Dynamics of Nuclear Pore Distribution in Nucleoporin Mutant Yeast Cells

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Abstract. To follow the dynamics of nuclear pore distribution in living yeast cells, we have generated fusion proteins between the green fluorescent protein (GFP) and the yeast nucleoporins Nup49p and Nup133p. In nup133Δ-dividing cells that display a constitutive nuclear pore clustering, in vivo analysis of GFP-Nup49p localization revealed changes in the distribution of nuclear pore complex (NPC) clusters. Furthermore, upon induction of Nup133p expression in a GAL-nup133 strain, a progressive fragmentation of the NPC aggregates was observed that in turn led to a wild-type nuclear pore distribution. To try to uncouple Nup133p-induced NPC redistribution from successive nuclear divisions and nuclear pore biogenesis, we devised an assay based on the formation of heterokaryons between nup133Δ mutants and cells either expressing or overexpressing Nup133p. Under these conditions, the use of GFP-Nup133p and GFP-Nup49p fusion proteins revealed that Nup133p can be rapidly targeted to the clustered nuclear pores, where its amino-terminal domain is required to promote the redistribution of preexisting NPCs.

Bidirectional exchange of molecules between the cytoplasm and the nucleus in eukaryotic cells is accomplished through nuclear pore complexes (NPCs)1 (Forbes, 1992; Fabre and Hurt, 1994). Anchored in the nuclear envelope, the NPCs of higher eukaryotes are macromolecular structures with an estimated molecular mass of 125 megadaltons (MD) (Reichelt et al., 1990). Their basic architecture, including a characteristic eightfold symmetry, is shared by the smaller 66 MD yeast NPC (Saccharomyces cerevisiae) (for reviews see Rout and Wente, 1994; Doye et al., 1995a; Gorsch et al., 1995; Grandi et al., 1995; Heath et al., 1995; Hurwitz and Blobel, 1995; Li et al., 1995; Goldstein et al., 1995; Siniossoglou et al., 1996). Similarly, overexpression of the human nucleoporin Nup153 was recently shown to inhibit mRNA trafficking (Bastos et al., 1996). Among the yeast nucleoporin mutants defective for mRNA export, nup84, nup85, nup120, nup133, nup145, and nup159 mutants all display a constitutive NPC clustering that is sometimes associated with alterations in the structure of the nuclear envelope (Doye et al., 1994; Wente and Blobel, 1994; Aitchison et al., 1995a; Gorsch et al., 1995; Heath et al., 1995; Li et al., 1995; Pemberton et al., 1995; Goldstein et al., 1996; Siniossoglou et al., 1996). However, in contrast to the phenotype observed in the nup153Δ null mutant in which a nuclear envelope seal over the NPC was suggested to directly inhibit nucleocytoplasmic traffic (Wente and Blobel, 1993), NPC clustering and mRNA export defects can be dissociated; in these nucleoporin mutants, the clustered pores are competent for poly(A)+ RNA export at the permissive temperature. Moreover, rat7-1/nup159-1 mutant cells recover a nearly normal NPC distribution within 1 h at 37°C, although cessation of mRNA export occurs at this restrictive temperature (Gorsch et al., 1995). Finally, a truncation of the amino-terminal domain of Nup133p that restores normal RNA export at 37°C does not correct the nuclear pore distribution defect (Doye et al., 1994).

Spatial heterogeneity in NPC distribution, including extreme situations consisting of large NPC-devoid regions of the nuclear envelope together with densely packed NPC clusters, have been described since the late 60s (for review...
results show that Nup133p can be targeted to the clustered clusters in dividing nup133. Two aspects of NPC dynamics: the movements of NPC ing (Doye et al., 1994; Li et al., 1995) enabled us to follow induced NPC redistribution, we developed an assay based in a GAL-nup133 strain. To further characterize Nup133p-the clustered NPCs upon induction of Nup133p expression (discussed in Markovics et al., 1974; Severs et al., 1976). Until recently, it was not possible to distinguish between these two hypotheses because the dynamic distribution of pores could not be directly observed. However, the recent advent of green fluorescent protein (GFP) technology now enables in vivo analysis of protein distribution. GFP and brighter GFP variants engineered by mutational analysis have been successfully used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization in various organisms (for reviews see Cubitt et al., 1995; Prasher, 1995). In addition, GFP-chimeras have been used to monitor subcellular events in living cells such as separation of the spindle pole bodies or movements of actin patches in yeast (Kahana et al., 1995; Doyle and Botstein, 1996; Waddle et al., 1996).

In this report, we used nucleoporins fused with GFP to monitor NPC distribution in vivo. The NUP133-disrupted (nup133−) strain that displays a constitutive NPC clustering (Doye et al., 1994; Li et al., 1995) enabled us to follow two aspects of NPC dynamics: the movements of NPC clusters in dividing nup133− cells and the redistribution of the clustered NPCs upon induction of Nup133p expression in a GAL-nup133 strain. To further characterize Nup133p-induced NPC redistribution, we developed an assay based on the formation of heterokaryons between nup133− mutants and cells expressing or overexpressing Nup133p. Our results show that Nup133p can be targeted to the clustered NPC and thereby promotes their rapid redistribution.

**Materials and Methods**

**Plasmid and Strain Construction**

DNA manipulations, including restriction analysis, fill-in reactions with Klenow fragment, and ligations, were performed essentially as described by Maniatis et al. (1982). Strains used in this study are listed in Table I. Yeast strains were grown in rich media (1% yeast extract, 2% bactopeteone, 30 mg/ml adenine) with either 2% dextrose (YPDA) or 2% galactose (YPGalA), or in synthetic complete media (SDC and SGalC) lacking specific amino acids (CSM medium; Bio 101, La Jolla, CA). Plasmid transfection, gene disruption, sporulation of diploid cells, and tetrad analysis were performed as described by Wimmer et al. (1992). To minimize autofluorescence, all ade2− ADE3 strains were transformed with plasmid pASZ11 containing ADE3 from (Storz and Linder, 1990).

To localize nucleoporins in vivo, a GFP S65T/V163A variant (Kahana and Silver, 1996) was used to tag Nup49p and Nup133p. Since the wild-type allele of GFP has not been used in this study, this GFP variant will be referred to as GFP in the manuscript.

The GFP-NUP49 fusion gene was constructed as previously described for ProtA-NUP49 (Wimmer et al., 1992). Briefly, an Nhel/XbaI fragment encoding GFP was obtained by PCR and fused in frame to the coding sequence of NUP49 at the unique Nhel site, thereby keeping the authentic NUP49 promoter. The fusion gene was inserted into a pUN100-LEU2 plasmid (Elledge and Davis, 1988).

To construct the GFP-NUP133 fusion gene, a BglII blunt-ended/EcoRI fragment encoding GFP and including a modified ATG start codon was generated by PCR. This fragment was inserted downstream of the blunt-ended Ndel site located at the ATG of NUP133, thereby keeping the authentic NUP133 promoter, and fused in frame to the coding sequence of NUP133 at an EcoRI site previously generated at the ATG of NUP133 (Doye et al., 1994). The GFP-nup133A fusion gene was generated by removing a 600-bp Ncol fragment encoding residues 44–226 of Nup133p as previously described (Doye et al., 1994). The resulting fusion genes were inserted either in the single copy plasmid pUN100-LEU2 or in the high copy number plasmid pRS424-TRPI (Christianson et al., 1992).

To construct the GAL-nup133 strain, a 1.7-kb fragment encoding TRPI-pGAL10-HA from plasmid pTIF (generous gift from C. Cullin, CGM, CNRS, Gil-sur-Yvette, France) was amplified by PCR using the following primers: NUP133F: ATGCGGCTGGTCTGGCAGGTTTGGGTTTTGTTAAGAAAAGATGTAC

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<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>VD1R</td>
<td>Mata ade2 ade3 his3 leu2 lys2 ura3 nup49::TRP1 (pCH1122-ADE3-URA3-NUP49)</td>
</tr>
<tr>
<td>nup133− α</td>
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<td>nup133− a</td>
<td>Mata ade2 his3 leu2 trpl ura3 nup133::His3</td>
</tr>
<tr>
<td>nup85− a</td>
<td>Mata ade2 his3 leu2 trpl ura3 nup85::His3</td>
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For disruption of the NUP133 gene in the kar1−1 strain EY93, the URA3 gene isolated as a blunt-ended BamHI fragment from plasmid yDpU (Berben et al., 1991) was used to replace a 1.9-kb Ncol fragment of NUP133 open reading frame from plasmid pUN100-NUP133, as previously described (Doye et al., 1994). A linear nup133−URA3 fragment was excised from the recombinant plasmid and used to transform the haploid strain EY93 by selection on SDC-ura. URA3− transformants were characterized for correct integration of TRPI-pGAL10-HA at the NUP133 gene locus by Southern analysis (Sherman, 1990). BMA411 diploids heterozygous for NUP133 were sporulated and tetrad analysis was performed.

**Microbiological Techniques**

To synchronize cells, the haploid strains RS453a and nup133− containing the pUN100-GFP-NUP49 and pASZ11 plasmids were grown at 25°C in SDC-Leu-ade medium to early-log phase (0.5 OD₆₀₀), 20 ng/ml of n-factor (Sigma Chemical Co., St. Louis, MO) was added to the medium, and cells
were further incubated for 2 h at 25°C. G1-arrested cells were collected by centrifugation and washed twice with SDC-leu-ade medium. Cells were further grown for 30 min in SDC-leu-ade medium at 25°C before being observed by confocal microscopy.

For mating and cytoduction experiments, ∼3 × 10^5 exponentially growing cells of each parent were mixed together and concentrated onto 25-mm nitrocellulose filters (type HA, 0.45 μm; Millipore Corp., Bedford, MA) using a syringe and a swinnex filter unit (Millipore Corp.). Filters were incubated on YPDA plates for 30 min to 1 h at 30°C. Cells were then resuspended by vortexing the filter in a sterile tube containing 1.5 ml of media and analyzed by fluorescence microscopy.

**Fluorescence Microscopy**

Indirect immunofluorescence microscopy on formaldehyde-fixed cells was performed according to Schimmang et al. (1989) with the following modifications: Yeast cells were fixed for 30 min in 3.7% formaldehyde and converted into spheroplasts using 0.2 mg/ml Zymolase 100 T (Seikagaku Corp., Tokyo, Japan). The spheroplasts were spotted on polylysine-coated slides and left to air dry for 5 min. Slides were immersed in methanol at −20°C for 6 min and then in aceton at −20°C for 30 s. After rinsing with PBS, the slides were incubated in mAb414 antibody (Aris and Blobel, 1989) (BAHC0, Richmond, CA) diluted 1:20 in PBS containing 1% BSA, followed by Rhodamine-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:100. For DNA staining, 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) was used. Fixed cells were visualized on a microscope (model DMR; Leica, Inc., Deerfield, IL) equipped with an HBO100 mercury lamp (Osram, Germany) and ×100/1.4 objective lens.

For confocal analysis, growing yeast cells were put in a Sykes Moore Chamber (Bellico, Vineland, NJ) filled with 1.2 ml SDC medium and centrifuged for 3 min at 1,500 g. Living cells were imaged at room temperature, 24−27°C, on an inverted microscope (Leica, Inc.) equipped with a ×100/1.4 objective lens, and scanning was performed with a True Confocal Scanner LEICA TCS 4D. GFP fluorescence signal being detected with the fluorescein channel. To compensate photobleaching during long-term experiments, laser power was progressively increased to yield images with similar intensities. Acquisition of eight focal planes, 0.3 to 0.8 μm apart, was routinely made. As indicated in figure legends, either individual sections or two-dimensional projections of images collected at all relevant z-axes is presented. Cells left in the chamber after an experiment proceeded to divide many times, which indicates that they were not damaged by the illumination. For kinetic analysis of heterokaryons, ∼10 independent heterokaryons were followed at various (10−25 min) intervals.

For random analysis of heterokaryons, 0.05% agarose was added to the medium, and 10 ml of cells was placed on a microscope slide and covered with a coverslip (18 × 18 mm). The cells were observed by fluorescence microscopy with a chilled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) on a microscope (Leica, Inc.) equipped with the following filter set: excitation, 450−490, dichroic, 510; emission, 515−560 (Filter L4; Leica, Inc., a ×100/1.4 objective lens). For these experiments, images of ∼100 individual heterokaryons were recorded.

**Results**

**GFP-Nup49p Is Functional and Localizes to the Nuclear Pore Complexes**

To examine in vivo the distribution of NPCs in wild-type and nucleoporin mutant cells, the yeast nuclear pore protein Nup49p was tagged with GFP. The single copy number plasmid, pUN100-GFP-NUP49, in which the GFP-NUP49 fusion gene is expressed under the NUP49 promoter, was transformed into the VD1R shuffling strain disrupted for the essential NUP49 gene and complemented with a wild-type NUP49 allele carried by plasmid pCH1122-NUP49. After selection on SDC-leu-ade plates, transformants displayed a typical red−white sectoring phenotype because of the loss of the ADE3-carrying pCH1122-NUP49 plasmid (data not shown), indicating that GFP-Nup49p rescues the disruption of NUP49. The expression of GFP-Nup49p was also analyzed by SDS-PAGE and Western blotting using anti-Nup49p and anti-GFP antibodies. As shown in Fig. 1A, an anti-Nup49p antibody recognized a single band corresponding to wild-type Nup49p in the VD1R strain (lane 3). Upon transformation with plasmid pUN100-GFP-NUP49, an additional band migrating at the expected size for the GFP-Nup49p fusion protein was detected by both anti-Nup49p and anti-GFP antibodies (lane 4). In white colonies, derived from the VD1R strain expressing GFP-Nup49p, wild-type Nup49p was no longer detected (lane 5), thereby confirming that GFP-Nup49p is able to rescue the otherwise lethal phenotype of a nup49 null mutant. As judged by the intensity of the signals obtained with anti-Nup49p, GFP-Nup49p displayed a similar expression level as compared to endogenous Nup49p when expressed either in wild-type (lanes 4 and 5) or in NUP133-disrupted cells (nup133<sup>−</sup>; lane 2).

The subcellular localization of GFP-Nup49p was analyzed in both wild-type and nup133<sup>−</sup> cells that display a constitutive NPC clustering phenotype (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995). Cells were fixed and immunofluorescence was performed using the monoclonal antibody mAb414 that recognizes nuclear pore antigens (Aris and Blobel, 1989) (Fig. 1B). In wild-type cells, a similar punctate staining around the nuclear envelope was detected with both GFP-Nup49p and the monoclonal antibody mAb414. The localization of GFP-Nup49p at the NPC was further confirmed by its colocalization with the mAb414 epitopes at the NPC clusters present in nup133<sup>−</sup> cells (Fig. 1B).

Similar results were obtained when GFP-Nup49p localization was examined in living cells using confocal microscopy (Fig. 1C). The preservation of the cell morphology further enabled the visualization of specific features of...
NPC distribution within wild-type cells, such as the lack of labeling in areas of close association between the nucleus and the vacuole (Severs et al., 1976). In agreement with the NPC-clustering phenotypes previously observed in nup133<sup>−</sup> and NUP85-disrupted (nup85<sup>−</sup>) cells (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995; Goldstein et al., 1996), GFP-Nup49p was localized within one or few clusters in nup133<sup>−</sup> cells (Fig. 1 C), whereas an intermediate phenotype with small aggregates, crescent-shaped and faint ringlike structures, could be observed in nup85<sup>−</sup> cells. In conclusion, these results indicate that GFP-Nup49p is functionally targeted to the NPC. Similarly, it has been recently reported that GFP-tagged Nup85p is functional and localized to the NPC (Goldstein et al., 1996).

The ability to follow GFP-Nup49p–expressing cells in vivo was essential to analyze the successive changes in nuclear envelope shape during cell division. In wild-type cells followed at 10-min intervals, insertion of the nucleus into the neck, nuclear elongation and migration into the bud, and the final bilobed shape of the nucleus during karyokinesis could be readily assessed (Fig. 2 A). Therefore, in cells displaying such a homogeneous NPC distribution, GFP-Nup49p provides an alternative tool to previously described fluorescent lipophilic dyes such as DiOC<sub>6</sub> (Koning et al., 1993) to monitor in vivo changes in nuclear shape and position.

In nup133<sup>−</sup> cells, changes in the distribution of the NPC clusters were observed within 10 min (Fig. 2 B, left). Just after cell division, as the nuclei moved towards the site of the future buds, the rotation of the nuclei and/or the modified distribution of NPC clusters within the nuclear envelope frequently led to aggregated NPCs that were positioned close to the budnecks (Fig. 2 B, right). In other cells, an elongated cluster of pores that spanned the two nuclei was seen during karyokinesis (Fig. 2 B, bottom). These results indicate a dynamic distribution of the NPC.

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**Figure 1.** Expression and localization of Nup49p tagged with GFP. (A) Immunoblotting analysis. Whole cell extracts were prepared from yeast strains either containing or not containing the pUN100-GFP-NUP49 plasmid: nup133<sup>−</sup> (lane 1), nup133<sup>−</sup> + pUN100-GFP-NUP49 (lane 2), VD1R (lane 3), VD1R + pUN100-GFP-NUP49 (lane 4), and VD1R + pUN100-GFP-NUP49 and lacking pCH1122-NUP49 (lane 5). Equivalent amounts of extracts from each strain were analyzed by SDS-PAGE and immunoblotting using an anti-Nup49p and an anti-GFP antibody. The positions of Nup49p and GFP-Nup49p and the molecular masses (kD) of a protein standard are indicated. (B) Colocalization of GFP-Nup49p and nuclear pore complex antigens. Wild-type (WT) and nup133<sup>−</sup> cells expressing GFP-Nup49p, which is detected in the fluorescein channel (GFP), were fixed and processed for indirect immunofluorescence using the monoclonal antibody mAb414 followed by rhodamine-labeled goat–anti mouse IgG. DNA staining and the Nomarski optics view are shown. (C) In vivo localization of GFP-Nup49p using confocal microscopy. Two successive sections (0.5 μm apart) are presented. A ringlike staining of the nuclear periphery, typical for nucleoporins, is observed in wild-type cells (WT). Arrows show absence of nuclear pore complexes in areas of close association between the nucleus and the vacuole. In nup133<sup>−</sup> and to a lesser extent nup85<sup>−</sup> strains, nuclear pores are clustered. Bars, 5 μm.
clusters within the nuclear envelope and further suggest that the localization of the NPC aggregates is, in general, not random, but rather permits the distribution of clustered NPCs to the daughter nucleus.

**Induction of Nup133p Expression in nup133<sup>−</sup> Cells Restores a Homogeneous Distribution of Nuclear Pore Complexes**

To investigate further the role of Nup133p in NPC distribution, a GAL-nup133 strain was constructed, in which Nup133p expressed under the control of the inducible GAL10 promoter was integrated at the NUP133 locus by homologous recombination. On galactose-containing medium (YPGalA), the GAL10 promoter is induced, and GAL-nup133 cells showed normal growth properties both at 24 and 37°C (Fig. 3 A). In agreement with the temperature-sensitive phenotype of NUP133-disrupted cells (Doye et al., 1994), GAL-nup133 cells displayed a growth defect at 37°C upon repression of the GAL10 promoter on glucose-containing plates (YPDA). However, a residual growth could be observed at 37°C, which correlated with the low level of Nup133p detected by Western blotting (Fig. 3 B), and probably reflected a leak of the GAL10 promoter. During galactose induction, Nup133p level increased after 4 h and was similar to wild-type Nup133p after about 8 h. GAL-nup133 cells grown overnight in galactose-containing medium reached a Nup133p-expression level similar to Nup133p when the protein was overexpressed from a high copy number plasmid.

NPC distribution was visualized in GAL-nup133 cells expressing GFP-Nup49p at various stages during galactose induction. When the GAL10 promoter was repressed, clusters of NPCs along the nuclear envelope were revealed...
by the GFP-Nup49p fluorescence (Fig. 3 C); however, the overall number of aggregates was higher when compared to nup133Δ cells (see Fig. 1 C). A further increase in the number of nuclear pore clusters was observed after 4 h growth in galactose-containing medium. Finally, a ringlike staining reflecting a homogeneous distribution of the NPCs appeared in some cells after 6 h, and was detected in most cells after growth for 8 h in galactose-containing medium. Since the intensity of the GFP signal directly reflects the amount of the fusion protein, the brighter fluorescence intensity of the NPC clusters when compared to the ringlike staining suggests that Nup133p induction finally leads to a homogeneous distribution of the NPCs within the nuclear envelope but does not alter their overall number. The correlation between the expression level of Nup133p (Fig. 3 B) and the number of NPC clusters (Fig. 3 C) suggests that induction of Nup133p expression induces a progressive fragmentation of the NPC aggregates that finally leads to a wild-type nuclear pore distribution. However, because of the delay (8 h) required to reach a wild-type NPC distribution, this final phenotype could reflect either the insertion in the nuclear envelope of de novo synthesized pores during successive cell divisions or the redistribution of preexisting nuclear pore complexes.

**Analysis of GFP-Nup133p Targeting to nup133Δ Nuclei**

To analyze whether Nup133p-induced NPC redistribution could be uncoupled from successive nuclear divisions, we devised an in vivo assay based on the use of heterokaryons. kar1 mutants that display nuclear fusion defects thus leading to the formation of heterokaryons have been previously used in *S. cerevisiae* for assaying the shuttling of proteins from one nucleus to another (Flach et al., 1994) or the targeting of Tub4p to the spindle pole body (Marshall et al., 1996). The basic design of our experiment was therefore to construct heterokaryons between nup133Δ cells and cells either expressing or overexpressing Nup133p, the kar1-1 mutation being carried by either of the two mating partners. We expected that Nup133p synthesized by the wild-type cell should be targeted to the nup133Δ nucleus upon zygote formation, thereby complementing the NPC clustering phenotype.

To follow the targeting of Nup133p during this mating process, a *GFP-NUP133* fusion gene, expressed under the authentic *NUP133* promotor, was inserted both in the single copy number plasmid pUN100 plasmid or the high copy number plasmid pRS424. As a further control, we tagged with GFP the previously characterized nup133ΔN mutant that is targeted to the NPCs but does not complement the clustering phenotypes of nup133Δ cells (Doye et al., 1994). Both GFP-Nup133p and GFP-nup133ΔNp fusion proteins were able to rescue the temperature-sensitive phenotype of nup133Δ cells (data not shown). On Western blots, the expression level of wild-type Nup133p and GFP-Nup133p with this antibody for the overexpressed GFP-Nup133p were hardly detectable with the anti-GFP antibody (data not shown), a comparable staining intensity was obtained with this antibody for the overexpressed GFP-Nup133p and GFP-nup133ΔNp proteins (Fig. 4 A, lanes 5’ and 6’). It should be noted that the anti-GFP antibody gave similar

**Figure 3.** Induction of Nup133p expression in GAL-nup133 cells. (A) Growth properties of GAL-nup133 cells. Two equivalent dilutions of GAL-nup133 cells were spotted on glucose (YPD)—or galactose (YPGalA)–containing plates and incubated for 3 d at 24°C or 2 d at 37°C. (B) Analysis of Nup133p expression levels by immunoblotting. Whole cell extracts were prepared from the following strains: nup133Δ (−), wild-type RS453 (+), nup133Δ carrying the high copy number plasmid pRS424-NUP133 (+ +), and GAL-nup133 grown overnight in YPDA medium (0) or shifted for 2, 4, 6, 8, and 24 h to YPGalA medium. Total protein corresponding to similar amounts of cells were analyzed by SDS-PAGE and immunoblotting using an anti-Nup133p antibody. Anti-Nop1p antibody was used to control the amounts of protein loaded. The molecular masses (kD) of a protein standard are indicated. (C) Analysis of NPC distribution during Nup133p induction. Cells from strain GAL-nup133 carrying plasmid pUN100-GFP-NUP49 were grown in SDC-leu medium (0) or shifted to SGalC-leu medium for 2, 4, 6, 8, and 24 h as indicated and analyzed by confocal microscopy. Each image is a two-dimensional projection of images collected at all relevant z-axes using the fluorescence channel. The fluorescent staining patterns reflect the intracellular distribution of NPCs.
staining intensities for GFP-Nup49p expressed on a centromeric plasmid and for overexpressed GFP-Nup133p or GFP-nup133ΔNp (Fig. 4 A, compare lane 7′ to lanes 5′ and 6′). Although variations in the transfer efficiency of the two proteins on nitrocellulose cannot be excluded, this result suggests that in yeast, the Nup49p nucleoporin is more abundant than Nup133p.

Confocal analysis of nup133- cells expressing GFP-Nup133p revealed a ringlike staining around the nuclear envelope, indicating that this fusion protein is targeted to the NPCs and complements the clustering phenotype of the nup133- mutant (Fig. 4 B, a). In agreement with Western blotting data, the fluorescent signal was, however, weaker as compared to the one obtained in GFP-Nup49p–expressing cells. As previously reported for the protein A–tagged mutant (Doye et al., 1994), GFP-nup133ΔNp expressed on a centromeric plasmid localized within NPC clusters (Fig. 4 B, b). Upon overexpression of either fusion, a heterogeneous signal was obtained that probably reflected the variable amount of copies of the 2μ plasmid present in each cell (Fig. 4 B, c and d). Cells that highly overexpressed GFP-Nup133p displayed not only a typical ringlike staining but also a very strong nuclear and cytoplasmic staining, whereas the vacuoles were not labeled (Fig. 4 B, c).

These various constructs were used to analyze the targeting of GFP-Nup133p to the nucleus of nup133- cells in heterokaryon assays. Fusions were first made between nup133- cells and nup133- kar1-1 cells expressing GFP-Nup133p on a centromeric plasmid (Fig. 5 A). Before fusion, the nup133- nucleus was not labeled, whereas GFP-Nup133p expression in the nup133- kar1-1 cell gave rise to a ringlike staining. Because of the low expression level of GFP-Nup133p, no more than three successive images could be recorded for each zygote. After heterokaryon formation, GFP-Nup133p labeling was initially restricted to one or two aggregates along the nuclear envelope of the nup133- nucleus. Within 10 to 20 min, the number of labeled aggregates increased (Fig. 5 A). At later stages, a homogeneous ringlike staining was observed (data not shown). As control, we also analyzed the targeting of GFP-Nup133p to wild-type nuclei by mating nup133- kar1-1 cells expressing GFP-Nup133p with wild-type cells. Under those conditions, a progressive and homogeneous labeling around the nuclear envelope of the wild-type nucleus was observed (Fig. 5 B). This result indicates that upon mating, GFP-Nup133p can be targeted to the clustered NPCs present in nup133- cells as well as to the randomly distributed NPC present in wild-type cells.

Using a similar procedure, targeting of overexpressed GFP-Nup133p was analyzed upon mating between nup133- kar1-1 and nup133- cells overexpressing GFP-Nup133p (Fig. 5 C). Because of the cytoplasmic staining associated with overexpression of GFP-Nup133p, early stages of cytoplasmic fusion could be visualized. Under these conditions, GFP-Nup133p, which was probably provided by the cytoplasmic pool, was rapidly targeted to a restricted area of the nuclear envelope. Within 10–20 min, a homogeneous labeling around the nup133- nucleus was observed. We examined the specificity of this targeting by mating nup133- kar1-1 cells with cells overexpressing GFP-Nup133p. As shown in Fig. 5 D, GFP-nup133ΔNp was also rapidly targeted to a defined area of the nuclear envelope, but unlike in the case of GFP-Nup133p, this labeling did not further spread along the nuclear envelope, even 60 min after cytoduction. Taken together, these results indicate that both fusions are efficiently targeted to both randomly distributed and clustered NPCs in which the binding sites for Nup133p are thus accessible. Furthermore, the appearance of a homogeneous labeling around the nuclear envelope of nup133- nuclei correlates with the amount of incorporated GFP-Nup133p and requires the presence of its amino-terminal domain.
Analysis of NPC Redistribution upon Mating between nup133<sup>2</sup> and Wild-Type Cells

The previous experiments suggested that Nup133p is able to induce the redistribution of the nup133<sup>2</sup> clustered NPCs. To further demonstrate that the homogeneous distribution of GFP-Nup133p around the nuclear envelope correlates with the redistribution of the nuclear pore complexes themselves, similar cytoduction experiments were performed, in which GFP-Nup49p expressed in the nup133<sup>2</sup> cells was used to follow NPC distribution.

In the first set of experiments, nup133<sup>2</sup> cells expressing GFP-Nup49p were mixed with kar1-1 cells and analyzed by confocal microscopy. Fig. 6A illustrates the GFP-Nup49p localization pattern of a typical heterokaryon at various stages after cell fusion. At early stages (0–12 min), NPC clusters on the nup133<sup>2</sup> nuclear envelope were brightly labeled with GFP-Nup49p, whereas the wild-type nucleus was not labeled. Subsequently, a decreased intensity of the GFP-Nup49p signal within the NPC clusters was found, coincident with the appearance of additional aggregates and the spreading of the fluorescent labeling along the nuclear envelope. Finally, nearly wild-type nuclear...
pore distribution was generally observed within 40 to 60 min. At this late stage, zygotes usually displayed a typical trilobed shape. In the mean time, a homogeneous albeit fainter labeling of the wild-type nucleus progressively appeared, indicating that GFP-Nup49p expressed by the nup133Δ cell can be targeted to the other nucleus.

Strikingly, when nup133− kar1-1 cells expressing GFP-Nup49p were mixed with yeast cells overexpressing Nup133p, the redistribution of the clustered NPCs could then be achieved within 10 min (Fig. 6 B). Intermediate stages could sometimes be recorded, in which small NPC aggregates were homogeneously distributed around the nuclear envelope (Fig. 6 B, right). The increased rate of NPC redistribution upon mating with cells overexpressing Nup133p was also revealed by the shape of the zygote in which no bud formation was yet detectable. In contrast, the progressive labeling of the wild-type nucleus occurred independently of the expression level of Nup133p (compare Fig. 6, A and B). These results were further confirmed by random analysis of ~100 individual heterokaryons recorded at various stages during the mating process. Schematic representation of the successive steps occurring
during either mating process are summarized in Fig. 7, A
and B. The fast rate of NPC redistribution observed when
overexpressed Nup133p is provided to the nup133
2
nucleus suggests that, under such conditions, the homoge-
neous GFP-Nup49p labeling reflects the redistribution of
preexisting NPCs.

To try to demonstrate that NPC redistribution can effec-
tively be uncoupled from de novo NPC biogenesis, a final
set of experiments were performed, in which GFP-Nup49
was provided by the wild-type nucleus together with wild-
type or overexpressed Nup133p. The rationale of this ex-
periment was that if NPC redistribution requires de novo
NPC biogenesis, this process should be stimulated by the
overexpressed Nup133p. Accordingly, the rate of incorpo-
ration of GFP-Nup49p in the nup133
2
nuclei should be
increased and intermediate stages with brightly labeled
aggregates should be observed. For these experiments,
random analysis of ~100 individual heterokaryons, re-
corded at various stages during the mating process, was
performed. Similar results were also obtained by kinetic
analysis of heterokaryons (data not shown). Heterokary-
ons representative of either mating experiment are respec-
tively presented in Fig. 7, C and D.

Figure 7. Schematic representation of GFP-Nup49p locali-
zation (green) and NPC distribution during the mating process. (A and B)
mating between nup133
2
cells expressing GFP-Nup49p and cells expressing or overexpressing Nup133p (see also Fig. 6). (C and D)
Analysis of GFP-Nup49p targeting to the nup133
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nucleus upon mating between nup133
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cells and cells expressing GFP-Nup49p to-
together with wild-type Nup133p (C) or overexpressed Nup133p (D). The localization of GFP-Nup49p and the shape of heterokaryons re-
corded on the CCD camera at various stages during the mating process are presented. The delay required for these processes, as esti-
imated through kinetic analysis of individual heterokaryons, is indicated on the left.

Furthermore, the rate of GFP-Nup49p incorporation within
the nup133
2
nucleus is similar to the one observed upon
targeting of GFP-Nup49p to wild-type nuclei (compare
Fig. 6, A and B, to Fig. 7 D). As summarized in Fig. 7,
these results indicate that under such mating conditions,
NPC redistribution occurs before the incorporation of
GFP-Nup49p in the nup133
2
nucleus.

Discussion

In this paper, we have demonstrated that it is possible to
GFP-tag Nup49p and Nup133p to produce functional fu-
sions that rescue lethal or temperature-sensitive pheno-
types of strains that contain deletion of the corresponding
genes. In both wild-type and nup133
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cells, GFP-Nup49p
colocalizes with antigens recognized by the antinucleoporin
mAb414. In addition, in nup133
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and nup85
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cells the flu-
orescent signal was concentrated within a restricted area
of the nuclear envelope corresponding to the clustered
NPCs; similar results were also obtained when GFP-
Nup133p is expressed in nup85
2
cells (data not shown).
This labeling is in agreement with the clustering phenotype
observed in nup133
2
and nup85
2
cells after freeze-fracture electron microscopy (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995; Goldstein et al., 1996). Fur-
thermore, when cells were not processed for immunostaining,
specific features of NPC distribution, such as the absence
of NPCs from areas in close association between the nu-
cleus and the vacuoles, could be observed by confocal mi-
croscopy. This staining is consistent with previous reports
using freeze-fracture electron microscopy, which showed
the absence of nuclear pores in nuclear envelope areas ad-
justant to the vacuole (Severs et al., 1976). Taken together,
these results clearly demonstrate that the in vivo localiza-
tion of the GFP-nucleoporin fusions faithfully reflects NPC distribution in both wild-type and nucleoporin mutant yeast cells.

Recently, a light microscopy method in which single NPCs can be visualized and their three-dimensional arrangement assessed in permeabilized 3T3 cells has been described (Kubitscheck et al., 1996). However, because of the higher density of NPCs in wild-type yeast cells (11–15 pores/μm²) as compared to 5 pores/μm² in 3T3 cells (Severs et al., 1976; Maul and Deaven, 1977; Kubitscheck et al., 1996), single yeast nuclear pores labeled with GFP-Nup49p cannot be visualized by this method (Kubitscheck, U., personal communication). Therefore, to analyze the dynamics of nuclear pore distribution in yeast, we have used the properties of NUP133-disrupted (nup133Δ) cells that display a constitutive NPC clustering that is not associated with drastic alterations in the structure of the nuclear envelope (Doye et al., 1994; Li et al., 1995). This strain enabled us to follow two aspects of NPC dynamics: the movements of NPC clusters in dividing nup133Δ cells and the redistribution of the clustered NPCs once Nup133p is provided to nup133Δ cells.

In dividing nup133Δ cells expressing GFP-Nup49p, apparent movements of NPC clusters could be visualized, whereas no major changes in their distribution were detected in cells in stationary phase (data not shown). In dividing cells, the dynamic distribution of the NPC clusters could thus be related to modifications in the nuclear shape and/or to the nuclear rotation that is required for correct positioning of the spindle pole bodies. It has also been reported that in S. cerevisiae, both nuclear surface area and nuclear pore number rapidly increase during nuclear division up until cell division (Jordan et al., 1977). The progressive spreading of NPC clusters along the nuclear envelope could therefore be induced by membrane formation if localized in between NPC clusters. Alternatively, lateral movements of NPC clusters within the nuclear envelope, possibly driven by interactions with as yet unidentified structures, may also be involved in this process (see below).

To induce the redistribution of the clustered NPCs present in nup133Δ cells, Nup133p was either expressed under the control of the inducible GAL10 promoter or provided to nup133Δ cells upon mating and subsequent cytoduction. Using both approaches, a correlation could be made between the expression level of Nup133p and the degree of nuclear pore clustering: an intermediate clustering phenotype could be observed in cells that expressed low levels of Nup133p, whereas random NPC distribution was only achieved once wild-type levels of Nup133p were reached. This result suggests that a threshold level of Nup133p is required for homogeneous NPC distribution in wild-type cells. The correlation between the amount of Nup133p provided to the nup133Δ nucleus upon cytoduction and the rate of NPC redistribution further demonstrates that Nup133p is directly involved in this process.

A major question raised in this study was to determine how Nup133p establishes an even NPC distribution, and whether this redistribution could be uncoupled from de novo NPC biogenesis and turnover. Indeed, upon mating between nup133Δ cells expressing GFP-Nup49p and wild-type cells, whereas a progressive redistribution of the clustered NPC was observed, a homogeneous labeling of the wild-type nucleus could be detected within 20 to 30 min. Similarly, when GFP-Nup49p was provided by the wild-type cell, its targeting to the nup133Δ-clustered NPCs could be observed. Similar results were also obtained upon analysis of GFP-Nup133p targeting to either wild-type or nup133Δ nuclei. This progressive labeling of the nucleus might correspond to the targeting of additional GFP-tagged nucleoporin subunits to the preexisting nuclear pores, to the exchange between GFP-fusion proteins and preexisting nucleoporins, or to the incorporation of GFP-Nup49p upon de novo synthesis of NPCs. Since nothing is known so far concerning the rate of NPC biogenesis in yeast heterokaryons, we cannot discriminate between the incorporation of GFP-tagged nucleoporins in either preexisting or newly assembled NPCs. Since the precise subcellular localization of Nup49p and Nup133p has not been analyzed so far, these two nucleoporins might thus be peripheral and accordingly easily exchangeable components of the NPC. In contrast, a set of abundant nucleoporins including Pom152p, Nic96p, Nup157p, Nup170p, and Nup188p have been proposed to form the octagonal core structure of the NPC (Aitchison et al., 1995b; Nebbress et al., 1996) and accordingly might only be incorporated during NPC assembly. Similar cytoduction assays, if extended to the analysis of such nucleoporins, may thus allow discrimination between exchange of subunits within preexisting NPCs and de novo NPC assembly. Therefore, we cannot exclude that the apparent nuclear pore redistribution occurring within the nup133Δ nucleus upon mating with wild-type cells may, at least in part, involve NPC biogenesis. Under these standard conditions, NPC redistribution can therefore not be uncoupled from a putative de novo assembly of nuclear pores.

In contrast, the use of yeast cells overexpressing Nup133p in such cytoduction experiments enables us to dissociate NPC redistribution from this putative de novo NPC biogenesis. Under these mating conditions, the amount of available Nup133p is no longer limiting. The rapid targeting of overexpressed Nup133p to the clustered NPCs, as visualized when Nup133p is tagged with GFP, correlates with the increased rate of NPC redistribution, which can occur within 10 min. This could imply that simultaneous degradation of the clustered NPCs and de novo biogenesis of randomly distributed nuclear pores could take place. However, since no detectable labeling of the wild-type nuclei could be observed within this short period, both processes should be specifically stimulated in the nup133Δ nuclei. In addition, since the rate of NPC redistribution correlates with the amount of Nup133p provided to the nup133Δ nucleus, these two processes should be directly induced by Nup133p. One would then expect Nup133p to play a key role in both NPC biogenesis and degradation. Yet, unlike Nsp1p and Nic96p, whose depletion/ mutation induces a decrease in nuclear pore density (Mutvei et al., 1992; Zabel et al., 1996), NUP133 disruption does not significantly alter the overall number of nuclear pores (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995). Furthermore, upon mating between nup133Δ cells and cells overexpressing Nup133p, analysis of GFP-Nup49p targeting revealed that the incorporation of GFP-Nup49p is not stimulated in the nup133Δ nuclei. In contrast, we could...
demonstrate that NPC redistribution occurs before the incorporation of GFP-Nup49p in the nup133\(^{-}\) nucleus. These results therefore indicate that NPC redistribution does not require de novo NPC biogenesis but rather corresponds to the redistribution of preexisting clustered NPCs. This rapid redistribution is most likely achieved through lateral movements of preexisting NPCs within the nuclear envelope.

The finding that NPCs can rapidly move within the nuclear envelope was rather unexpected. Although rapid movements of transmembrane proteins that may result from membrane fluidity have been frequently reported, NPCs are elaborate structures embedded in two membranes, the inner and the outer nuclear membrane. NPC in higher eukaryotes were also shown to be physically anchored in the nuclear lamina (Aaronson and Blobel, 1974). In addition, filamentous structures associated with the baskets and projecting deeply inside the nucleus (Franke and Scheer, 1970; Scheer et al., 1976; Ris and Malicki, 1993) and a nuclear lattice that is thought to connect the distal rings of NPC baskets (Goldberg and Allen, 1992) have been observed in amphibian oocytes. Filaments protruding from the cytoplasmic side of the NPCs may also anchor the nuclear pores within the cytoskeleton (Scheer et al., 1976; for review see Goldberg and Allen, 1995). Similarly, both cytoplasmic and nuclear filaments associated with the nuclear pore complexes have been observed in S. cerevisiae (Allen and Douglas, 1989; Rout and Blobel, 1993). Although not demonstrated in higher eukaryotes, lateral movements of NPCs within the nuclear envelope may therefore require specific modifications of these NPC-anchoring structures. For example, drastic changes in NPC distribution are found during apoptosis (Falcieri et al., 1994). This may be related to nuclear lamina disassembly which has also been observed to occur during the active phase of apoptosis (Lazebnik et al., 1993). However, since no lamins have yet been identified in yeast, lateral mobility may be a specific characteristic of yeast NPCs. Finally, the rapid redistribution of preexisting NPCs, so far only demonstrated in our cytoduction assays, may reflect a specific feature of nup133\(^{-}\) cells in which interactions between NPCs and putative anchoring structures would be impaired.

Various factors have been previously suggested to influence nuclear pore distribution in eukaryotic cells (discussed in Markovics et al., 1974; Severs et al., 1976). One factor is the presence of condensed chromatin in close proximity to the inner nuclear membrane. In addition, the vacuolar membrane was proposed to constitute one constraint on nuclear pore distribution in yeast (Severs et al., 1976). Unlike in these various models, changes in NPC distribution that occur in nucleoporin mutants have not been found associated with major changes in vacuolar or chromatin organization: in these mutants, pore-free areas are not associated with vacuolar membranes. In addition, immunolocalization of Rap1p, a telomere binding protein (Klein et al., 1992), revealed a normal telomere distribution in nup133\(^{-}\) cells (Gotta et al., 1996). As an alternative explanation, the orientation of the clustered NPCs may reflect a higher order structure within the nucleus since NPC clusters in nup120-disrupted cells were generally found opposite to the nucleolus (Aitchison et al., 1995a).

Among the nucleoporins that affect NPC distribution, Nup84p, Nup85p, and Nup120p are tightly associated within a nuclear pore subcomplex (Siniossoglou et al., 1996). NPC clustering may thus correspond to a specific defect shared by a subset of proteins associated within one or a few NPC substructures. A weak homology has been previously reported between the amino-terminal domain of Nup133p that encompasses our nup133-AN deletion and a central domain within Nup120p (Aitchison et al., 1995a). In addition, nup133\(^{-}\) and nup120\(^{-}\) cells display a similar constitutive clustering phenotype that is not associated with major alteration of the nuclear envelope at permissive temperature (Doye et al., 1994; Aitchison et al., 1995a; Heath et al., 1995; Li et al., 1995). Although phenotypical analysis of the corresponding nup120 deletion mutant would be necessary to test this hypothesis, the interaction of Nup133p and Nup120p with a common partner may be required for inducing or maintaining the homogeneous distribution of NPCs in the nuclear envelope. Our future goal will be to identify such component(s) interacting with the amino-terminal domain of Nup133p and required for homogeneous NPC distribution.

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