A Conditional Allele of the Novel Repeat-containing Yeast Nucleoporin RAT7 / NUP159 Causes Both Rapid Cessation of mRNA Export and Reversible Clustering of Nuclear Pore Complexes

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Abstract. In a screen for Saccharomyces cerevisiae genes required for nucleocytoplasmic transport of messenger RNA, we identified the RAT7 gene (ribonucleic acid trafficking), which encodes an essential protein of 1,460 amino acids. Rat7p is located at the nuclear rim in a punctate pattern characteristic of nucleoporins. Furthermore, the central third of Rat7p contains 22 XXFG and three XFXFG degenerate repeats that are similar to signature GLFG and XFXFG repeats present in a majority of yeast and some mammalian nucleoporins sequenced to date. Shift of a strain bearing the temperature-sensitive rat7-1 allele from 23°C to 37°C resulted in rapid (within 15 minutes) cessation of mRNA export, but did not cause concomitant cytoplasmic accumulation of a reporter protein bearing a nuclear localization signal. This suggests that Rat7p may play a direct role in nucleocytoplasmic export of RNA. Immunofluorescence and thin section electron microscopy revealed that in rat7-1 cells grown at 23°C, the majority of nuclear pore complexes (NPCs) were clustered on one side of the nucleus. No ultrastructural abnormalities of the nuclear envelope were seen. Interestingly, shifting rat7-1 cells to 37°C for 1 h caused the NPCs to disperse, restoring near wild-type NPC distribution. After this temperature shift, the mutant Rat7p was no longer detectable by immunofluorescence.
manner with the 3′ end of the particle in the lead (Mehlin et al., 1992). The very large diameter of these Balbiani ring RNP particles requires that they unfold as they pass through the NPC (Scheer et al., 1988; Mehlin et al., 1992).

NPCs span the nuclear envelope at sites where the inner and outer nuclear membranes are fused. NPCs are composed of ~100 distinct proteins and have an estimated molecular mass of 125 megadaltons (mD) in Xenopus and 65 mD in yeast (Reichelt et al., 1990; Rot and Blobel, 1993). The structure of the NPC is believed to be similar in all eukaryotic organisms (Maul, 1977) and has been determined using EM and high-resolution image reconstruction (Unwin and Milligan, 1982; Hinshaw et al., 1992; Akey and Radermacher, 1993). Briefly, the NPC is thought to consist of two coaxial rings that are coplanar with the inner and outer membranes of the NE and are connected by eight “spokes” that extend inward and delimit a central channel. The outermost region of the spokes are located within the lumen of the NE where, presumably, they serve to anchor the NPC in the pore membrane. Eight short fibrils emanate from the cytoplasmic ring into the cytoplasm. Eight longer fibrils protrude from the nuclear ring into the nucleoplasm, where their ends are connected by a smaller ring which forms the base of a basket-like structure (Jarnik and Aebi, 1991; Ris, 1991). There is evidence that NPCs are attached to the nuclear lamina via their nuclear rings (Stewart and Wytoczyk, 1988; Akey, 1989) and to a separate “nuclear envelope lattice” via the distal basket ring (Goldberg and Allen, 1992).

At present, the sequences of only 8 vertebrate and 11 yeast NPC proteins (also called nucleoporins) are known (for review see Rout and Wente, 1994). Two nucleoporin subfamilies have been defined on the basis of highly repeated sequence motifs. Yeast Nup49p/Nsp49p, Nup100p, Nup16p/ Nsp16p (Wente et al., 1992; Wimmer et al., 1992), and Nup45p (Fabre et al., 1994; Wente and Blobel, 1994) all contain GLFG (gly-leu-phe-gly) repeats, whereas degenerate XFXFG (X-phe-X-phe-gly) repeats exist in vertebrate p62 (Starr et al., 1990; Carreno-Fonseca et al., 1991; Cordes et al., 1991), Nup153p (Sukegawa and Blobel, 1993) and Pom121p (Halling et al., 1993), and in yeast Nuplp (Davis and Fink, 1990), Nsplp (Hurt, 1988), and Nup2p (Loeb et al., 1993). The functional significance of these repeats is unclear, though in some cases they can be deleted (Nehrbass et al., 1990; Loeb et al., 1993) without affecting cell viability.

Individual yeast nucleoporins have been implicated in nucleocytoplasmic transport. Nup100p, Nup16p, and Nup45p all contain a novel RNA binding motif that has been shown to mediate binding to homopolymeric RNA in vitro, suggesting that these proteins could interact directly with RNA during its transport through the NPC (Fabre et al., 1994). Depletion of Nup45p from wild-type cells results in the gradual impairment of both poly(A)+ RNA export and, somewhat later, protein import (Fabre et al., 1994). Yeast strains bearing various conditional alleles of NUP1 also show defects in both of these processes (Bogerd et al., 1994). Strains in which NUP133 is mutated or partially deleted have been shown to accumulate poly(A)+ RNA in their nuclei after shift to 37°C (Doye et al., 1994; Li et al., 1995). Two different ts alleles of NSP49/NUP49 have been shown to affect poly(A)+ RNA export and protein import differentially (Doye et al., 1994). Inhibition of protein import was reported for wild-type cells in which NSP1 expression had been shut off for >10 h (Mutvei et al., 1992). Despite these findings, none of the nucleoporins has been shown to be directly involved in translocation of substrate through the NPC.

The known yeast nucleoporins have been identified using several distinct experimental approaches including screening of λgt11 expression libraries with antibodies raised against rat liver nuclear envelopes or yeast nucleoskeleton preparations (Davis and Blobel, 1986; Hurt, 1988; Wente et al., 1992; Loeb et al., 1993; Wente and Blobel, 1994), selection for genes whose products interact with the proteins encoded by mutant alleles of the nucleoporins NSP1 (Wimmer et al., 1992; Fabre et al., 1994) and NSP49 (Doye et al., 1994) or RPA190 (encoding the largest subunit of RNA polymerase I) (Yano et al., 1992), and biochemical isolation (Grandi et al., 1993; Wozniak et al., 1994). None of these approaches selected for nucleoporins with specific transport functions.

Here we describe a novel yeast nucleoporin identified on a functional basis. We cloned RAT7 (ribonucleic acid trafficking) by complementation of the temperature-sensitive growth defect of a mutant Saccharomyces cerevisiae strain isolated in a screen for strains with temperature-dependent defects in poly(A)+ RNA export. The RAT7 gene is essential and encodes a 1,460-amino acid protein containing 25 XXFG and 3 XFXFG degenerate repeats. 12 of the XXFG repeats are embedded in four nearly perfect tandem 26-amino acid repeats. Antiserum to the repeat domain of Rat7p decorated the nuclear periphery in a punctate pattern characteristic of NPC proteins. Shift of rat7Δ cells to the nonpermissive temperature of 37°C resulted in rapid (within 15 min) accumulation of poly(A)+ RNA in the nucleus, but did not cause concomitant cytoplasmic accumulation of a reporter protein containing a nuclear localization signal. Thin section EM and indirect immunofluorescence of rat7Δ cells grown at 23°C revealed NPCs clustered toward one region of the nuclear envelope. Partial dispersal of these clustered NPCs occurred within 1 h of a shift of mutant cells to 37°C, and under these conditions, the mutant Rat7p could no longer be detected. The rapid induction of nuclear accumulation of poly(A)+ RNA when mutant cells were shifted to 37°C along with the lack of a detectable effect on protein import are consistent with a direct role for Rat7p in mRNA export.

Materials and Methods

Yeast Strains, Growth Conditions, and Genetic Methods

Table I lists the yeast strains used in this study. The strains were grown in rich media (1% yeast extract, 2% bactopeptone) with either 2% dextrose (YPD), 2% raffinose (YPR), or 2% galactose (YPG), or in synthetic complete media lacking specific amino acids (Rose et al., 1989). Temperature shifts were performed by brief centrifugation to collect cells and resuspension in prewarmed media. Yeast cells were transformed by electroporation (BioRad Laboratories, Melville, NY) and were allowed to recover in rich media with 1 M sorbitol at 23°C for at least 1 h before plating. General genetic manipulations of yeast cells, including strain crosses, sporulation, and tetrad dissection, were performed according to Rose et al. (1989).

Generation and Screening of Temperature-sensitive Mutants

UV mutagenesis of yeast strains FY86 and FY23 (provided by Dr. F. Win-
Table 1. Yeast Strains

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Comments</th>
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<tr>
<td>FY23</td>
<td>MATα ura3-52 trpl1Δ63 leu2Δ1</td>
<td>Wild type; derived from S288C; obtained from</td>
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<tr>
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<td>Dr. Fred Winston</td>
</tr>
<tr>
<td>FY86</td>
<td>MATα ura3-52 his3Δ2000 leu2Δ1</td>
<td>Wild type; derived from S288C; obtained from</td>
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<tr>
<td></td>
<td></td>
<td>Dr. Fred Winston</td>
</tr>
<tr>
<td>LGtsso230</td>
<td>MATα ura3-52 his3Δ2000 leu2Δ1 rat7-1&quot;</td>
<td>Original rat7&quot; isolate</td>
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<td>LGY101</td>
<td>MATα ura3-52 his3Δ2000 leu2Δ1 rat7-1&quot;</td>
<td>Segregant from 3rd LGtsso230 × FY23 backcross</td>
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<td>LGY103</td>
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<td>LGY105</td>
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<td>Genetics Course, 1990</td>
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<tr>
<td>CHS 5-8</td>
<td>MATα his5-131</td>
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In Situ mRNA Localization Assay

Cells shifted to 37°C for specified times were collected by centrifugation in a 37°C room, resuspended in 37°C 0.1 M K2HPO4, pH 6.5, 4% formaldehyde, and incubated for 90 min at 37°C with gentle agitation. Fixation was ended by washing twice with phosphate buffer (0.1 M K2HPO4, pH 6.5) and once with wash buffer (0.1 M K2HPO4, pH 6.5, 1.2 M sorbitol) at 23°C. Cells were resuspended in 1 ml wash buffer and stored at 4°C for up to 18 h. Processsing of fixed cells was carried out essentially as described previously (Amberg et al., 1992) with a modification of the permeabilization step. All incubations and washes were performed at 23°C unless otherwise noted. Cells in 1 ml of wash buffer were treated with 300 μg 100T Zymolyase (Seikagaku America Inc., Rockville, MD) until 90% of the cells were no longer refractile (appearing grey) as visualized by phase contrast microscopy (15–60 min depending on the strain and length of temperature shift). These spheroplasted cells were washed gently, resuspended in wash buffer, and allowed to adsorb to polylysine-coated wells of teflon-faced slides for 10 min. Adhered cells were incubated successively with phosphate buffer, phosphate buffer with 0.1% NP-40, and phosphate buffer (5 min each), followed by 0.1 M triethanolamine, pH 8.0 for 2 min, triethanolamine with 0.25% acetic anhydride for 10 min, and 4× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 5 min. Cells were incubated in prehybridization solution (50% formamide, 10% dextran sulfate, 4× SSC, 1× Denhardt’s solution, 125 μg of tRNA/ml, 500 μg of denatured sonicated salmon sperm DNA) at 37°C for 1 h in a humid chamber. Oligo dT20 was endlabeled with digoxigenin-dUTP using terminal deoxynucleotidyl transferase as described previously (Amberg et al., 1992). Hybridization with prehybridization solution containing 500 pg/ml of digoxigenin-labeled (dT20) probe was carried out for 18 h at 37°C in a humid chamber. Cells were washed for 1 h in 2× SSC, 1 h in 1× SSC, 30 min in 0.5× SSC at 37°C, and 30 min in 0.5× SSC. The cells were equilibrated in antibody wash (ABW) 1 (0.1 M Tris, pH 9.0, 0.15 M NaCl) for 5 min and blocked in ABW containing 5% heat-inactivated FCS and 0.3% Triton X-100 for 1 h. The cells were incubated in ABW containing 5% FCS, 0.3% Triton X-100, and fluoresceinlabeled antidigoxigenin Fab fragments (Boehringer Mannheim Corp., Indianapolis, IN) for 4 h in the dark. Unbound antibody was removed by washing with ABW1 first for 10 min, then for 30 min, followed by washing with ABW2 (0.1 M Tris, pH 9.5, 0.1 M NaCl, 50 mM MgCl2) first for 10 min, and then for 30 min. The nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (10 μg/ml in ABW2) for 5 min and then washed twice (5 min each) with ABW2. The slides were mounted under 90% glycerol, 1× PBS containing 1 mg of p-phenylene-diamine per ml, and stored at ~20°C.

Cloning and Sequencing of RAT7

The RAT7 gene was cloned by complementation of the ts growth phenotype of strain LGTso1 with a YCP50-based S. cerevisiae genomic library (a generous gift of Dr. Phil Hieter, Johns Hopkins University, Baltimore, MD). A 7.2-kb restriction fragment common to three overlapping, complementing clones was subcloned into pMOB (Gold Biotechnology, St. Louis, MO), and the resulting plasmid was introduced into a bacterial strain (DPWC) harboring TNI000 for random transposon insertion (Gold Bio-technology). With primers complementary to both transposon ends, sequence was generated from both strands by the dideoxy chain termination method (Sanger et al., 1977) using denatured, double-stranded DNA, [α-35S]dATP (New England Nuclear, Boston, MA) and Sequenase version 2.0 (United States Biochemical Corp., Cleveland, OH.). The DNA and predicted protein sequences were compared to the GenBank and EMBL data bases using FASTA and BLAST programs (Altschni et al., 1990; Pearson, 1994). The accession number for this sequence is L40634.

Disruption of RAT7 and Targeted Integration of LEU2 at the RAT7 Locus

To facilitate both construction of a rat7 null allele and targeted integration of LEU2 adjacent to the RAT7 locus, a 7.5 kb EcoRI genomic DNA fragment from complementing clone pLG1 was subcloned into YIpplac28 (Gietz and Sugino, 1988) from which polylinker restriction sites between and including 1,068-bp EcoRI/NsiI fragment from plasmid pLG4 was digested with EcoRI to generate a 4.5-kb linear RAT7::HIS3 disruption fragment, which
was used to transform diploid yeast strain LGY105. Southern blot analysis of His* transformants identified heterogeneous diploid strains with correct replacement of one allele of \(\text{RAT7}^{*}\). One such strain was transformed with pLG4 (\(\text{RAT7}^{*}\) under control of its own promoter on a URA3-marked CEN plasmid) yielding LGY106, which was sporulated and dissected onto YPD plates. Strains arising from spores were replica plated onto plates lacking histidine and onto plates containing 5-fluoroorotic acid (PCR Inc., Gainesville, FL) and grown at 3°C. The presence of the \(\text{RAT7}^{*}\) locus was performed as described previously (Guthrie and Fink, 1991). LEU2-marked pLG5 was linearized at a unique PstI site within the \(\text{RAT7}^{*}\) ORF and introduced into a haploid rat7Δ strain. Southern blot analysis of Leu* transformants was performed to identify strains that had homologously recombined linear DNA into the genomic \(\text{RAT7}^{*}\) locus, thereby abrogating temperature sensitivity. Positive strains were crossed with a strain carrying the rat7Δ allele and sporulated. Spores were tested for growth on YPD plates at 37°C or on plates lacking leucine at 23°C.

**Preparation of Rat7p Antiserum**

To generate a Rat7p antigen, we constructed a plasmid encoding glutathione S-transferase (GST) fused in frame with amino acids 486-779 of Rat7p. The ends of a Scal-Aval fragment from the \(\text{RAT7}^{*}\) ORF were blunt-ended using the Klenow fragment of DNA polymerase I, and the fragment was inserted into pGEX-5X (Pharmacia Corp., Piscataway, NJ) that had been linearized with Scal. Escherichia coli DH5a were transformed with the resulting plasmid. Fusion protein was expressed in and purified from bacteria acetate-grown cells by published methods (Ausubel et al., 1988). Briefly, a 3-liter culture was grown to an OD600 of 0.8 and isoprropyl \(\beta\)-D-thiogalactopyranoside (IPTG) was added as an induction of the fusion protein. Cells were grown an additional 4 h and then lysed by sonicating. Fusion protein was purified from the cell extract by chromatography on a glutathione-agarose column (Sigma Chemical Co., St. Louis, MO) and eluted with reduced glutathione (Sigma Chemical Co.). Guinea pigs were inoculated with 50 \(\mu\)g of purified fusion protein and then given 25-\(\mu\)g boosts after 2, 3, 7, and 11 wk. Cocalico Biologicals (Reamstown, PA) performed all immunizations and bleeds.

**Epitope Tagging of Rat7p**

To prepare myc-tagged Rat7p, a 120-bp DNA fragment encoding three consecutive myc epitopes (EQKLISEEDL) was released from pKK1 (obtained from G. Fink, Massachusetts Institute of Technology, Cambridge, MA) by BamHI digestion and inserted into an unique BamHI site corresponding to amino acid 623 in the \(\text{RAT7}^{*}\) ORF. To prepare hemagglutinin (HA)-tagged Rat7p, we synthesized two complementary oligonucleotides, which, when annealed, encoded the HA epitope (YPYDVPDYA) and have PstI ends. The oligonucleotides were heated to 90°C, slowly cooled to permit annealing, and inserted into the unique PstI site (corresponding to amino acid 456) in the \(\text{RAT7}^{*}\) ORF. Subclones were screened to identify those with the tags in the coding orientation. This yielded pLG8, a LEU2-marked CEN plasmid encoding myc-tagged Rat7p. One such strain was transformed with pLG8 (\(\text{RAT7}^{*}\) under control of its own promoter on a URA3-marked CEN plasmid) yielding LGY106, which was sporulated and dissected onto YPD plates. Strains arising from spores were replica plated onto plates lacking histidine and onto plates containing 5-fluoroorotic acid (PCR Inc., Gainesville, FL) and grown at 3°C.

**Immunofluorescence Procedures**

Indirect immunofluorescence was performed as described previously (Copeland and Snyder, 1993). Cells were fixed by adding 0.1 vol of 37% formaldehyde to cell cultures and incubating with gentle agitation for 1 h at 23°C. After three washes with 1.2 M sorbitol, 50 mM K2HPO4, pH 7.5 (solution A), cells were resuspended in 1 ml of solution A containing 300 \(\mu\)g 100T Zymolase (Seikakagu America Inc.) and incubated at 23°C for 10-60 min until spheroplasts were generated. These spheroplasts were washed gently once with solution A and adhered to poly-l-lysine coated 12 δm2 slides. Cells in these wells were washed sequentially with PBS containing 0.1% BSA (solution B), solution B containing 0.1% NP-40, and solution B with a 5-min incubation per wash. Cells were incubated with primary antibody in solution B overnight at 4°C and then washed again with solution B, solution B containing 0.1% NP-40, and solution B. Cells were incubated with secondary antibody in solution B for 2 h at 23°C and then washed once with each solution B, solution B containing 0.1% NP-40 and 10 \(\mu\)g of DAPI per ml, solution B containing 0.1% NP-40, and solution B. Cells were mounted as for the mRNA localization assay. Anti-myc epitope antibody (mAb RL1, kindly provided by Dr. J. Michael Bishop, University of California at San Francisco, San Francisco, CA). mAb RL1 was a generous gift of Dr. Larry Gerace (Scripps Institute, La Jolla, CA). Anti-ß-galactosidase antibody was purchased from Sigma Chemical Co. Dr. John Aris (University of Florida, Gainesville, FL) kindly provided anti-Nablp/Npl3p mAb IE4. Secondary antibodies included FITC-labeled goat anti-mouse IgM, FITC-labeled horse anti-mouse IgG, and FITC-labeled goat anti-guinea pig IgG (H+L) were all obtained from Vector Laboratories, Inc. (Burlingame, CA).

**Results**

**Rapid Nuclear Accumulation of mRNA in a Strain Bearing the Temperature-sensitive rat7Δ Allele**

To identify yeast genes involved in nucleocytoplasmic messenger RNA export, we generated ts strains of Saccharomyces cerevisiae and screened them to identify strains that accumulated poly(A)+ RNA in their nuclei following a shift to 37°C or shifted to 37°C for 1 h before addition of label. To measure incorporation of radioactivity into TCA-precipitable material, aliquots of each culture were removed after 30, 60, 90, 120, and 240 min of labeling and precipitated with 10% TCA. Precipitates were collected on TCA-pretreated glass filters (GF/A), washed sequentially with 10% TCA and 95% ethanol, and dried. 35S-Methionine incorporation was measured by scintillation counting.
clear accumulation of poly(A)+ RNA have been named RAT genes.

One of the strains isolated by this assay, LGtsot230, bears the ts rat7-1 allele, which caused rapid nuclear accumulation of mRNA in cells shifted to 37°C. Even at 23°C, rat7-1 cells displayed a partial mRNA mislocalization phenotype, with more than half of the cells exhibiting a somewhat brighter fluorescent signal in the nuclear region than in the cytoplasm (Fig. 1 B). After a 15-min shift to 37°C, nearly 100% of rat7-1 cells had nuclei that were dramatically brighter than their cytoplasm (Fig. 1 C). While nuclear fluorescence remained bright, the cytoplasmic fluorescence faded within 2 h at 37°C (Fig. 1 D), suggesting that little or no new mRNA reached the cytoplasm after the shift to 37°C. Almost 2 h at 37°C (Fig. 1 B). After a 15-min shift to 37°C, nearly 100% of fluorescent signal in the nuclear region than in the cytoplasm more than half of the cells exhibiting a somewhat brighter displayed a partial mRNA mislocalization phenotype, with clear accumulation of poly(A)+ RNA have been named RAT genes.

To determine whether the mRNA export block was reversible, rat7-1 cells were shifted to 37°C for 2 h and then returned to 23°C for 60 min before processing for in situ hybridization (Fig. 1 F). These cells had discernibly more cytoplasmic signal than cells examined after growth at 37°C for 2 h (compare Fig. 1, F and D). Their cytoplasmic signal was comparable to, if not brighter than, that seen in rat7-1 cells grown continuously at 23°C (compare Fig. 1, F and B). Although their nuclei were still bright, these cells appeared to have resumed exporting mRNA at 23°C. Viability data mirror these findings. Almost immediately after a shift to 37°C, rat7-1 cells stopped dividing without a specific terminal morphology. When returned to 23°C within 24 h, >80% of the cells were able to resume growth and form colonies.

We compared the growth rates of mutant and wild-type cells at both permissive and restrictive temperatures (Fig. 2). Consistent with the partial mRNA mislocalization phenotype exhibited by rat7-1 cells grown at 23°C, these cells had a doubling time at 23°C of 160 min, which is ~10-15% longer than the 140-min doubling time of wild-type cells grown at the same temperature. Mutant cells rapidly ceased growth after a shift to 37°C.

It would be expected that interruption of mRNA export would lead to a decline in the rate of protein synthesis. To examine this, cultures of wild-type and rat7-1 cells that had been grown to early log phase at 23°C were either left at 23°C or shifted to 37°C. After an additional hour of growth, [35S]methionine was added to all cultures and the rate of incorporation into protein examined. Wild-type cells incorporated [35S]methionine into protein at approximately the same rate at both 23°C and after a shift to 37°C. Although cells carrying the rat7-1 mutation displayed a near wild-type rate of incorporation of [35S]methionine into protein at 23°C, the rate fell to <10% of the wild-type rate within 60 min of a shift to 37°C (data not shown).

Cloning and Sequencing of RAT7

The RAT7 gene was cloned by complementation of the ts growth phenotype of yeast strain LGY101 using an S. cerevisiae genomic library. LGY101 is a segregant from the third backcross of the original rat7-1 isolate, LGtsot230. Temperature sensitivity and nuclear accumulation of poly(A)+ RNA cosegregated in 24 out of 24 segregants tested from the third backcross, indicating that a single mutant allele confers both phenotypes. Those library plasmids that corrected the temperature-sensitive growth defect of strain LGY101 were tested for their ability to restore mRNA export to rat7-1 cells grown at 37°C. All clones that could were related, based on restriction endonuclease digestion patterns. Sequencing of a region common to three different complementing clones revealed an open reading frame encoding a protein of 1,460 amino acids (Fig. 3) with a predicted molecular mass of 159 kDa. This was consistent with Northern blot analysis, which indicated that the DNA fragments sequenced hybridized to a transcript of 4.4 kb (data not shown). Expected consensus sequences for transcription and translation initiation signals were found in the sequence upstream of the RAT7 ORF.

Analysis of the primary amino acid sequence revealed that Rat7p contains three different classes of repeats, most of which are clustered in the central third of the protein. As shown in Figs. 3 and 4, there are 25 degenerate XXFG repeats and 3 degenerate XFXFG repeats. 12 of the XXFG repeats are found within four nearly perfect 26-amino acid repeats. Repeats containing the uncommon combination of phenylalanine (F) followed by glycine (G) have been found in a majority of sequenced yeast nucleoporins. Nup49p/Nsp49p, Nup100p, Nup16p/Nsp116p (Wente et al., 1992; Wimner et al., 1992), and Nup145p (Fabe et al., 1994; Wente and Blobel, 1994) all contain GLFG repeats. Nsp1p (Hurt, 1988), Nuplp (Davies and Fink, 1990), and Nup2p (Loeb et al., 1993) contain multiple XFXFG degenerate repeats that form the cores of 9 amino acid repeats that are loosely related in Nuplp and Nup2p and highly conserved in Nsp1p. Examination of the Rat7p XFXFG repeats (Fig. 4) reveals that PSFG and SAFG are found most frequently. There is no clear consensus sequence for the Rat7p XFXFG repeats. 25% of Rat7p amino acids are charged, with 50% more acidic residues than basic resulting in a low isoelectric point of 4.5. The charges are distributed fairly evenly throughout the protein. Secondary structure programs predict that the COOH-terminal third of the protein is largely a helical, unlike the central repeat region, which is rich in proline (P). Furthermore, within the COOH-terminal third is a region (amino acids 1,281-1,301) predicted to have the potential to form coiled-coil interactions (Lucas et al., 1991). The segments from amino acids 1,303-1,316 and 1,392-1,412 have a lower but significant probability of also forming coiled-coil interactions. The NH2-terminal third has no apparent distinguishing characteristics. Analysis of the protein sequence did not reveal any functional motifs such as RNA binding domains, transmembrane domains, or zinc fingers. There are many potential phosphorylation sites within Rat7p.

A FASTA search of the SwissProt and EMBL data bases for proteins with homology to Rat7p yielded five yeast nucleoporins among the highest scoring sequences. Nup49p/Nsp49p shares 25.8% amino acid identity with Rat7p over a stretch of 151 residues. Nuplp, Nup2p, and Nup145p have 16-19% identity over 380-510 amino acids, and Nsp1p has 21% identity over 408 amino acids. In all five cases, the regions of homology map to the repeat region of Rat7p between amino acids 480 and 980 (see Fig. 5). Two vertebrate nucleoporins, Nup153p and Nup214p, showed ~25% identity with the Rat7p repeat region over 313 and 195 amino acids. The protein sequence did not reveal any functional motifs such as RNA binding domains, transmembrane domains, or zinc fingers. There are many potential phosphorylation sites within Rat7p.
RAT7 is an Essential Gene on Chromosome IX

To determine whether RAT7 is an essential gene, we performed standard gene replacement and plasmid rescue (Guthrie and Fink, 1991). We removed 4,036 bp out of the 4,380-bp RAT7 ORF and replaced them with the HIS3 gene (Fig. 5). Haploid cells bearing the disrupted allele were unable to grow without a plasmid-borne RAT7 gene, indicating that RAT7 is essential for mitotic growth of S. cerevisiae.

The chromosomal location of RAT7 was determined by hybridizing labeled RAT7 DNA to an ordered set of λ clones representing most of the yeast genome (obtained from Riles, L., Washington University, St. Louis, MO, and M. V. Olson, University of Washington, Seattle, WA). The RAT7 probe recognized λ clone 4210, which maps to the left arm of chromosome IX midway between the centromere and telomere. We confirmed this physical mapping with genetic mapping of the rat7-1 allele against his5-131. The HIS3 gene has also been mapped to λ clone 4210. Based on the segregation data from the genetic cross (10 parental ditypes, 2 tetratypes, 0 nonparental ditypes), the calculated distance between the two loci is ~10 cM. Agreement between physical and genetic mapping data suggested that the gene we cloned was RAT7 rather than a suppressor of the rat7-1 allele.

Figure 1. Temperature shift of rat7-1 cells to 37°C rapidly induces a reversible block in mRNA export. Wild-type and rat7-1 cells were grown to mid-log phase in rich media at 23°C and then subjected to various temperature conditions before fixation and in situ hybridization with a digoxigenin-tagged oligo (dT) probe for localization of poly(A) + RNA. A–D and F show fluorescence signal from representative fields of cells that were probed with FITC-conjugated anti-digoxigenin antibody after hybridization. E shows DAPI staining for the same field of cells as in D. (A) Wild type, 2 h at 37°C; (B) rat7-1, 33°C; (C) rat7-1, 15 min at 37°C; (D and E) rat7-1, 2 h at 37°C; (F) rat7-1, 2 h at 37°C followed by 1 h at 23°C. Identical photographic and printing conditions were used for A–D and F. Shorter exposure times were used for the single DAPI-stained image (E).
Figure 3. Nucleotide sequence of Rat7 and predicted protein sequence of Rat7p. 5904 nucleotides including the 4,380-nucleotide Rat7 open reading frame are presented. The predicted Rat7p amino acid sequence is shown in single-letter code, and the last amino acid in each line is numbered. XFXG repeats are underlined. XFXFG repeats are bracketed and in bold. Direct repeats of 26 amino acids are shaded gray. The amino acids included in the GST-Rat7 fusion protein are bracketed by >> and <<. These sequence data are available on April 13, 2017.
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Analytically for the discrepancy between the predicted and apparent molecular weight of Rat7p. Thus far, glycosylation of yeast translational modifications.

Figure 5. Schematic representation of the Rat7p locus and modifications made to it. The 4,380-bp Rat7 ORF is indicated by the long shadowed box. DNA flanking the ORF is represented by thin horizontal lines. Within the ORF, a striped section indicates the region used for the Rag7 fusion. For the gene disruption, DNA in between the diagonal lines was replaced with the HIS3 gene as shown. An asterisk marks the BamH1 site into which DNA encoding three tandem “myc” epitopes was inserted. All restriction sites used in this work are shown. Numbers below the ORF indicate the positions of amino acids corresponding to the sequence junctions shown. The repeat region, which contains all of the repeats except for three NH2-terminal XXFG repeats, is marked with a double headed arrow.

To determine whether rat7-1 cells have rapidly induced protein import defects, we analyzed the distribution of a karyophilic reporter protein in rat7-1 cells. Indirect immunofluorescence was performed to monitor the distribution of a fusion protein containing Escherichia coli β-galactosidase and the first 33 amino acids of yeast histone H2B (containing its nuclear localization signal) (Moreland et al., 1987). As a positive control for defects in nuclear protein import, we used strain LDY97, which carries the sup1-166 allele. This

Analysis of the Distribution of a Karyophilic Reporter Protein in rat7-1 Cells Shifted to the Restrictive Temperature

The NPC supports bidirectional transport of macromolecules across the nuclear envelope. The mutation in the rat7-1 allele could alter NPC structure in a way that affects all nucleocytoplasmic transport. If this were the case, we would expect that defects in protein import in rat7-1 cells would occur with kinetics similar to those observed for defects in mRNA export. Alternatively, the rat7-1 mutation could specifically affect RNA export functions of the NPC.
strain has been shown to have temperature-dependent defects in both mRNA export and protein import (Bogerd et al., 1994). Wild-type rat7-1 and nupl-106 cells were transformed with a plasmid encoding the reporter protein under the control of GAL10 promoter elements, facilitating high levels of expression upon induction with galactose and repression when glucose is present. However, nupl-106 cells require >2 h at the nonpermissive temperature to develop an RNA export block, and never show poly(A)+ RNA accumulation in more than a minority of the cells. Therefore, if handled identically to rat7-1 cells, the nupl-106 cells would continue to export reporter protein mRNA after the temperature shift, while rat7-1 cells would cease mRNA export rapidly. To control for this difference, we limited reporter gene transcription to 90 min in all cell types by adding glucose at the end of the induction period.

All three transformed strains were grown to early log phase at 23°C in YPR, rich media containing 2% rafinose (a noninducing carbon source). For unshifted samples, cells were pelleted and resuspended in YPG (2% galactose) to induce reporter gene transcription. After 1.5 h of induction, cells were transferred to YPD (2% dextrose) and grown for 1.5 h more at 23°C before fixation. In both wild-type (Fig. 8 A) and rat7-1 cells (Fig. 8 B), reporter protein was detected exclusively in the nucleus, based on colocalization of FITC (Fig. 8) and DAPI staining (data not shown). By contrast, in nupl-106 cells, reporter protein accumulated in the nucleus but was also clearly detected in the cytoplasm (Fig. 8 C), suggesting a partial protein import block in these mutant cells cultured at 23°C. Since rat7-1 cells show moderate accumulation of mRNA in their nuclei when grown at 23°C (see Fig. 1 B), the lack of detectable cytoplasmic accumulation of the reporter protein provides evidence that the moderate defect in mRNA export in rat7-1 cells does not reflect an overall impairment of nucleocytoplasmic trafficking.

To examine protein import under nonpermissive conditions, nupl-106 cells were shifted to 36°C for 2 h before galactose induction because it has been shown that these cells require 3 h to show a protein import block (Bogerd et al., 1994). After 2 h of incubation at 36°C, we induced reporter gene expression for 1.5 h at the same temperature. After this induction period, cells were incubated in YPD an additional 1.5 h at 36°C before processing for indirect immunofluorescence. 36°C was used as the non-permissive temperature because we have found that the GAL10 promoter does not function efficiently at 37°C. We have performed the in situ mRNA localization assay on rat7-1 cells shifted to 36°C and saw no difference from mutant cells shifted to 37°C; data not shown.) nupl-106 cells shifted to 36°C showed significantly stronger staining for the reporter protein in their cytoplasm than when grown continuously at 23°C (compare Fig. 8, F and C). In some cells, the cytoplasm was so bright that a distinct nuclear signal could no longer be discerned.

In rat7-I cells, it was necessary to induce the reporter gene at the permissive temperature since the very rapid block to mRNA export at 36°C precluded the production of detectable quantities of reporter protein when induction was performed at 36°C (data not shown). Cultures of wild-type and rat7-I cells that had been induced at 23°C for 1.5 h were transferred to 36°C YPD for an additional 1.5 h before fixation. There was no obvious temperature-dependent cytoplasmic accumulation of the reporter protein in either wild-type or rat7-I cells (Fig. 8, D and E). This absence of detectable cytoplasmic reporter protein in rat7-I cells shifted to 36°C is compatible with the possibility that rapid development of the mRNA export block in rat7-I cells at 36°C is not a consequence of a nonspecific structural collapse of the NPC. This conclusion is supported by the absence of cytoplasmic accumulation of two endogenous nuclear proteins, Noplp and Npl3p, either in cells maintained at 23°C or after a 1-h shift to 37°C (data not shown). However, due to the very rapid

Figure 7. Immunolocalization of the Rat7 protein. Indirect IF was performed on wild-type cells using antisera raised against a GST-Rat7 fusion protein bearing 292 amino acids from the repeat region (see Figs. 2 and 4) in the central third of Rat7p. A shows the fluorescent Rat7p staining pattern. B shows the same field of cells stained with DAPI.
Figure 8. Nuclear import of an overexpressed karyophilic reporter protein in wild-type, rat7-1, and nup1-106 cells. Reporter protein consisting of the histone 2B nuclear localization signal fused to β-galactosidase was overexpressed in three different strains and immunolocalized with anti-β-galactosidase antibody after growth at 23°C or 36°C. All strains were grown to mid-log phase in medium containing raffinose, a noninducing carbon source. Cells shown in F were preshifted to 36°C in the same medium. Overexpression of the GAL10 promoter-driven reporter gene was induced by growth on galactose-containing medium for 1.5 h at 23°C (A-D) or 36°C (F). After induction, cells were transferred to glucose-containing medium and grown at 23°C (A-C) or 36°C (D-F) for 1.5 h before processing for indirect IF. (A and D) Wild type; (B and E) rat7-1; (C and F) nup1-106.

cessation of mRNA export in rat7-1 cells shifted to 36°C, we cannot be certain that the amount of reporter protein synthesized after the shift to 36°C was sufficient to detect cytoplasmic accumulation. Clearly, rat7-1 cells do not have a defect in retention of nuclear proteins at the nonpermissive temperature, but additional studies will be required to evaluate the ability of rat7-1 cells to transport karyophilic proteins to the nucleus.

rat7-1 Mutants Have Aberrant Distribution of NPCs

To determine whether the mutation(s) in the rat7-1 allele produced any structural and/or localization defects associated with NPCs, immunofluorescence (IF) and EM studies were carried out with mutant cells cultured either at 23°C or shifted to the nonpermissive temperature of 37°C. For IF studies, we used the RL-1 antibody (Snow et al., 1987), which recognizes both mammalian and S. cerevisiae NPC proteins (Copeland and Snyder, 1993). Wild-type cells that were grown at either 23°C or shifted to 37°C and stained with RL-1 exhibited punctate staining of moderate intensity all around the nuclear perimeter (Fig. 9 A). In contrast, rat7-1 cells grown at 23°C and stained with RL-1 showed a very strong, continuous signal that was localized to one region of the nuclear periphery (Fig. 9 B). Punctate staining...
Figure 9. Indirect IF of NPCs in wild-type and rat7-1 cells. Wild-type and rat7-1 cells were grown continuously at 23°C or shifted to 37°C for 1 h before fixation and processing for indirect IF. Cells were stained with monoclonal antibody RL-1 (Snow et al., 1987), which recognizes NPC epitopes from various species including rat and S. cerevisiae. The fluorescent staining patterns in each of the four panels reflect the intracellular distribution of NPCs. (A) Wild type, 23°C; (B) rat7-1, 23°C; (C) wild type, shifted to 37°C for 1 h; (D) rat7-1 shifted to 37°C for 1 h.

around the remainder of the nuclear rim was greatly reduced in intensity, relative to wild-type cells, or was altogether absent. The intensification and melding of signal around part of the nuclear periphery together with the reduction of signal elsewhere suggested that the epitopes detected by RL-1 were clustered together in one region of the nuclear envelope. The degree of "clustering" varied from cell to cell, with staining extending between one quarter and two thirds of the way around the nuclear periphery in a majority of cells. A low percentage of cells displayed immunofluorescence in a single small spot, while even fewer retained an almost wild-type appearance (see cell marked with arrow in Fig. 9 B). In all cases, the observed signal was found adjacent to the DAPI-stained region, indicating that the RL-1 antibody staining was at the nuclear periphery (data not shown).

Surprisingly, rat7-1 cells that were shifted to 37°C for 1 h displayed RL-1-reactive antigens distributed around the nuclear periphery in a pattern more similar to that of wild-type cells (Fig. 9 D) than that of mutant cells at 23°C. Fewer than 20% of the cells examined exhibited residual clustering. To determine whether this change in NPC distribution seen when rat7-1 cells were shifted to 37°C could be correlated with alterations in the association of Rat7p with NPCs, we performed indirect IF using the anti-Rat7p antibodies described above. In cells cultured at 23°C, Rat7p was detected at the nuclear periphery in both wild-type (Fig. 10 A) and rat7-1 cells (Fig. 10 B). As expected, NPCs appeared clustered in mutant cells. When mutant cells that had been shifted to 37°C for 1 h were stained with these antibodies, >95% of the cells showed no staining for Rat7p at the nuclear periphery (Fig. 10 E). In contrast, wild-type cells showed a normal punctate nuclear rim staining pattern under these conditions (Fig. 10 D). When these cells were stained with the RLI antinucleoporin antibody, the same pattern was seen as is shown in Fig. 9 D (data not shown).

To further substantiate the observations made via indirect IF, wild-type and rat7-1 mutant cells were prepared for viewing by thin section EM. Cells were grown continuously at 23°C or shifted to 37°C for 1 h before fixation. Examination of wild-type cells exposed to both temperatures showed that the NPCs were more or less evenly distributed around the nuclear envelope (Fig. 11, A and C). In agreement with the IF studies, rat7-1 cells grown at 23°C had many but not all NPCs clustered together within the plane of the nuclear envelope (Fig. 11 B). This clustering dispersed to some extent upon shifting the cells to 37°C (Fig. 11 D), as also noted by indirect IF. Whereas many rat7-1 cells grown at 23°C had clusters of 6–10 NPCs, relatively few rat7-1 cells shifted to 37°C had clusters of >4 NPCs. Other than the clustering of

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Figure 10. Indirect IF to determine whether mutant Rat7p/Nup159p is present in NPCs of rat7-1 cells. Wild-type and mutant rat7-1 cells were grown to early log phase and either maintained at 23°C or shifted to 37°C for 1 h before fixation and processing for indirect IF. Cells were stained with anti-Rat7p antibodies reactive with the repeat domain of Rat7p/Nup159p and with FITC-conjugated goat anti-guinea IgG. (A) Wild type, 23°C, FITC; (B) rat7-1, 23°C, FITC; (C) the same field of cells as in B stained with DAPI; (D) wild type, shifted to 37°C for 1 h, FITC; (E) rat7-1, shifted to 37°C for 1 h, FITC; (F) the same field of cells as in E stained with DAPI.

Discussion

We have identified a novel S. cerevisiae nucleoporin, Rat7p/Nup159p, on the basis of its involvement in mRNA export. We cloned the RAT7/NUP159 gene by complementation of the ts growth defect of a mutant yeast strain that accumulated poly(A)⁺ RNA in its nuclei within 15 min of a shift to the restrictive temperature. 2 h after the shift, nuclear poly(A)⁺ RNA concentration remained elevated, but cytoplasmic poly(A)⁺ RNA was no longer detectable, indicating that export of newly synthesized mRNA had been blocked. The gene is essential for yeast viability and is predicted to encode a 1,460-amino acid protein with a molecular mass of 159 kDa.

Several lines of evidence show that Rat7p/Nup159p is a...
Figure 11. Electron micrographs of the nuclear region of wild-type and rat7-I cells grown at 23°C or shifted to 37°C for 1 h. Cells were grown to early log phase and prepared for ultrastructural analysis as described in Materials and Methods. (A) Wild type, 23°C; (B) rat7-I, 23°C; (C) wild type, shifted to 37°C for 1 h; (D) rat7-I, shifted to 37°C for 1 h. Arrows denote representative NPCs in each cell. Large arrowheads denote a cluster of NPCs in a rat7-I cell grown at 23°C. n, nucleus. Bar, 1 μm.

nucleoporin. First, as seen by indirect IF, antibody specific for Rat7p/Nup159p stained the nuclear periphery in a punctate pattern diagnostic of NPCs. Second, the Rat7p/Nup159p amino acid sequence contains multiple tetrapeptide and pentapeptide repeats closely related to repeats previously found in many yeast and several mammalian nucleoporin sequences. The only proteins in the data base at present that contain these repeats are NPC proteins. Several yeast and
vertebrate nucleoporins share significant amino acid homology with Rat7p/Nup159p within its repeat region. Third, by both IF and EM analyses, cells bearing the ts rat7-1 allele exhibited aberrant NPC distribution with the majority of NPCs clustered toward one side of the nucleus. NPC clustering has been reported for yeast strains bearing partial deletions of nucleoporins NUP145 and NUP133 (Doye et al., 1994; Wente and Blobel, 1994). Finally, the rat7-1 allele is synthetically lethal with a ts-sensitive allele of RAT3 (Gorsch, L. C., Wente and Blobel, 1994). Finally, the rat7-1 allele is synthetically lethal with a ts-sensitive allele of RAT3 (Gorsch, L. C., Wente and Blobel, 1994).

Repeat Motifs of Rat7p/Nup159p

Many of the yeast nucleoporins sequenced to date have been assigned to subfamilies based on the presence of highly repeated GLFG or XFXFG motifs in their protein sequences. These repeats have been defined not only by their amino acid sequences but also by the amino acids that separate them. GLFG repeats are generally separated by spacer sequences of five or more amino acids rich in asparagine, glutamine, serine, and threonine. Rat7p/Nup159p contains 25 XXFG degenerate repeats that differ from GLFG repeats both in their primary sequences and in their spacer sequences. As shown in Fig. 4 A, the majority of the tetrapeptide repeats are either PSFG or SAFG. Though repetitive with serines, the spacer sequences are not unusually rich in asparagine, glutamine, or threonine and often consist of only a single amino acid. Rat7p/Nup159p also contains three XFXFG degenerate repeats that differ from those in other nucleoporins in that they are far less abundant, do not form the cores of longer repeats, and do not have highly charged spacer sequences (Fig. 4 B).

Various monoclonal antibodies raised against rat liver nuclear envelopes have been shown to recognize multiple members of either the GLFG or XFXFG nucleoporin subfamilies (Aris and Blobel, 1989; Davis and Fink, 1990; Wente et al., 1992). Presumably, these antibodies bind an epitope common to the repeat regions of these proteins. By contrast, polyclonal antisera raised against a GST-Rat7/Nup159 fusion protein bearing 19 XXFG repeats and 2 XFXFG repeats detected only a single band on a Western blot of total yeast protein. The repeat region of Rat7p/Nup159p appears to be antigenically unique, suggesting that the repeats in Rat7p/Nup159p are not present in other yeast nucleoporins. 12 of the 19 XXFG repeats included in the fusion protein are embedded in 4 nearly perfect 26-amino acid repeats (see Fig. 3). Such highly conserved, extended repeats have not been found in other yeast or metazoan nucleoporins and may impose a higher order structure on the shorter repeats.

NPC Clustering

We have shown by IF and thin section EM that rat7-1 cells grown at 23°C have an aberrant distribution of NPCs. Instead of being evenly spaced around the nuclear envelope as in wild-type cells, the majority of NPCs in mutant cells are clustered together in one region of the nuclear periphery. More than 50% of rat7-1 cells grown at 23°C exhibited some nuclear accumulation of poly(A)+ RNA, perhaps as a consequence of reduced mRNA export through clustered NPCs.

Transfer of rat7-1 cells to 37°C for 1 h exacerbated this mRNA export defect dramatically while, paradoxically, restoring near wild-type distribution of NPCs. Thus, NPC clustering by itself can occur (as in rat7-1 cells at 23°C) without causing a powerful block to mRNA export or dramatic reductions in growth rate; conversely, a wild-type or nearly wild-type distribution of NPCs (as in rat7-1 cells at 37°C) does not ensure that other NPC functions, in this case mRNA export, occur normally. The finding that Rat7p could not be detected in NPCs after a shift of mutant cells to 37°C (Fig. 10) suggests that loss of Rat7p from mutant NPCs permitted them to regain a more normal distribution within the nuclear envelope but dramatically decreased their ability to export poly(A)+ RNA.

Mutations in five nucleoporin genes identified to date result in altered NPC distribution and/or nuclear envelope morphology. These phenotypes—nuclear envelope herniation, nuclear envelope projections, and nuclear pore clustering—seem to reflect three separable defects. (a) In nupl45ΔN cells, which lack most of the amino half of NUP145, NPCs are found in "grapelike" clusters where they aggregate into amorphous masses (Wente and Blobel, 1994). Within these clusters, NPCs underlie successive herniations in the nuclear envelope. In nupl16ΔA cells shifted to 37°C, NPCs are distributed in a wild-type pattern but have membranous seals overlying their cytoplasmic faces (Wente and Blobel, 1993). The nuclear envelope herniations associated with NPCs in these mutants have been suggested to reflect a defect in the attachment of the NPC to the surrounding pore membrane and a weakening of the boundary that normally separates the two (Wente and Blobel, 1994). (b) Various nupl mutants have misshapen nuclei with projections of the nuclear envelope extending from the body of the nucleus into the cytoplasm. Within these deformed nuclear envelopes, NPCs have essentially wild-type morphology and spacing (Bogerd et al., 1994). These nuclear envelope projections have been taken to indicate a dissociation of the NPCs from underlying nucleoskeletal elements (Bogerd et al., 1994). (c) Both deletion and mutation of NUP133 result in NPC clusters that are not associated with gross perturbations of the nuclear envelope (Doye et al., 1994; Li et al., 1995). Likewise, in rat7-1 cells grown at 23°C, NPCs are indistinguishable from those in wild-type cells except for their spacing. They remain aligned in the plane of the nuclear envelope, which itself appears normal. Thus, although NPC clustering and nuclear envelope deformation were both seen in nupl145ΔN cells, NPC clustering can occur independently, as in rat7-1 cells.

Altered interactions between NPCs and either cytoskeletal or nucleoskeletal structures could account for the NPC clustering in rat7-1 cells. These structures could be the yeast nuclear lamina (Allen and Douglas, 1989), the nuclear envelope lattice that is thought to connect the distal rings of NPC baskets in Xenopus laevis (Goldberg and Allen, 1992), or cytoskeletal elements. This hypothesis predicts that Rat7p/Nup159p would be a component of one or more structures at the periphery of the NPC. For example, Rat7p/Nup159p could be a nuclear basket component involved in anchoring of the NPC to the lattice. Alternatively, Rat7p/Nup159p could be a component of either the cytoplasmic ring of the NPC or cytoplasmic filaments that emanate from the cytoplasmic face of the NPC. It is also possible that Rat7p/
Nup159p does not interact directly with these structures but could influence interactions between other NPC components and these structures.

The most unusual aspect of NPC clustering in rat7-1 cells is the apparent restoration of nearly wild-type NPC distribution within an hour of shift to the nonpermissive temperature. This could reflect turnover of preexisting pores followed by the insertion into the nuclear envelope of new NPCs distributed normally. Alternatively, redistribution of preexisting NPCs may be occurring. Experiments are in progress to distinguish between these alternatives. Because so little is known about NPC biogenesis and turnover, it is difficult to understand the role of the mutant Rat7p in NPC distribution. Since mutant cells shifted to 37°C showed loss of mutant Rat7p from the nuclear periphery, a role for the mutant protein in NPC clustering at 23°C seems possible. This would be consistent with a gain of function for mutant Rat7p and might have been expected to be a dominant phenotype. However, NPC clustering was not seen in rat7/T rat7-1 heterozygous diploids. Perhaps NPCs in heterozygotes contain primarily wild-type Rat7p in their NPCs. This could be due either to differences in the stability of mutant and wild-type Rat7p such that most of the Rat7p in heterozygotes might be wild-type protein or to differences in the ability of the two forms of Rat7p to become part of NPCs. NPCs are normally found clustered in some types of metazoan cells, indicating that physiological mechanisms must exist to permit NPC clustering (for examples, see Fawcett, 1981). Yeast strains with NPC clustering should be useful tools for studying NPC biogenesis and distribution.

**Rat7p/Nup159p Transport Function(s)**

Does Rat7p/Nup159p play a direct role in mRNA export? In a recent review about mRNA export, Elliott et al. (1994) described the type of mutant that is most likely to define a gene whose product plays a primary role in mRNA export. They suggested that a nucleoporin mutant with rapid effects on mRNA export, no rapid effect on protein import, and as few additional phenotypes as possible would be the ideal candidate. Of nucleoporin mutants characterized to date, the rat7-1 mutant best approximates this description. In 100% of rat7-1 cells shifted to 37°C, mRNA export was inhibited very rapidly without causing cytoplasmic accumulation of a karyophilic reporter protein. Is alleles of four other nucleoporin genes including NUP1 (Bogerd et al., 1994), NUP133 (Doye et al., 1994; Li et al., 1995), NUP49 (Doye et al., 1994), and NUP116 (Wente and Blobel, 1993) also cause nuclear retention of poly(A)⁺ RNA, but three of these (NUP1, NUP133, and NUP49) require a minimum of 3 h under restrictive conditions for the phenotype to develop. Furthermore, for alleles of all three of these genes, only a fraction of the cells show accumulation of poly(A)⁺ RNA under restrictive conditions. The slow kinetics and partial penetrance with which poly(A)⁺ RNA accumulates in these strains makes it difficult to distinguish between primary defects in mRNA export and indirect effects resulting from impairment of other processes, such as NPC assembly or protein import. Mutant alleles of NUP1 cause protein import defects in addition to defects in RNA export and neither defect appears rapidly following a temperature shift (Bogerd et al., 1994). In vivo repression of NUP145 expression gradually inhibited both RNA export and protein import, but cells that accumulated nuclear poly(A)⁺ RNA were detected several hours before cells showing cytoplasmic accumulation of a karyophilic protein (Fabre et al., 1994). The rapidly induced mRNA export block seen in nup116Δ cells has been shown to be an indirect consequence of membrane formation over the cytoplasmic face of nuclear pores (Wente and Blobel, 1993).

The data presented here do not eliminate the possibility that Rat7p/Nup159p also plays a direct role in protein import. First of all, in the experiment shown in Fig. 8, we do not know whether the amount of H2B-lacZ produced after the temperature shift was sufficient to permit its detection in the cytoplasm. The perfect control strain for this experiment does not exist; it would have the same rapid block in mRNA export and a rapid block in nuclear protein import. The strongest evidence that rat7-1 cells do not have a dramatic protein import defect is the finding that normal nuclear proteins are not mislocalized in mutant cells at either 23°C or 37°C, even though modest or dramatic defects, respectively, in mRNA export were seen at these two temperatures. Even if the rat7-1 allele shows no protein import defect, Rat7p might play a role in protein import as well as in mRNA export. Analysis of additional conditional alleles of rat7 NUP159 should help to clarify this possibility. Doye et al. (1994) described two is alleles of NUP49; in one, the mutation affected RNA export to a much greater extent than protein import; in the other, protein import was more rapidly impaired than was RNA export.

How the RNA export defect of rat7-1 is related to its unusual NPC clustering defect remains mysterious. Obviously, these defects could reflect two distinct properties or functions of Rat7p/Nup159p, but other explanations are also possible. Future experiments to determine where Rat7p/Nup159p localizes within the NPC and with what it interacts, both genetically and physically, should help to clarify the precise functions of the Rat7p/Nup159p protein and the mechanisms underlying both cessation of mRNA export and reversible NPC clustering in rat7 mutant cells.

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