* coding region was deleted by removal of the 2565-bp *Nsi*I-*Xba*I fragment from the middle of the

Table 1. Yeast strain genotypes

Strain	Genotype	Source
W303α	*MATα ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	
W303a	*MATa ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	
L40	*MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA::(lexAop)8-LacZ*	Gift from S. Hollenberg
HF7C	*MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal80-538 gal4-542 LYS2::GAL1$_{uas}$-GAL1$_{TATA}$-HIS3 URA3::GAL4$_{17mers(3X)}$-CYC1$_{TATA}$-lacZ*	Gift from H. Feilotter
YCH130	*MATa ade2-1 ade3::HISG ura3-1 his3-11,15 leu2-3,112 TRP1 lys2 can1-100*	Hardy, 1996
SWY122	*MATa nup145-7::LEU2 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	Wente and Blobel, 1994
SWY127	*MATa nup116-5::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can 1-100* + pSW131 (*NUP116 URA3 CEN*)	Wente and Blobel, 1994
SWY1013	*MATa gle2::HA-TRP1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can 1-100*	this study
SWY1016	*MATa gle2::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW532 (*gle2-G222D LEU2 CEN*)	this study
SWY1017	*MATα nup100::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1022	*MATα nup100::HIS3 ade2-1 ade3::HISG ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1024	*MATa nup100::HIS3 ade2-1 ade3::HISG ura3-1 his3-11,15 leu2-3,112 TRP1 lys2 can1-100*	this study
SWY1030	*MATα nup100::HIS3 ade2-1 ade3::HISG ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW201 (*NUP100 ADE3 URA3 CEN*)	this study
SWY1032	*MATa nup100::HIS3 ade2-1 ade3::HISG ura3-1 his3-11,15 leu2-3,112 TRP1 lys2 can1-100* + pSW201 (*NUP100 ADE3 URA3 CEN*)	this study
SWY1096	*MATα nup100::HIS3 gle1-1 ade2-1 ade3::HISG ura3-1, his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1110	*MATa nup100::HIS3 gle2-1 ade2-1 ade3::HISG ura3-1, his3-11,15 leu2-3,112 TRP1 lys2 can1-100*	this study
SWY1136	*MATα gle2-1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1143	*MATα gle1-1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1197	*MATα gle2-1 nup145-7::LEU2 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW190 (*NUP145 URA3 CEN*)	this study
SWY1201	*MATα gle1-1 nup145-7::LEU2 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW190 (*NUP145 URA3 CEN*)	this study
SWY1215	*MATα gle2-1 nup116-5::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW131 (*NUP116 URA3 CEN*)	this study
SWY1216	*MATα gle1-1 nup116-5::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW131 (*NUP116 URA3 CEN*)	this study
SWY1221	*MATa gle1-1 gle2-1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 TRP1 LYS2 can1-100* + pSW411 (*GLE1 URA3 CEN*)	this study
SWY1225	*MATa gle2::HIS3 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100*	this study
SWY1228b	*MATa/α GLE2/gle2::HIS3 ade2-1/ade2-1 ADE3/ADE3 ura3-1/ura3-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 LYS2/LYS2 can1-100/can1-100*	this study
SWY1298	*MATα gle1-1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW190 (*NUP145 URA3 CEN*)	this study
SWY1299	*MATα gle2-1 ade2-1 ADE3 ura3-1 his3-11,15 leu2-3,112 trp1-1 LYS2 can1-100* + pSW190 (*NUP145 URA3 CEN*)	this study

open reading frame (ORF) and coincident insertion of the 1247-bp *Nsi*I-*Xba*I *HIS3*-containing fragment from pJJ217 to make pSW180. Visible His$^+$ transformants were selected after the integrative transformation (Rothstein, 1991) of W303α cells with the 1780-bp *Eco*RI-*Xho*I fragment of pSW180 containing the *nup100::HIS3* construct. Southern blot analysis identified the strain (SWY1017) with the correct replacement of *NUP100*. This strain was mated to YCH130 (gift from C. Hardy), sporulated, and dissected to create the strains SWY1022 and SWY1024.

A 5155-bp *Sal*I-*Bam*HI fragment from pDK255 (gift from D. Koshland) containing *ADE3* was cloned into *Sal*I-*Bam*HI of pBSKS(+) to make pSW198. *ADE3* was reisolated as an *Xba*I-*Xho*I fragment from pSW198 and subcloned into *Xba*I-*Xho*I of pSW132 to make pSW201 (*NUP100 ADE3 URA3 CEN*). pSW201 was transformed into SWY1022 and SWY1024, creating the strains SWY1030 and SWY1032, respectively. These strains produced colonies exhibiting a high degree of sectoring when grown on nonselective medium. Ethylmethane sulfunate (EMS) mutagenesis of SWY1030 and SWY1032 was conducted as described by Lawrence (1991). SWY1030 and SWY1032 were grown in SD-ura liquid to saturation at 30°C and then washed in sterile H_2O and resuspended to a density of 5×10^7 cells/ml in 50 mM KPO_4, pH 7.0. EMS was added to a final concentration of 3% and cells were incubated at 30°C to 30% viability before quenching with 5% sodium thiosulfate. After one wash in H_2O, cells were diluted in H_2O and plated to YPD at ~325 colony-forming units/plate. Approximately 71,000 colonies from SWY1030 and 60,000 colonies from SWY1032 were screened for the nonsectoring phenotype (Sec$^-$) after 7 days of incubation at 23°C. Nonsectoring colonies were streaked to YPD plates and incubated for 7 days at 23°C to confirm the Sec$^-$ phenotype before plating to medium containing 1 mg/ml 5-fluoroorotic acid (5-FOA) to assess dependency on pSW201. Sec$^-$ and 5-FOA-sensitive strains were transformed with pSW78 (*NUP100 LEU2 CEN*; Wente *et al.*, 1992) or with pRS315 (*LEU 2 CEN*; Sikorski and Hieter, 1989) to identify and eliminate those strains retaining pSW201 for reasons unrelated to synthetic lethality with *nup100::HIS3*. Thirteen mutagenized strains derived from SWY1030 and 15 strains derived from SWY1032 were complemented for sectoring by pSW78 but not by pRS315. These strains were mated in all pairwise combinations and divided into four complementation groups (Table 2) by assaying for Sec$^-$ and 5-FOA-sensitive diploids. Members of each complementation group were tested for conditional growth on YPD at 15 and 37°C. All members of each complementation group were transformed with pSW226 (*NUP133 LEU2 CEN*), pSW229 (*POM152*

LEU2 CEN), pSW75 (*NUP116 LEU2 CEN*), pSW62 (*NUP49 LEU2 CEN*), pLG8 (*RAT7/NUP159.myc LEU2 CEN*; Gorsch *et al.*, 1995), or pRS315 and assayed for the ability to sector. Similarly, all members of each complementation group derived from SWY1030 (*trp1-1*) were transformed with pLDB107 (*NUP1 TRP1 CEN*; Belanger *et al.*, 1994), pKBB11 (*SRP1 TRP1 CEN*; Belanger *et al.*, 1994), pSW232 (amino terminal 100 kDa of *NUP145* in pRS314), or pRS314 (*TRP1 CEN*; Sikorski and Hieter, 1989) and tested for the ability to sector. The potentially novel *gle1*, *gle2*, and *gle3* alleles were backcrossed to SWY1030 or SWY1032 to confirm single-gene segregation patterns of mutant phentoypes. *gle1* and *gle2* alleles were crossed away from *nup100::HIS3* by mating to W303, sporulating, and dissecting single mutant haploid progeny.

To analyze potential synthetic lethal interactions with other previously characterized GLFG-containing nucleoporins, SWY122 was mated to SWY1298 and SWY1299. The resulting diploids were sporulated and then dissected to construct the haploid strains SWY1201 and SWY1197. The haploid strains SWY1216 and SWY1215 were constructed in an analogous manner from mating SWY1143 and SWY1136 with SWY127. Likewise, SWY1221 resulted from sporulation and dissection of an appropriate diploid. Since the *gle1-1*, *gle2-1*, and *nup116-5::HIS3* alleles were each temperature sensitive, SWY1216, SWY1215, and SWY1221 were backcrossed to W303 to confirm the presence of both mutations by independent assortment of the respective temperature-sensitive alleles. All double-mutant strains were patched to YPD plates along with W303α, SWY1143, SWY1136, SWY127, and SWY122 (transformed with pSW190) as control strains.

Cloning, Deletion, Mutation, and Epitope Tagging of GLE2

SWY1110 was transformed with a yeast genomic library in the vector p366 (American Type Culture Collection, stock 77162, deposited by P. Hieter). Approximately 9760 transformants were screened for the ability to sector after 7 days of growth at 23°C on SD-leu plates. Sectoring colonies were plated to YPD at 37°C for 2 to 3 days to test for complementation of the temperature-sensitive (TS^-) phenotype. One transformant grew very well at 37°C and was plated to 5-FOA to confirm its ability to lose pSW201. The rescuing library plasmid (pSW396) was then recovered from the yeast cells and transformed into DH5α cells. After reisolation from bacteria, pSW396 was transformed into SWY1096 and SWY1110 to retest its ability to rescue or not rescue the 5-FOA-sensitive, Sec^-, and TS^- phenotypes of these strains.

Using oligonucleotide primers hybridizing to the *tet* gene sequence immediately flanking the insertion point of the genomic fragment in pSW396, the ends of the complementing library fragment were sequenced with the dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase kit version 2.0 (United States Biochemical, Cleveland, OH). The resulting DNA sequence was compared with the database sequence. To create pSW408 (*NUP157 LEU2 CEN*), two oligonucleotides (289 and 290) were used in the polymerase chain reaction (PCR) with genomic yeast DNA to create *Bam*HI restriction sites flanking the ORF 808 bp upstream and 204 bp downstream, respectively, for insertion into pRS315. In a similar manner, pSW606 (*PHD5 LEU2 CEN*) was constructed using oligonucleotides (348 and 349) positioned 995 bp upstream and 307 bp downstream of *PHD5*. pSW406 (*GLE2 LEU2 CEN*) was constructed using oligonucleotides (287 and 288) positioned 748 bp upstream and 260 bp downstream to create a *Xba*I restriction fragment for insertion into pRS315. Likewise, oligonucleotides (233 or 235) with a *Bgl*II site at the initiation methionine for Gle2p were used to create restriction fragments for insertion into pGEX-3X (Pharmacia, Uppsala, Sweden), pMAL-cRI (Maina *et al.*, 1988), or pCH358 (Hardy, 1996), generating pSW424, pSW431, and pSW432, respectively. The pSW432 construct expressing a Gal4p-activation domain ($Gal4_{AD}$)-Gle2p fusion protein complemented the severe growth phenotype of *gle2Δ* strains.

The *gle2-G222D* mutation was made by site-directed mutagenesis with two oligonucleotides (322 and 323) and the PCR. The mutated DNA was subcloned into pRS315, yielding pSW532. The site of the mutation was confirmed by DNA sequence analysis. The *gle2* null strain was made using the method of Baudin *et al.* (1993) with pBM2815 and two 64-mer oligonucleotides (5′HISΔ = 229; 3′HISΔ = 230). This generated an ~1100-bp *HIS3* fragment flanked on the 5′ end with 45 bp of sequence from bp +9 to −36 of *GLE2* and on the 3′ end with 45 bp of sequence from bp 1103–1147. The fragment was transformed into the diploid W303. The heterozygous null genotype *GLE2/gle2::HIS3* (SWY1228b) was confirmed by genomic colony PCR and by complementation with pSW407.

To epitope tag *GLE2*, oligonucleotides (231 and 232) were used in the PCR to create a restriction fragment consisting of the *GLE2* ORF flanked by a 5′ *Sac*I site and an in-frame 3′ *Bgl*II site replacing the GLE2 stop codon. A 240-bp *Bgl*II-*Eco*RI restriction fragment from pCH606 (gift from C. Hardy) encoding three repeats of the HA epitope YPYDVPDYA was fused in-frame to the 3′ end of the *GLE2* fragment generated above. An ~1-kb *Eco*RI-*Sal*I fragment carrying the yeast ADH terminator from pCH583 (gift from C. Hardy) was then inserted directly 3′ to the HA repeats. The entire *GLE2*-HA(3X)-tADH fusion construct was subcloned into *Sac*I-*Sal*I of the integrating vector pRS304 to yield pSW426. The *GLE2*-HA tagged construct was targeted to the endogenous *GLE2* locus in diploid W303 cells by transformation of *Cla*I-digested pSW426 and selecting for Trp^+ integrative transformants. Proper insertion was confirmed by genomic colony PCR. The diploid was sporulated and dissected to yield the tagged haploid strain (SWY1013) and used for localization.

Gle2p Antibodies and NPC Fractionation Analysis

Full-length Gle2p was fused in-frame behind the maltose-binding protein (MBP; pSW431), expressed, and purified from DH5α bacterial cells as follows. Fusion protein synthesis was induced by growth in LB-rich/amp media (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose, 50 μg/ml ampicillin) to logarithmic phase, the addition of isopropyl β-D-thiogalactopyranoside to 0.3 mM, and continued growth at 37°C for 4 h. Bacteria were harvested and lysed by treating with 1 mg/ml lysozyme in 300 mM NaCl, 50 mM sodium phosphate (pH 8) for 30 min on ice and sonicated for a total of 15 min (cycles of 1-min bursts, 1-min rests). The lysate was centrifuged at 4°C for 30 min at 10,000 × *g*. The protein concentration was diluted to 50 mg/ml, and the fusion protein was purified using amylose resin (New England Biolabs, Beverly, MA) according to the manufacturer's directions. The antigen was sent to Cocalico for production of rabbit antiserum WU849. Antiserum to the full-length Gle2p was purified by affinity chromatography over a glutathione *S*-transferase (GST)-Gle2p Affi-Gel 10 (Bio-Rad, Hercules, CA) column that was made by coupling with bacterially expressed, purified GST-Gle2p fusion protein. GST-Gle2p was purified from DH5α cells transformed with pSW424 via induction with 0.3 mM isopropyl β-D-thiogalactopyranoside and cell lysis as described above, but with the final step utilizing glutathione agarose (Sigma, St. Louis, MO) chromatography.

An enriched fraction of detergent-solubilized NPCs was prepared from the wild-type diploid W303 strain as described by Rout and Blobel (1993), except the protocol was scaled proportionally down to begin with 18 l of early logarithmic phase cells. Total yeast cell lysates were made as described by Yaffe and Schatz (1984). Protein samples were separated by electrophoresis in a 9% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose membranes. Blots were probed with affinity-purified rabbit polyclonal anti-Nup116p carboxyl-terminal antibody (Iovine *et al.*, 1995) at a 1:2500 dilution (1 h at room temperature) or affinity-purified rabbit polyclonal anti-Gle2p antibody at a 1:25 dilution (2 h at room temperature). All dilutions were made in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST)/2% milk. With washes between, the blots were then incubated with affinity-purified alka-

line phosphatase-conjugated anti-rabbit IgG (Promega, Madison, WI; diluted 1:7500 in TBST) for 1 h. Blots were developed via color visualization with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate (Promega).

Immunofluorescence and Electron Microscopy

W303α and SWY1136 (*gle2-1*) cells were prepared for immunofluorescence experiments as described by Wente *et al.* (1992). Cells were grown to early log phase in YPD liquid at 23°C and were shifted to 37°C for varying times before fixation. Fixed cells were incubated with the appropriate antibody [undiluted tissue culture supernatant (tcs) MAb D77 (Henriquez *et al.*, 1990) for detection of Nop1p; 1:100 diluted tcs MAb 5B5 (gift from M. Rout and J. Kilmartin) for detection of Nsr1p; 1:50 diluted anti-β-galactosidase MAb from Boehringer Mannheim (Mannheim, Germany); undiluted tcs MAb 12CA5 (Berkeley Antibody, Berkeley, CA) for detection of HA-Gle2p; undiluted tcs MAb 414 (Davis and Blobel, 1986) for detection of NPCs] for 16 h at 4°C. After washing with 40 mM K_2HPO_4, 10 mM KH_2PO_4, 150 mM NaCl, 0.1% NaN_3, 0.1% Tween 20, and 2% nonfat dry milk (M buffer) alone, detection of bound antibody was accomplished by incubation with affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Cappel Laboratories, Organon Teknika, Durham, NC) at a 1:100 dilution for 1 h at room temperature. The final washes in M buffer and then 1% bovine serum albumin-phosphate-buffered saline were followed by mounting with 90% glycerol, 1 mg/ml *p*-phenylenediamine, and 0.05 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) at pH 8.0. Photographs were taken with the 100× objective on an Olympus microscope with Kodak T-MAX 400 film.

Preservation of both protein and membrane structures in the electron microscopic studies was obtained using the protocols described in Wente and Blobel (1993). Briefly, W303α and SWY1136 cells were grown to early logarithmic phase in YPD at 23°C before shifting to 37°C for 3.5 h and immediate fixation by resuspension of the cell pellet in 40 mM K_2HPO_4-KH_2PO_4 (pH 6.5) and 0.5 mM $MgCl_2$ containing 2% glutaraldehyde-2% formaldehyde (incubation 30 min on ice). After cell wall digestion and osmium postfixation (Byers and Goetsch, 1991), the samples were embedded in Epon. Thin sections collected on nickel grids coated with formvar, stabilized with carbon, were contrasted by staining with uranyl acetate and Reynold's lead. Specimens were visualized with a Zeiss-902 electron microscope, and photographs were recorded with Kodak electron microscopy film.

Nuclear Transport Assays

For analysis of nuclear export, the localization of poly(A)$^+$ RNA was detected by in situ hybridization. Early logarithmic phase cultures of W303α or SWY1136 were grown in YPD at 23°C before shifting to 37°C for either 0 min, 30 min, 1.5 h, 3.5 h, or 5 h. The cells were processed as described for immunofluorescence through the methanol/acetone dehydration step. The cell-coated slides were rinsed with sterile 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7), and the subsequent in situ hybridization steps were conducted exactly as described by Forrester *et al.* (1992). The oligonucleotide $(dT)_{30}$ probe was end labeled by terminal transferase (Life Technologies, Gaithersburg, MD) with digoxigenin-11-dUTP (Boehringer Mannheim; Amberg *et al.*, 1992). Antidigoxigenin-fluorescein Fab fragments were obtained from Boehringer Mannheim. Photographs were taken with the 100× objective on an Olympus microscope with Kodak T-MAX 400 film.

Nuclear import capacity was assessed by monitoring the localization of Nop1p, Nsr1p, and a *GAL1*-induced green fluorescence protein (GFP)-histone H2B1 fusion protein. Localization of Nop1p and Nsr1p was assessed in temperature-arrested *gle2* mutant cells by indirect immunofluorescence microscopy as described above. Alternatively, the *gle2* mutant strains were transformed with pJON280 (*GAL1-GFP-H2B1 URA3*; Schlenstedt *et al.*, 1995) and grown to early logarithmic phase at 33°C in SC-ura/raffinose media before shifting to growth in galactose for 7 h at 35°C. The cells were then directly observed with fluorescence microscopy for nuclear versus cytoplasmic localization of GFP-H2B1.

Two-Hybrid Interaction Analysis

Two-hybrid interactions were assayed either with $LexA_{BD}$ and $Gal4_{AD}$ fusions in yeast strain L40 or with $Gal4_{BD}$ and $Gal4_{AD}$ fusions in yeast strain HF7C. Strains were tested for β-galactosidase expression levels using the filter assay described in the study of Breeden and Nasmyth (1985). Plasmids tested for Gle2p interactions include pSW365 (full-length Srp1p in $LexA_{BD}$; gift from K. Mishra), pSW310 (full-length Kap95p in $LexA_{BD}$; Iovine *et al.*, 1995), pSW262 (the GLFG region of Nup116p in $Gal4_{BD}$; Iovine *et al.*, 1995), pSW546 (the carboxyl-terminal region of Nup116p in $Gal4_{BD}$; gift from A. Ho), pSW392 (the GLFG region of Nup100p in $Gal4_{AD}$; Murphy and Wente, 1996), pSW423 (the carboxyl-terminal region of Nup100p in $Gal4_{BD}$), pSW259 (the amino terminal region of Nup145p in $Gal4_{BD}$; gift from J. Emtage), pSW257 (the carboxyl-terminal region of Nup145p in $Gal4_{BD}$; gift from J. Emtage), pSW379 (full-length Nup133p in $Gal4_{BD}$; gift from M. Bucci), pSW457 (full-length Gle1p in $LexA_{BD}$; Murphy and Wente, 1996), and pSW485 (full-length Rip1p in $LexA_{BD}$; Murphy and Wente, 1996).

RESULTS

nup100 Synthetic Lethal Screen

To isolate and characterize novel factors required for nuclear transport, a genetic screen was conducted in the yeast *Saccharomyces cerevisiae*. The *nup100*Δ strain was a good candidate for a synthetic lethal screen because the *nup100*Δ mutation has no obvious growth defects (Wente *et al.*, 1992) and *nup100* mutated alleles had not been reported from other genetic screens. Mutations that were lethal in combination with the *nup100*Δ mutation were isolated using a colony-sectoring assay (Bender and Pringle, 1991), which relies on the difference in colony color between *ade2* strains (red) and *ade2 ade3* strains (white). Haploid *ade2 ade3 nup100*Δ strains carrying a *NUP100 ADE3 URA3 CEN* plasmid formed red/white-sectored colonies on rich medium, reflecting their capacity to lose the *NUP100 ADE3* plasmid. Haploid yeast strains of opposite mating types (SWY1030 and SWY1032) were independently mutagenized with EMS until ~30% of the cells remained viable and screened for the loss of the ability to sector (red colonies). The nonsectoring (Sec$^-$) colonies were tested for sensitivity to 5-FOA and consequently dependence on the *NUP100 URA3* plasmid. A total of 28 mutants were isolated from a total of ~130,000 colonies.

The mutants were placed into complementation groups by mating pairs of the respective *MATα* and *MATa* strains and selecting for Lys$^+$Trp$^+$ diploids. Mutants that formed 5-FOA-sensitive and Sec$^-$ diploids were placed in the same complementation group (Table 2). We previously observed that the combination of *nup116*Δ and *nup100*Δ alleles rendered cells inviable at all growth temperatures (Wente *et al.*, 1992; Wente and Blobel, 1994). Thus, one of the complemen-

Table 2. Complementation analysis of *nup100* synthetic lethal alleles

	Genetic		Molecular		
Group	No. of alleles	Temperature sensitivity	*NUP100*	*NUP116*	Other NUPS
nup116	19	All−	+	+	−
gle1	5	All+	+	−	−
gle2	3	All+	+	−	−
gle3	1	−	+	−	−

5-FOA sensitive, nonsectoring (Sec$^-$) strains from the *nup100Δ* synthetic lethal screen were divided into four complementation groups based on genetic and molecular criteria. Strains were mated in all pairwise combinations and assayed for 5-FOA sensitive, Sec$^-$ diploids. Members of each genetic complementation group were then tested for molecular complementation (+) of their mutant phenotypes by transformation with a panel of plasmids bearing known nucleoporins (see MATERIALS AND METHODS).

tation groups was likely comprised of *nup116* mutations. It was also possible that we had identified mutant alleles of known nucleoporin genes whose interaction with *nup100* had not been documented. Therefore, representatives of each complementation group were tested for restoration of the sectoring phenotype with plasmids bearing *NUP116*, *NUP49*, *NUP145*, *SRP1*, *NUP1*, *POM152*, *RAT7/NUP159*, or *NUP133*. The largest complementation group (19 alleles) was rescued by *NUP116*. For the other three complementation groups, sectoring was not restored with any of the plasmids. Therefore, these groups were designated *gle* (for GLFG lethal) mutants.

Growth of mutant strains at a variety of temperatures was examined to identify conditional alleles. The five *gle1* alleles and three *gle2* alleles were all temperature sensitive with varying degrees of severity, whereas the single *gle3* allele was viable at all growth temperatures. When the mutants were backcrossed to wild-type parental *ade2 ade3 nup100Δ* haploids, the Sec$^-$ phenotype segregated 2:2 from sporulated diploids, indicating that each mutation resided in a single gene.

Genetic Interactions of GLE1 and GLE2 with NUP116 and NUP145

Because *NUP100*, *NUP116*, and *NUP145* share significant amino acid sequence similarities outside their GLFG regions and because they possess apparent functional redundancies (Fabre *et al.*, 1994; Wente and Blobel, 1994), we predicted that screening for novel genes synthetically lethal with *nup100Δ* would also identify genes related to *NUP116* and *NUP145*. Similarly, genes synthetically lethal with *nup100Δ* may also interact with one another. To test these hypotheses, the phenotypes of the temperature-sensitive *gle1* and *gle2* mutants in combination with *nup116* or *nup145* mutations, and with each other, were evaluated. Synthetic lethal tests with *gle3* were not conducted because conditional or marked *gle3* alleles were not available. Double-mutant strains were constructed bearing *CEN/URA3* plasmids covering one of the two mutations (see MATERIALS AND METHODS). As shown in Figure 1, these strains were patched to YPD and then assayed for synthetic lethality by their ability to grow at 23°C after replica plating to medium containing 5-FOA (which is toxic in the presence of *URA3*). Cells carrying both *gle1-1* and *nup116Δ* were unable to lose a *CEN/URA3* plasmid bearing *NUP116*, indicating that these two mutations were synthetically lethal. In contrast, *nup116Δ gle2-1*, *nup145ΔN gle1-1*, *nup145ΔN gle2-1*, and *gle1-1 gle2-1* double mutants were viable at 23°C.

To test for lethality at higher temperatures, patches growing on 5-FOA were replica plated to YPD and incubated at 30, 34, and 37°C (Figure 1). The viability of cells with both *gle1-1* (with a restrictive temperature of 34°C) and *nup145ΔN* (a deletion of the amino ter-

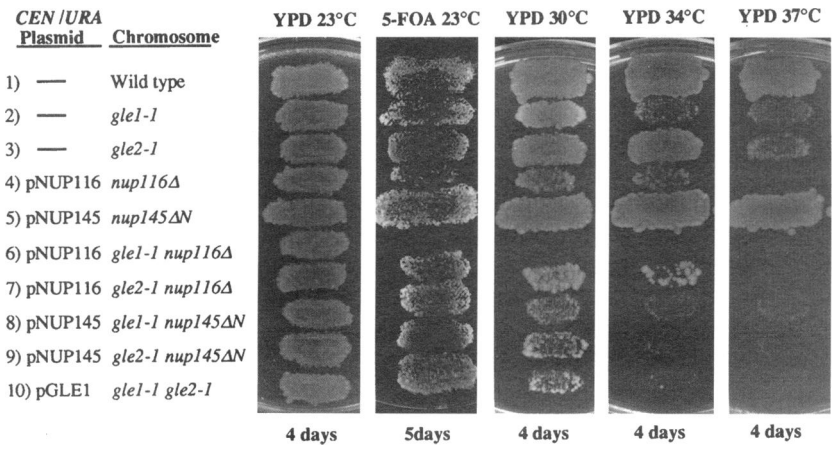

Figure 1. Synthetic lethal analysis with *gle1* and *gle2*. Mutant alleles of *GLE1* and *GLE2* display varying degrees of synthetic lethality with mutations in the genes encoding GLFG nucleoporins Nup116p and Nup145p. Double-mutant haploid strains harboring *CEN/URA3* plasmids with a wild-type copy of one of the mutated genes were generated as described in MATERIALS AND METHODS. These strains were patched to YPD at 23°C and incubated for 4 days before replica plating to medium containing 5-FOA. After 5 days at 23°C, the patches on 5-FOA were replica plated to YPD and incubated at 30, 34, and 37°C for 4 days.

minal half of *NUP145* which is not temperature sensitive; Wente and Blobel, 1994) mutations was unchanged from that of *gle1-1* alone. In contrast, cells carrying both the *gle2-1* and *nup145ΔN* mutations were dead at 34°C, a temperature at which strains carrying *gle2-1* alone or *nup145ΔN* alone grew very well. Double *gle1-1 gle2-1* mutants were sick at 30°C when compared with the corresponding single mutant strains. However, *gle1* and *gle2* were not strongly synthetically lethal because growth of the double *gle1-1 gle2-1* strain did not cease until 34°C (the same as *gle1-1* alone). The observation that *GLE1* interacted strongly with *NUP116* and less so with *NUP145*, whereas almost the opposite was true of *GLE2*, suggested that *GLE1* and *GLE2* were potentially distinct in function. Further characterization of *GLE1* is reported elsewhere (Murphy and Wente, 1996) and discussed below. In this report, the characterization of *GLE2* is detailed.

GLE2 Encodes a 40.5-kDa Polypeptide with Similarity to Schizosaccharomyces pombe RAE1

The wild-type *GLE2* gene was identified by complementation of the *gle2-1* mutant nonsectoring phenotype with a *CEN LEU2* yeast genomic library. Sectoring strains were subsequently plated to YPD and assayed for growth at 37°C because genomic inserts containing *NUP100* could rescue the nonsectoring phenotype but not the temperature sensitive phenotype. One sectoring transformant grew very well at 37°C. The library plasmid in this strain was isolated and retransformed into both *gle1-1 nup100Δ* and *gle2-1 nup100Δ* cells. As expected, only the *gle2-1 nup100Δ* mutant strain was rescued.

By DNA sequence analysis and comparison to the database from the yeast genome sequencing project, the library plasmid insert contained the region of chromosome V shown in Figure 2A. The library fragment was truncated such that the ORF encoding a polypeptide with a predicted molecular mass of 122 kDa was not flanked by any promoter sequence. Moreover, a plasmid (pSW606) harboring only *PHD5* (a gene required for filamentous growth, accession number U51431) did not complement the *gle2*-1 Sec^- and TS^- phenotypes. Therefore, two likely complementing genes remained. One of the ORFs encoded the amino terminal portion of a known nucleoporin, Nup157p (Aitchison *et al.*, 1995b). However, a plasmid harboring only full-length *NUP157* also did not rescue the *gle2-1* Sec^- and TS^- phenotypes. A second likely candidate for *GLE2* was an ORF spanning 1098 bp that is designated YER107c in the database and encodes a 40.5-kDa protein with striking similarity to *RAE1* from *S. pombe*. When this ORF was tested, it was sufficient for complementation of the *gle2-1* mutant. In addition, the phenotype of a null strain for this ORF was identical to that of the *gle2-1* and *gle2-G222D* mutants in terms of RNA export inhibition and nuclear pore complex clustering (see below and our unpublished observations). Therefore, this gene was designated *GLE2*.

The amino acid sequence similarity between Gle2p and Rae1p extends over their entire length (Figure 2B). Alignment of the sequences gave an overall match of 82% (with 52% identical and 32% similar). The four putative β-transducin/WD repeats in Rae1p (Brown *et al.*, 1995) are largely conserved in Gle2p (underlined, Figure 2B). Mutation of the glycine residue at position 219 in Rae1p to glutamic acid results in a temperature-sensitive RNA export mutant (Brown *et al.*, 1995). By site-directed mutagenesis, the glycine residue at position 222 in Gle2p was changed to an aspartic acid, and the mutated gene was expressed in a *gle2Δ* background (see below). By most criteria, including temperature-dependent NPC clustering and poly(A)$^+$ RNA export defects (see below), the *gle2-G222D* mutant behaved the same as the *gle2-1* mutant (the strongest temperature-sensitive allele isolated in the *nup100Δ* screen). However, the temperature-sensitive phenotype of the *gle2-G222D* allele was rescued by growth on media containing 1 M sorbitol, whereas *gle2-1* cells were not (Figure 2C). Such suppression by 1 M sorbitol has been reported for temperature-sensitive mutant alleles of *RAT2/NUP120*, *RAT7/NUP159*, *RAT3/NUP133*, and *NUP116* (Heath *et al.*, 1995).

In *S. pombe*, *RAE1* is an essential gene (Brown *et al.*, 1995). To test whether *GLE2* encoded an essential gene product, the chromosomal copy of *GLE2* was replaced by homologous recombination with *HIS3*. Sporulation and dissection of a heterozygous *gle2::HIS3* diploid strain resulted in the recovery of only two viable His^- spores per tetrad after growth for 5 days at 23°C. However, after 11 days at 23°C *gle2Δ* His^+ microcolonies appeared. These *gle2Δ* cells grew very slowly at 23°C, and they were markedly temperature sensitive. The phenotype of the disruption was rescued by the presence of a single-copy *URA3* plasmid bearing an intact *GLE2* gene. Although *GLE2* is not strictly essential, it is required for normal cell growth.

Gle2p Is Associated with NPCs

The localization of Rae1p in *S. pombe* cells is reported as cytoplasmic (Brown *et al.*, 1995). The subcellular localization of epitope-tagged-Gle2p was determined by indirect immunofluorescence microscopy. Three tandem repeats of the HA epitope from the influenza virus hemagglutinin protein were fused in-frame at the carboxyl-terminus of Gle2p. The epitope-tagged gene was chromosomally integrated in place of the wild-type gene, and it sustained wild-type growth at all temperatures. At short fixation times, the Gle2p-HA was concentrated in a punctate pattern at the nuclear periphery (Figure 3). This staining pattern

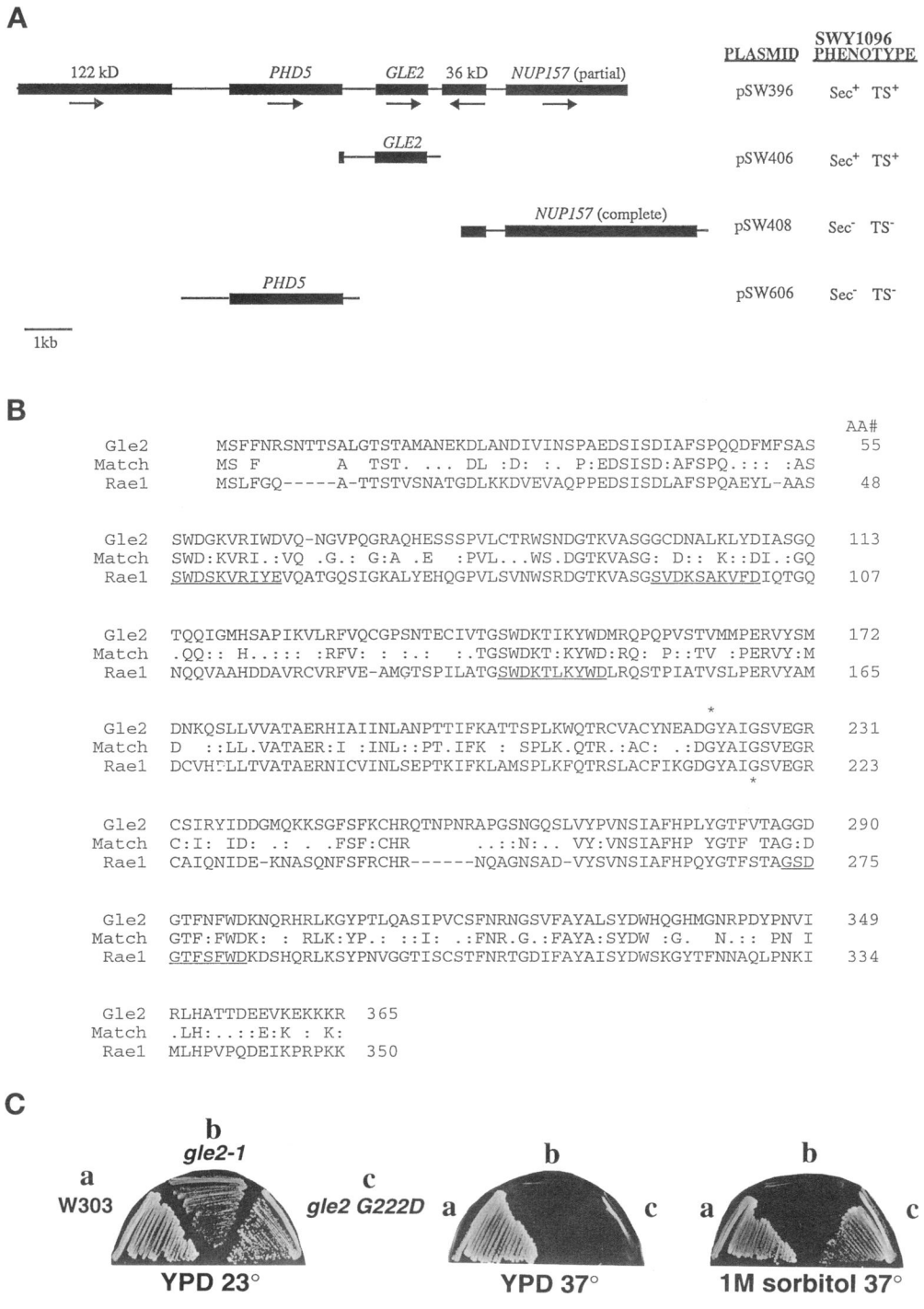

Figure 2. Cloning of *GLE2*. (A) A fragment sufficient for rescuing the *gle2-1* nonsectoring (Sec$^-$) and temperature-sensitive (TS$^-$) phenotypes was isolated from a yeast genomic plasmid library (pSW396) in strain SWY1096 (*nup100Δ gle2-1*). The library insert contained three complete ORFs, one ORF without a promoter, and one partial ORF encoding ~75% of Nup157p. The *PHD5*, full-length *NUP157*, and a putative *GLE2* were each individually subcloned in *CEN/LEU2* plasmids by PCR amplification of genomic DNA with oligonucleotides flanking the promoter and termination sequences. A plasmid harboring *GLE2* alone was capable of complementing the *gle2* mutant strain. (B) Alignment of the Gle2p sequence with the *S. pombe* Rae1p. An ALIGN analysis (Dayhoff *et al.*, 1983) between the full-length proteins revealed significant homology. The middle line designates identical residues (capital letter) and conserved/similar residues (:/.) between the two sequences. The four underlined blocks of 10 residues in Rae1p mark the carboxyl-terminal end of four putative β-transducin/WD repeats (Brown *et al.*, 1995). Changing the starred glycine residues at position 222 in Gle2p and 219 in Rae1p, respectively, to aspartic acid (C) and glutamic acid (Brown *et al.*, 1995) generates temperature-sensitive mutations. Accession number YER107C. (C) *gle2-1* and *gle2-G222D* mutants display differential temperature sensitivity. Wild-type (W303a), *gle2-1*, and *gle2-G222D* mutant strains were plated on YPD at 23 and 37°C for 3 days or on YPD supplemented with 1 M sorbitol at 37°C for 3 days. The temperature sensitivity of *gle2-G222D* but not *gle2-1* was suppressed by 1 M sorbitol.

is typical of yeast nuclear pore complexes (Davis and Fink, 1990). However, a low level of cytoplasmic staining was also detected.

To confirm the NPC localization, polyclonal antibodies were raised against a bacterially expressed MBP-Gle2p fusion protein. The antibodies were affinity purified, and their staining pattern in immunofluorescence analysis was identical to that of the Gle2p-HA (although weaker overall staining was observed; our unpublished observations). The anti-Gle2p antibody was also used to test for the presence of Gle2p in an enriched preparation of deter-

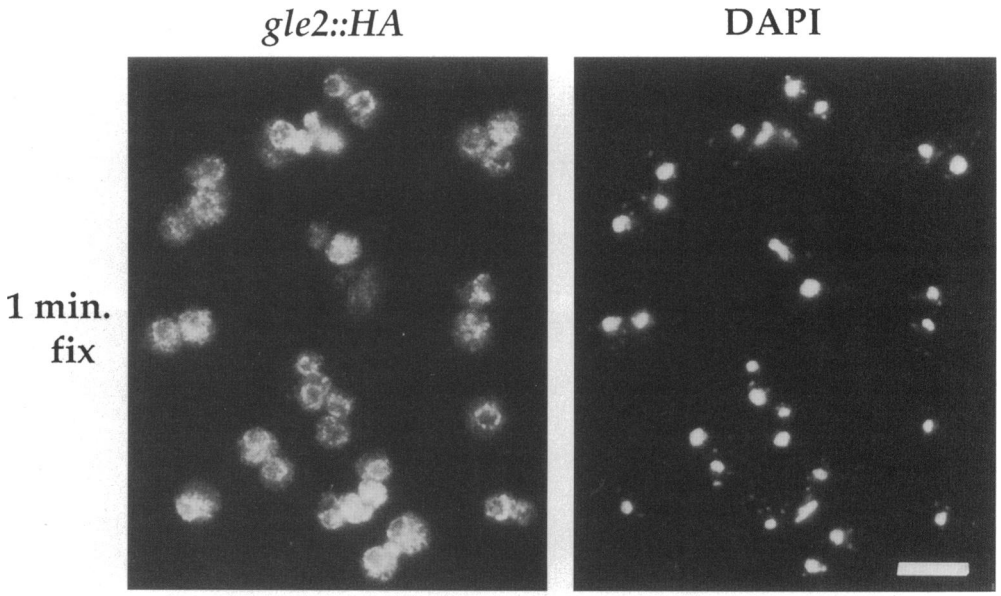

Figure 3. Gle2p is localized both in the cytoplasm and at NPCs. *GLE2* was tagged at its carboxyl-terminus with a sequence encoding three repeats of the HA epitope. The tagged gene was chromosomally integrated in place of wild-type *GLE2* (SWY1013). The *gle2-HA* cells were grown to an early logarithmic phase in YPD liquid medium at 23°C before fixation in methanol/formaldehyde as described in MATERIALS AND METHODS. Fixed cells were incubated with MAb 12CA5, and binding was detected with a FITC-conjugated goat anti-mouse IgG. Punctate, peripheral nuclear rim staining characteristic of NPC localization was predominant, although cytoplasmic staining was also above background levels. Coincident DAPI staining is shown to the right. Bar, 5 μm.

gent-solubilized yeast NPCs. An immunoblot (Figure 4) with samples of both total yeast cell lysate and enriched NPCs was divided in half and probed with either the anti-Gle2p antibody or an affinity-purified polyclonal antibody recognizing Nup116p (Iovine *et al.*, 1995). Gle2p migrated with an apparent molecular mass of 40 kDa and was present in the enriched NPC fraction. Interestingly, the Gle2p band appeared as a doublet in the NPC sample. The physical difference between these Gle2p bands has not been determined.

GLE2 Is Required for poly(A)$^+$ RNA Export

To test for perturbations of NPC function, the *gle2-1*, *gle2Δ*, and *gle2-G222D* mutants were assayed for nuclear export and import capacity. They behaved the same in all assays tested. Nuclear export was monitored in *gle2* cells shifted to 37°C for various periods of time by in situ localization of poly(A)$^+$ RNA. When compared with the diffuse cytoplasmic staining in wild-type cells, nuclear accumulation of poly(A)$^+$ RNA was detected in at least 25% of the *gle2-1* cells even at 23°C (Figure 5). The nuclear accumulation was noticeably enhanced with incubation at 37°C. This export defect is similar to that reported for *rae1-1 S. pombe* cells. Interestingly, in the majority of the *gle2* cells, the poly(A)$^+$ RNA staining was enhanced in the periphery of the nucleus. Depending on the focal plane (Figure 5, e, g, and i), the staining was ring-like and/or punctate. This discrete nuclear localization pattern is very similar to that reported for temperature-arrested *nup116Δ* cells (Wente and Blobel, 1993).

To monitor protein import capacity, two different assays were used. First, the distribution of endogenous Nsr1p and Nop1p was determined by indirect immunofluorescence microscopy. For both, the signal remained concentrated in the crescent-shaped nucleolus of temperature-arrested *gle2* cells and cytoplasmic staining remained at background levels (our unpublished observations). Therefore, the import of these two proteins was not perturbed in the *gle2* mutants. Second, a GFP-histone H2B1 fusion protein under the control of the *GAL1* promoter was expressed in *gle2* cells. After growth to early logarithmic phase in raffinose media at 33°C, GFP-histone production was induced in galactose media at a semipermissive temperature of 35°C. Samples were collected and examined directly by fluorescence microscopy. The subcellular distribution of the GFP-histone remained exclusively nuclear (our

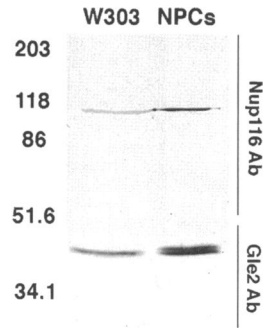

Figure 4. Gle2p is present in a detergent-solubilized, enriched NPC preparation. Samples of total yeast lysate and an enriched preparation of detergent-solubilized NPCs were prepared from W303 cells, separated electrophoretically on a 9% SDS-polyacrylamide gel, and transferred to nitrocellulose. The blot was divided in half, and the top half was probed with an affinity-purified antibody recognizing Nup116p. The bottom half was probed with an anti-Gle2p affinity-purified antibody. The nucleoporin Nup116p (upper 116-kDa band) was coenriched with NPCs. A single band of ~40 kDa, corresponding to Gle2p, was present in both the W303 lysate and enriched NPC fraction. The Gle2p band was a doublet in the enriched NPC fraction.

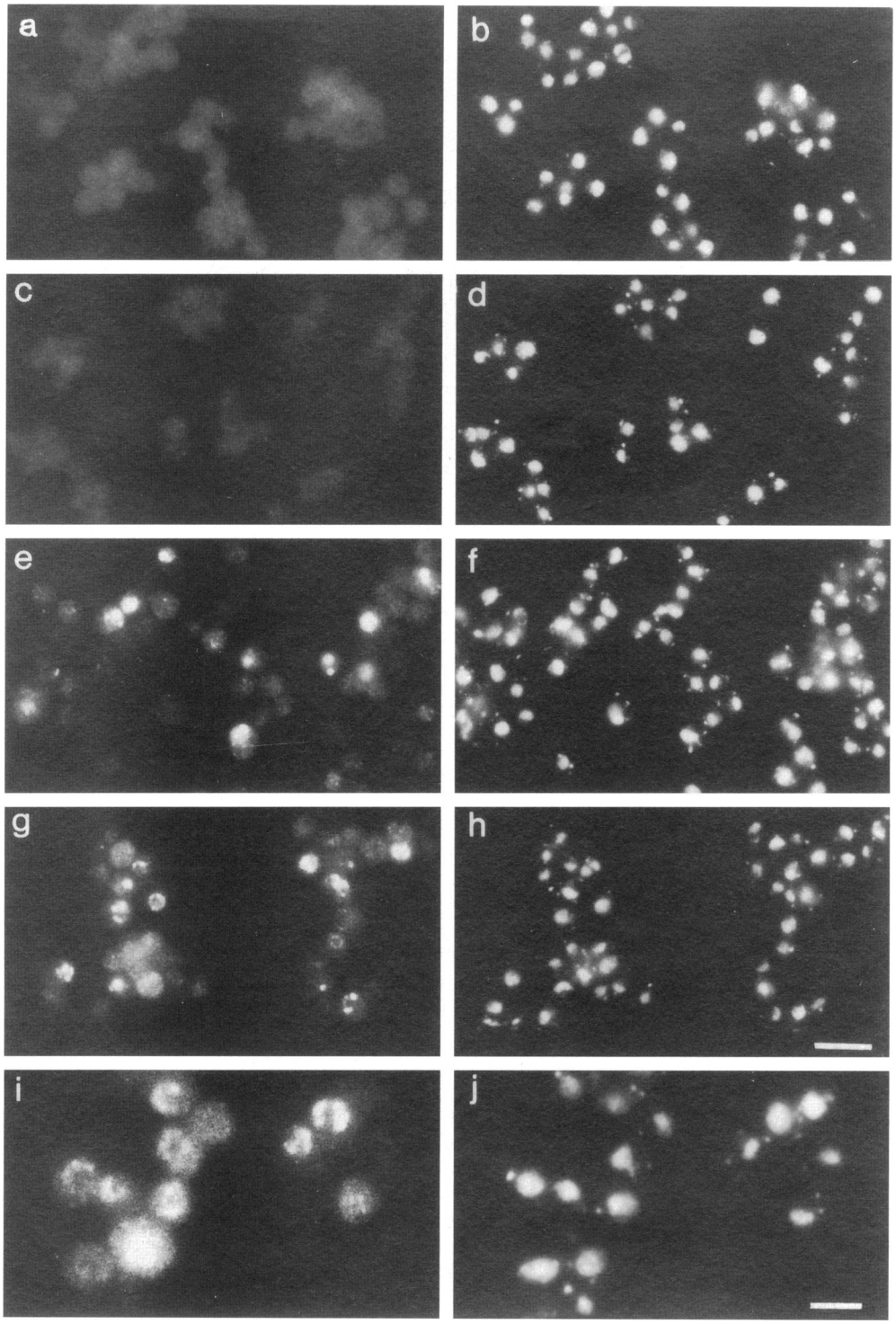

Figure 5. Poly(A)$^+$ RNA export is blocked in *gle2-1* cells. Wild-type (W303; a–d) and *gle2-1* cells (e–j) were grown to early logarithmic phase in YPD at 23°C. Aliquots were maintained at either 23°C (a, b, e, and f) or shifted to 37°C (c, d, g, and h–j) for 30 min before methanol/formaldehyde fixation. Fixed cells were processed for in situ hybridization with a digoxigenin-labeled oligonucleotide poly(dT) probe as described in MATERIALS AND METHODS (a, c, e, g, and j). Coincident DAPI staining is shown (b, d, f, h, and i). The nuclei of *gle2-1* cells accumulated poly(A)$^+$ RNA in ~25% of the cells at 23°C (e). This increased to ~50% of the cells after a 30-min shift to the nonpermissive temperature, 37°C (g and i). The poly(A)$^+$ RNA signal often appeared in a punctate, ring-like pattern around the nuclear periphery (g and shown at higher magnification in i). Bars: a–h, 5 μm; i and j, 2.5 μm.

unpublished observations). Thus, protein import was not noticeably diminished. This suggested that Gle2p is specifically required for a NPC export function.

NPC and Nuclear Envelope Morphology Is Altered in gle2 Mutants

In *S. cerevisiae*, many RNA export mutants also exhibit morphological perturbations of the NPC and/or nu-

clear envelope (Wente and Blobel, 1993; Bogerd *et al.*, 1994; Doye *et al.*, 1994; Aitchison *et al.*, 1995a; Gorsch *et al.*, 1995; Heath *et al.*, 1995; Li *et al.*, 1995; Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996). Freeze-fracture and thin-section electron microscopic analysis showed wild-type NPCs distributed over the nuclear surface at a density of ~15 NPC/μm^2 (Severs *et al.*, 1976; Jordan *et al.*, 1977). Mutations in *NUP145* (Wente and Blobel, 1994), *NUP133* (Doye *et al.*, 1994; Li *et al.*, 1995; Pemberton *et al.*, 1995), *NUP120* (Aitchison *et al.*, 1995a; Heath *et al.*, 1995), *NUP159* (Gorsch *et al.*, 1995), *NUP84* (Siniossoglou *et al.*, 1996), and *NUP85* (Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996) alter this distribution such that clusters of NPCs are concentrated in discrete patches of the nuclear envelope. To analyze *gle2* cells, NPCs were localized by indirect immunofluorescence with the antinucleoporin MAb 414. In wild-type cells grown at 23 or 37°C, NPC staining was distinctly punctate and localized around the entire nuclear circumference (Figure 6, right). The same was true for *gle2* cells grown at 23°C (Figure 6, top left). However, when *gle2* cells were grown at 37°C, the majority of the immunofluorescence signal was present in single foci (Figure 6, bottom left). These putative NPC clusters were not observed until ~1.5 h after the shift to 37°C, with the majority of cells showing clusters by 3.5 h.

To confirm the presence of NPC clusters in *gle2* cells, thin-section electron microscopic analysis was performed. At 23°C, the NPC and nuclear envelope structure in *gle2* cells was identical to that of wild-type cells (Figure 7A and B). However, after growth at 37°C at least three different perturbations were observed in *gle2* cells. Surprisingly, not only were the NPC-like structures clustered (see the three arrowheads at 12:00 and the nuclear envelope region between the arrows, Figure 7C), but most of the clustered NPCs were also associated with a membrane-herniated structure similar to that found with the NPCs in temperature-arrested *nup116Δ* cells (between the arrows, Figure 7C; Wente and Blobel, 1993). In micrographs at higher magnification (Figure 7, E–H), the herniated-NPCs in the cluster still appeared to be individually attached to the inner nuclear membrane. However, a membrane continuous with the inner membrane apparently sealed over the cytoplasmic face, and electron-dense material was accumulated between the cytoplasmic face of the NPC and the membrane, resulting in a "herniated" structure (arrow, Figure 7E).

Besides the clusters of herniated NPC structures, a third distinct morphological perturbation was also observed in the *gle2-1* temperature-arrested cells. In many cases, additional layers of double membranes studded with densities resembling NPCs were observed in the nucleus juxtaposed below the cluster of herniated NPCs (arrow, Figure 7, F–H). This structure

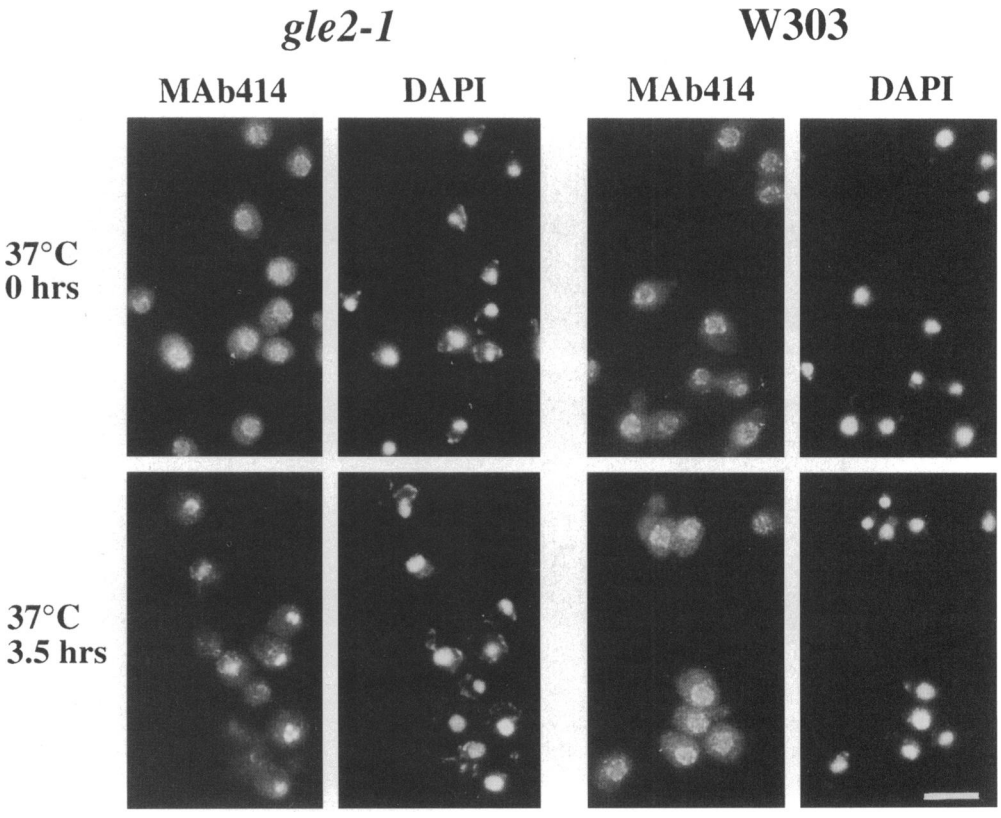

Figure 6. Temperature-dependent clustering of NPCs in *gle2-1* cells. Methanol/formaldehyde-fixed cells were incubated with MAb 414 and binding was detected with a FITC-conjugated goat anti-mouse IgG. In wild-type (W303) cells at 23 and 37°C and in *gle2-1* cells at 23°C, antinucleoporin staining was nuclear, distinctly punctate, and localized to the nuclear periphery. In contrast, the staining in *gle2-1* cells shifted to 37°C for 3.5 h was concentrated into bright tight clusters along the nuclear periphery. Bar, 5 μm.

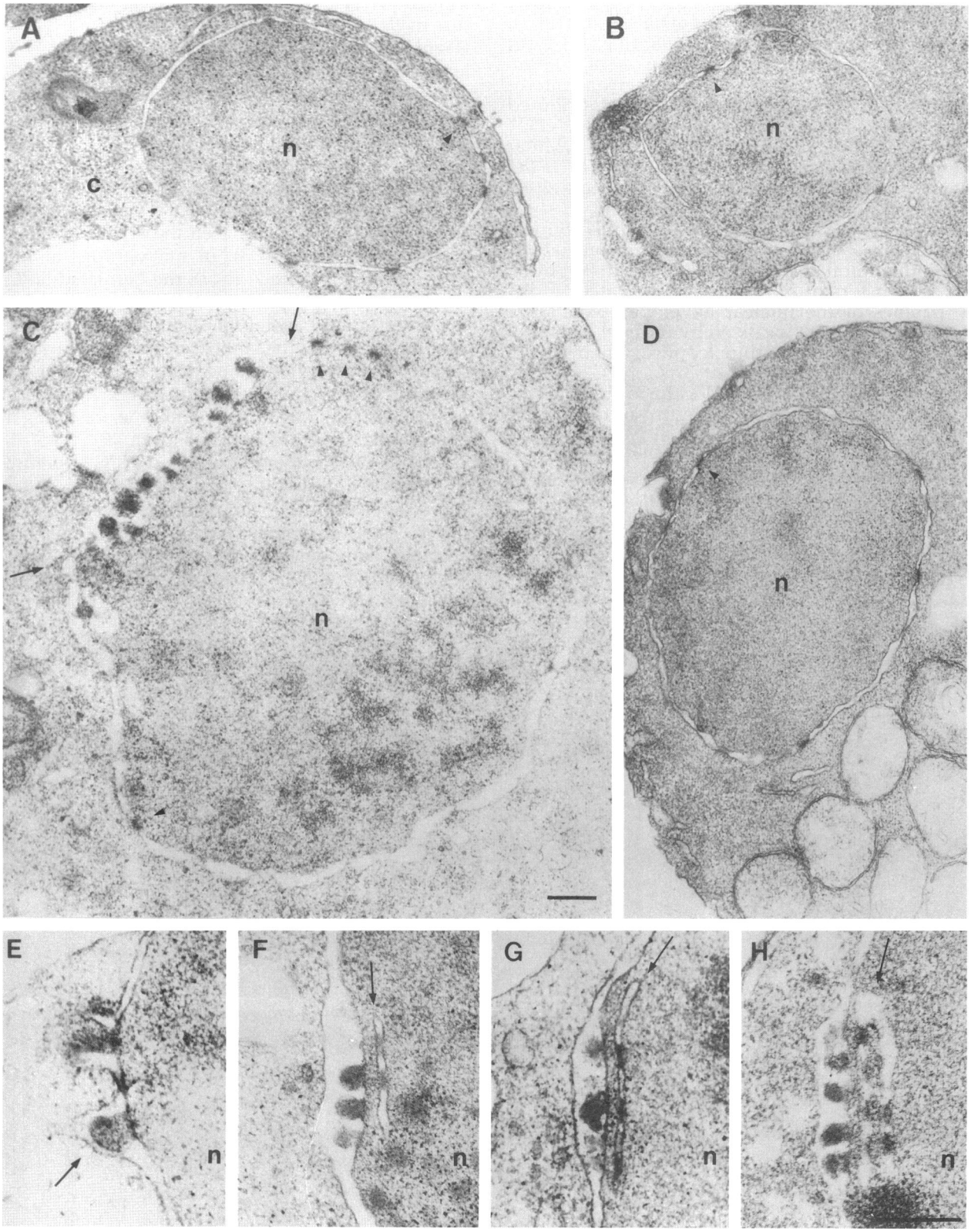

Figure 7.

is similar to the intranuclear annulate lamellae-like structures observed in *nup116Δ* cells (Wente and Blobel, 1993) and when *NUP170* is overexpressed in *pom152Δ* cells (Aitchison *et al.*, 1995b). The NPC clusters, herniations, and intranuclear annulate lamellae were not found in wild-type cells grown at 37°C (Figure 7D).

Gle2p Interacts with Srp1p and Rip1p in the Two-Hybrid Assay

It was possible that Gle2p and Nup100p physically interacted because we identified *GLE2* in a genetic screen with a *nup100* mutation. This is true for Gle1p (Murphy and Wente, 1996). To test this, the yeast two-hybrid assay was conducted (Fields and Song, 1989). In addition to Nup100p, we coincidentally tested whether Gle2p interacted with any of several other nuclear transport factors or nucleoporins studied in our laboratory. For these experiments, full-length Gle2p was fused to the activation domain of Gal4p ($Gal4_{AD}$-Gle2p) and transformed into yeast strains harboring plasmids expressing fusions to the LexA DNA-binding domain ($LexA_{BD}$) or the DNA-binding domain of Gal4p ($Gal4_{BD}$). Alternatively, Gle2p was fused to $Gal4_{BD}$ and tested with fusions to $Gal4_{AD}$. The plasmid expressing $LexA_{BD}$-Orc2p (Hardy, 1996) was used to confirm the specificity of the interactions. If two proteins interact in the reporter yeast cell, transcriptional activation of a *lacZ* gene with upstream LexA or Gal4p DNA-binding sites would occur via coincident juxtaposition of the DNA-binding and activation domains (Fields and Song, 1989). The level of β-galactosidase expression was measured with a filter assay, and no interaction was detected between Gle2p and Nup100p, Nup116p, Nup145p, Nup133p, Kap95p, or Gle1p (our unpublished observations). Surprisingly, Gle2p specifically interacted with both Srp1p and Rip1p (Figure 8).

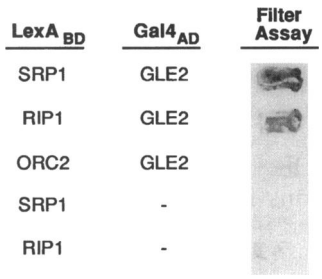

Figure 8. Interaction of Gle2p with Srp1p and Rip1p in the yeast two-hybrid assay. Yeast strain L40 was cotransformed with plasmids expressing the indicated activation domain and DNA-binding domain constructs. β-galactosidase activity was detected with a filter-binding assay. The $LexA_{BD}$-Orc2 (Hardy, 1996) was used to confirm the specificity of the interactions, and the $LexA_{BD}$-Srp1p and $LexA_{BD}$-Rip1p constructs did not have detectable activation capacity alone. The $Gal4_{AD}$-Gle2 plasmid complemented the *gle2Δ* phenotype (our unpublished observations).

DISCUSSION

We report here the identification of *S. cerevisiae GLE2* in a genetic screen for nucleoporin-interacting factors. Gle2p possesses striking amino acid sequence similarity to the protein encoded by *RAE1*, a gene required for normal poly(A)$^+$ RNA export in *S. pombe* (Brown *et al.*, 1995) It has been suggested that the *rae1-1* mutant RNA export phenotype in *S. pombe* cells is an indirect consequence of a role for Rae1p in the cell cycle or cytoskeletal machinery (Brown *et al.*, 1995). Our characterization studies in *S. cerevisiae* demonstrate both a genetic and physical basis for Gle2p function at the NPC. Mutations in *GLE2* are synthetically lethal, with a null allele of the gene encoding the GLFG nucleoporin Nup100p. They also display diminished RNA export capacity and marked alterations of NPC/nuclear envelope structure. Moreover, localization and subcellular fractionation analysis shows that Gle2p is physically associated with NPCs.

The *gle2* temperature-dependent clustering of NPCs is an unusual observation. For all of the other NPC-clustering mutants, except *nup159* cells, the NPCs are clustered constitutively at all growth temperatures. In *nup159* cells, the phenomenon is actually the opposite of that in *gle2* cells: *nup159* NPCs are clustered at 23°C, whereas at 37°C their distribution is over the entire nuclear surface (Gorsch *et al.*, 1995). The herniated clustered NPCs in *gle2* cells are distinct from the successive interconnected NPCs found in grape-like aggregates of constitutively clustered NPCs in *nup145*, *nup120*, *nup84*, and *nup85* mutant cells (Wente and Blobel, 1994; Aitchison *et al.*, 1995a; Heath *et al.*, 1995; Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996). Cells deleted for *NUP116* form herniations when shifted to the restrictive temperature (Wente and Blobel, 1993); however, unlike *gle2* mutants, *nup116Δ* NPCs do not become clustered. Interestingly, *gle2* cells at the restrictive temperature form intranuclear annulate lamellae, which are present in *nup116Δ* cells at all growth temperatures (Wente and Blobel, 1993). We have not determined whether the three types of *gle2* NPC struc-

Figure 7 (cont). Thin-section electron micrographs of wild-type and *gle2-1* cells. The wild-type (W303a; B and D) and gle2-1 cells (A, C, and E–H) were grown at 23°C to early logarithmic phase in YPD and sampled before A and B and after C–H shifting to growth at 37°C for 2.5 h for fixation, embedding, and staining for protein and membrane structures. At 23°C, the NPCs and the nuclear envelope morphology in wild-type (B) and *gle2-1* (A) cells was similar. Arrowheads point to examples of NPCs. At 37°C, the wild-type (D) cell structure was unchanged; however, three perturbations were observed in *gle2-1* cells. The *gle2-1* NPCs were clustered (C, between the arrows and the three arrowheads at 12:00), and the majority were also sealed on their cytoplasmic face by a membrane herniation filled with electron-dense staining material (C, between arrows; E, at arrow). Intranuclear double membranes with NPC-like structures, as in F–H (at arrow), were often beneath the clustered, herniated NPCs of the nuclear envelope. c, cytoplasm; n, nucleus. Bars: A–D, 0.25 μm; E–H, 0.25 μm.

tural perturbations occur coincidentally or sequentially after the shift to growth at 37°C. Although *rae1-1* cells have not been analyzed for NPC/nuclear envelope structural perturbations, we predict that perturbations similar to those of *gle2* exist. In *gle2* cells, the appearance of the NPC structural perturbations is markedly delayed compared with the rapid and penetrant poly(A)$^+$ RNA export defect. In fact, at the permissive temperature in *gle2* cells there are no noticeable structural perturbations, yet export is inhibited. Therefore, the structural abnormalities are likely pleiotropic defects of the *gle2* mutant phenotype, and the primary Gle2p function is in nuclear transport.

The association of Gle2p with NPCs indicates that it may be a structural component of the NPC. In addition, the morphological perturbations observed in *gle2*-arrested cells (NPC clusters, herniations, and intranuclear annulate lamellae) have only been previously reported for mutant alleles of bonafide nucleoporins (discussed above). However, the cytoplasmic staining in our immunofluorescence experiments suggests that Gle2p's localization is more dynamic than that expected for a nucleoporin. Moreover, localization of an epitope-tagged Rae1p in *S. pombe* cells is primarily cytoplasmic (Brown *et al.*, 1995). Similar cytoplasmic and NPC localization is reported for the yeast NLS receptor, Srp1p (Yano *et al.*, 1992; Aitchison *et al.*, 1995a; Koepp *et al.*, 1996). If its primary function is in nuclear export, the cytoplasmic localization of Gle2p may seem paradoxical. However, similar observations have been reported for Rna1p, a GTPase-activating protein for Gsp1p/Ran/TC4 transport activity (Bischoff *et al.*, 1995; Corbett *et al.*, 1995). Rna1p is localized both in the cytoplasm and at NPCs, and the *rna1-1* mutant has a rapid and penetrant poly(A)$^+$ RNA export block (Hopper *et al.*, 1990; Amberg *et al.*, 1992; Koepp *et al.*, 1996). In contrast to the *gle2* mutants, the *rna1-1* mutant also has an import defect (Corbett *et al.*, 1995). Thus, Gle2p may be a nuclear transport factor that is concentrated at its site of action, the NPC.

The role for Gle2p in nuclear transport may be revealed by determining its protein-protein interactions at the NPC. The two-hybrid interactions of Gle2p with Srp1p and Rip1p at this point are only suggestive. Experiments to analyze whether these interactions are direct and to test putative roles for these interactions in nuclear transport are currently underway. The interaction of Gle2p with Rip1p, in combination with the strong export block in *gle2* cells, implies an export function for Gle2p. The yeast Rip1p was identified in a two-hybrid screen for Rev-interacting proteins (Stutz *et al.*, 1995). The Rip1p-Rev interaction requires the NES motif in Rev (Stutz *et al.*, 1995). The cellular RNA export mediator Gle1p also directly interacts with Rip1p, and the NES in Gle1p is necessary for both this interaction and for poly(A)$^+$ RNA export (Murphy and Wente, 1996). The dynamics of Rip1p localization during the export of NES-bearing proteins have not been determined. The Gle2p interaction with Rip1p could also be due to an NES-like sequence in Gle2p. However, there are no matches in Gle2p to the leucine-rich [L(X)nLXXLXL/I] NES motif found in Rev, protein kinase inhibitor, TFIIIA, and Gle1p. We are testing whether the putative WD repeats in Gle2p directly or indirectly mediate Rip1p connections to the NPC.

Srp1p (also known as Kap60p (Enenkel *et al.*, 1995)) is the yeast homologue of the vertebrate NLS receptor/importin α/karyopherin α/hSRP1 (Gorlich *et al.*, 1994; Loeb *et al.*, 1995; Moroianu *et al.*, 1995a; Weis *et al.*, 1995). Srp1p binds to NLS-bearing proteins in the cytoplasm and is required for docking of a NLS protein-Srp1p-Kap95p trimeric complex to the NPC (reviewed in Gorlich and Mattaj, 1996). During the import process, Srp1p is translocated through the NPC, and when the import complex disassembles in the nucleoplasm, Srp1p must be recycled to the cytoplasm (Gorlich *et al.*, 1995; Moroianu *et al.*, 1995b). It is possible that Srp1p binds to an NLS-like sequence in Gle2p and mediates the nuclear import of Gle2p. If this step is essential for Gle2p export activity, Srp1p would have an indirect role in mediating nuclear export. However, the *srp1- 31* mutation does not have a poly(A)$^+$ RNA export defect (Loeb *et al.*, 1995). It also seems unlikely that the Srp1p-Gle2p interaction simply reflects the import of Gle2p itself because the only motif in Gle2p that resembles a NLS is the carboxyl-terminal KKKR sequence, and Gle2p is not predominantly nuclear localized. Alternatively, Gle2p could facilitate a specific step of the Srp1p import cycle. However, we have not detected an inhibition of total import capacity in the *gle2-1*, *gle2-G222D*, or *gle2*Δ mutants. This may reflect an inability to measure perturbations of import rates with the in situ assays. A provocative hypothesis is that Gle2p facilitates the recycling of Srp1p from the nucleus to the cytoplasm and that diffusive export of Srp1p in *gle2* mutants is sufficient to maintain import at levels which appear to be wild type with in situ assays. The mechanism for recycling import factors from the nuclear face of the NPC to the cytoplasm is unknown.

Thus far, we have isolated two potential transport factors, Gle1p and Gle2p, from a synthetic lethal screen with the *nup100*Δ mutation. If the yeast GLFG nucleoporins Nup116p and Nup100p are localized exclusively at the nucleoplasmic face of the NPC, in a similar manner as the vertebrate GLFG nucleoporin rat-Nup98p (Radu *et al.*, 1995b), these genetic interactions may reflect unique roles for Nup116p and Nup100p in nuclear export processes. Continued analysis of the interactions between

these proteins will provide an excellent opportunity to elucidate the mechanism of nuclear transport at the molecular level.

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