Nuclear Pore Complex Clustering and Nuclear Accumulation of Poly(A)⁺ RNA Associated with Mutation of the
Saccharomyces cerevisiae RAT2 / NUP120 Gene

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Abstract. To identify genes involved in the export of messenger RNA from the nucleus to the cytoplasm, we used an in situ hybridization assay to screen temperature-sensitive strains of Saccharomyces cerevisiae. This identified those which accumulated poly(A)⁺ RNA in their nuclei when shifted to the non-permissive temperature of 37°C. We describe here the properties of yeast strains carrying mutations in the RAT2 gene (RAT - ribonucleic acid trafficking) and the cloning of the RAT2 gene. Only a low percentage of cells carrying the rat2-1 allele showed nuclear accumulation of poly(A)⁺ RNA when cultured at 15°C or 23°C, but within 4 h of a shift to the nonpermissive temperature of 37°C, poly(A)⁺ RNA accumulated within the nuclei of approximately 80% of cells. No defect was seen in the nuclear import of a reporter protein bearing a nuclear localization signal. Nuclear pore complexes (NPCs) are distributed relatively evenly around the nuclear envelope in wild-type cells. In cells carrying either the rat2-1 or rat2-2 allele, NPCs were clustered together into one or a few regions of the nuclear envelope. This clustering was a constitutive property of mutant cells. NPCs remained clustered in crude nuclei isolated from mutant cells, indicating that these clusters are not able to redistribute around the nuclear envelope when nuclei are separated from cytoplasmic components. Electron microscopy revealed that these clusters were frequently found in a protuberance of the nuclear envelope and were often located close to the spindle pole body. The RAT2 gene encodes a 120-kD protein without similarity to other known proteins. It was essential for growth only at 37°C, but the growth defect at high temperature could be suppressed by growth of mutant cells in the presence of high osmolarity media containing 1.0 M sorbitol or 0.9 M NaCl. The phenotypes seen in cells carrying a disruption of the RAT2 gene were very similar to those seen with the rat2-1 and rat2-2 alleles. Epitope tagging was used to show that Rat2p is located at the nuclear periphery and co-localizes with yeast NPC proteins recognized by the RL1 monoclonal antibody. The rat2-1 allele was synthetically lethal with both the rat3-1/nup133-1 and rat7-1/nup159-1 alleles. These results indicate that the product of this gene is a nucleoporin which we refer to as Rat2p/Nup120p.

Nuclear pore complexes (NPCs) play a central role in the exchange of macromolecules between the nuclear and cytoplasmic compartments of eukaryotic cells (for reviews see Rout and Wente, 1994; Davis, 1995; Doye and Hurt, 1995). NPCs are large complex structures which perforate the nuclear envelope; they have a mass of approximately 125 million daltons in metazoan cells, 65 million daltons in yeast cells, and may contain as many as 100 distinct polypeptides (Reichelt et al., 1990; Rout and Blobel, 1993). When examined by high resolution electron microscopy, NPCs are seen to have an eightfold rotational symmetry. Three-dimensional image reconstruction suggests that NPCs contain nuclear and cytoplasmic rings with spokes radiating inwardly from these rings to define a central channel where the transporter apparatus is thought to be located (Unwin and Milligan, 1982; Akey, 1990, 1991; Reichelt et al., 1990; Jarnik and Aebi, 1991; Hinshaw et al., 1992; Akey and Radermacher, 1993). Filamentous structures have been observed projecting from NPCs into both the cytoplasm and the nucleoplasm (Maul, 1977; Richardson et al., 1988; Allen and
Douglas, 1989; Jarnik and Aeby, 1991; Goldberg and Allen, 1992; Ris and Malecki, 1993). The nucleoplasmic filaments are organized into a basket-like structure called the fishtrap, which may contain sites for docking of macromolecules being exported from the nucleus. In metazoan cells, NPCs appear to be physically anchored to the nuclear lamina (Stewart and Whytock, 1988; Akey, 1989). NPCs possess 9-nm aqueous channels that permit small molecules including ions and metabolites to cross the nuclear envelope by passive diffusion. Translocation of larger molecules across the NPC, including proteins and RNAs, is an active process that is energy dependent, saturable, and requires specific signals in the molecules being transported. Recent studies indicate that the small GTP-binding protein Ran/TC4 is one of the factors responsible for protein import in vertebrate cells (Melchior et al., 1993; Moore and Blobel, 1993; Ren et al., 1993) and that its yeast homologs, Gsp1p and Gsp2p, play a similar role in Saccharomyces cerevisiae (Schlenstedt et al., 1995). These proteins are also likely to be involved in export from the nucleus to the cytoplasm since accumulation of poly(A)$^+$ RNA in the nuclei of yeast cells was observed in cells carrying mutations affecting Gsp1p, or Rna1p, its GTPase activating protein Rna1p, of Prp20p, its guanine nucleotide exchange factor (Amberg et al., 1992; Forrester et al., 1992; Kadokawa et al., 1992; Amberg et al., 1993; Schlenstedt et al., 1995).

Immunological, biochemical and genetic approaches have been taken to identify nucleoporins. Isolated rat liver nuclear envelopes were used to generate monoclonal antibodies reactive with proteins associated with the nuclear envelope, and many of these antibodies recognize short repeated peptide motifs common to many nucleoporins (Davis and Blobel, 1986; Park et al., 1987; Snow et al., 1987). This has facilitated the purification of specific nucleoporins and the cloning of the genes or cDNAs encoding these proteins. Many of these antibodies to rat liver nuclear envelopes also recognize nucleoporins from S. cerevisiae (Aris and Blobel, 1989; Copeland and Snyder, 1993) permitting their use in cloning of yeast nucleoporins (Davis and Fink, 1990; Wente et al., 1992; Loeb et al., 1993). Other nucleoporins have been identified based on their co-purification with repeat-containing nucleoporins (Snow et al., 1987; Finlay et al., 1991; Buss and Stewart, 1995; Grandi et al., 1993, 1995b).

Genetic approaches in yeast have proved valuable in the identification of nucleoporins. We described a genetic approach to identify gene products important for the nucleocytoplasmic export of poly(A)$^+$ RNA in yeast (Amberg et al., 1992). We screened a bank of temperature-sensitive mutants of S. cerevisiae by in situ hybridization for those which accumulated poly(A)$^+$ RNA in their nuclei when shifted to the nonpermissive temperature of 37°C. This led to the identification of additional yeast nucleoporins (Gorsch et al., 1995; Li et al., 1995). Synthetic lethal genetic screen have also been a productive pathway to the identification of yeast nucleoporins (Wimmer et al., 1992; Doye et al., 1994; Fabre et al., 1994). Furthermore, the ability to purify yeast NPCs has accelerated the identification of additional S. cerevisiae nucleoporins (Rout and Blobel, 1993; Wozniak et al., 1994; Hurwitz and Blobel, 1995; Kraemer et al., 1995; Pemberton et al., 1995).

Three major repeat-containing classes of nucleoporins have been identified (Davis, 1995). The XFXFG class includes metazoan p62, nup153, nup214, and pom121, and the yeast nucleoporins Nup1p, Nup2p, and Nsp1p (Davis and Fink, 1990; Nehrbass et al., 1990; Loeb et al., 1993). A second class of nucleoporins contains GLFG repeats and includes p98 from N. crassa (Powers et al., 1995), and Nup49p, Nup57p, Nup100p, Nup116p, and Nup145p from S. cerevisiae (Wente et al., 1992; Wimmer et al., 1992; Wente and Blobel, 1993; Fabre et al., 1994; Wente and Blobel, 1994; Grandi et al., 1995b). A third class of nucleoporins contain degenerate XXFG repeats. Within this group, yeast Rat7p/Nup159p contains 25 XXFG repeats, with SAFG and PSFG being most common (Gorsch et al., 1995). Very similar repeats are found in rat nup214 (Kraemer et al., 1994; Panté et al., 1994). To date, the only yeast nucleoporins identified that lack repeat motifs are Nic96p (Grandi et al., 1993), Rat3p/Nup133p (Doye et al., 1994; Li et al., 1995; Pemberton et al., 1995), and Nup82p (Grandi et al., 1995a; Hurwitz and Blobel, 1995).

We report here the identification of Rat2p/Nup120p, an additional yeast nucleoporin lacking repeat motifs. We initially isolated the rat2-1 strain by screening UV-mutagenized yeast for strains which were temperature sensitive for growth and accumulated poly(A)$^+$ RNA in their nuclei at the nonpermissive temperature. We cloned the RAT2 gene by complementation. It encodes a 120-kD protein which we show by indirect immunofluorescence to colocalize at the nuclear rim with known yeast NPC components. Yeast strains bearing mutant alleles of RAT2 show constitutive clustering of NPCs. This clustering is retained in isolated crude nuclei, suggesting that connections to the cytoskeleton are not required for proper distribution of NPCs. While these strains accumulate poly(A)$^+$ RNA in their nuclei following a shift to 37°C, protein import does not appear to be affected by mutation or disruption of this gene. Mutant strains were able to grow at high temperatures (37°C) in the presence of 1 M sorbitol. Under these conditions, the block to export of poly(A)$^+$ RNA was partially suppressed but clustering of NPCs in mutant cells was retained. A modest disruption of the nucleolus was seen in mutant strains shifted to the nonpermissive temperature, but synthesis and processing of rRNA continued following the temperature shift. Synthetic lethality was seen between strains carrying mutant alleles of RAT2/NUP120 and strains with mutations in RAT3/NUP133 or RAT7/NUP159, indicating genetic interactions among these nucleoporins.

**Materials and Methods**

**Yeast Cell Culture, Strains, Media, Mutant Isolation, and Cell Fractionation**

Table I lists the yeast strains used in this work. The plasmids used are listed in Table II. S. cerevisiae were cultured by standard methods (Rose et al., 1989; Sherman, 1991). Wild-type strains FY23 and FY86, GAL2 derivatives of S288C carrying the ura3-52 allele, were obtained from F. Winston (Harvard Medical School, Boston, MA). Strains K31-10A, containing the mutant metl4 allele, and strain JMS010, containing an integra-
Table I. Yeast Strains Used in This Study

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<th>Strains</th>
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<td>Provided by F. Winston</td>
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<td>EE1b</td>
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The isolation of rat mutants (ribonucleic acid trafficking) has been previously described (Amberg et al., 1992). Briefly a collection of 1200 temperature-sensitive strains generated by mutagenesis with ultraviolet light was screened microscopically after in situ hybridization with digoxigenin-tagged oligo-dT15 to localize poly(A)^+ RNA, followed by incubation with fluoresceinlabeled anti-digoxigenin antibodies. Strains showing fluorescent signal in the nucleus following growth at 37°C were re-purified and several were chosen for further study.

For temperature-shift experiments, cells cultured in liquid media at 25°C were grown overnight at room temperature. Cell density was determined with a hemacytometer. Duplicate cultures of each strain were diluted to between 4 and 10^7 cells/ml in YPD, re-counted, and incubated at 23°C. After 4 h, an aliquot of each culture was removed and counted and one culture from each strain was shifted to 37°C. Duplicate samples were removed from each culture every 4 h and cell concentration determined by counting. To examine reversion, cells were removed from each culture after 28 h and plated on YPD plates. Plates were incubated at 23° or 37°C for a week and colony numbers determined daily. To study the effect of high osmolarity on the growth and properties of wild-type and various mutant strains, either NaCl or sorbitol were added to media to final concentrations of 0.9 or 1.0 M, respectively. Isolation of a crude nuclear fraction for indirect immunofluorescence was carried out by the short protocol described by Mrzayyan et al. (1992).

Table II. Plasmids Used in This Study

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<td>gene A* (RAT2) in YCplac33</td>
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Bacterial Plasmids

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<td>pCH11</td>
<td>disruption of RAT2 by HIS3 in pCH4</td>
<td>This study</td>
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</table>

* These letters correspond to the open reading frames shown in Fig. 3 A.
S. cerevisiae DNA (Sac 3A partial digestion products) cloned into a LEU2-GEN plasmid (a gift from P. Hieter, Johns Hopkins Univ., Baltimore, MD). This library was transformed into the mutant strain by electro-

proportion and LEU+ colonies were selected at 37°C. Plasmid DNA was isolated from these strains (Rose et al., 1989), transformed into Escheri-

chiae coli, and plasmids isolated for further analyses. Plasmids were tested for their ability to re-transform the mutant strain to LEU+ and grow at 37°C. Plasmids able to transform the strain contained overlapping yeast DNA segments. Different plasmid clones overlapped by ~7.2 kb. Subclones containing a 6.1-kb SacI-Sall fragment of pCH2 complemented both the temperature sensitivity as well as the aberrant RNA localization phenotype of the rat2-1 strain.

A 1.0-kb Dral fragment common to all complementing plasmids was used to probe a blot of electrophoretically separated yeast chromosomes and several ordered plasmids and lambda phages containing alpha sequences, and transformed by elec-

troporation into wild-type strain FY86 and into YCC282 carrying the

RA72 strain.

Disruption of the RAT2 Gene

To disrupt the RAT2 gene, the entire RAT2 open reading frame except for the first 86 and last 60 amino acids was replaced with the HIS3 gene. pCH4, containing the complete RAT2 open reading frame, was digested with NcoI and PstI, and a 7.2-kb fragment was isolated after electrophore-

sis (see below). The resulting plasmid, pCH11, was digested with SphI and Xmal to release a 7.2-kb fragment of pCR4, containing the complete HIS3 gene, to create pCH7. This plasmid was linearized by digestion with Clal, which cleaves within the open reading frame. The resulting plasmid, pCH11, was digested with SphI and Xmal to release a fragment containing RAT2 sequences flanking the inserted HIS3 gene. Diploid wild-type cells (strain LGY105) were transformed with this fragment by electroporation and colonies able to grow on SC-HIS media were selected. Southern analysis was used to show that the fragment had inte-

grated into the RAT2 locus (data not shown). The resulting strain was transformed with plasmid pCH3 which contains the complete gene, the entire gene was located 2.5 cM from the centromere on the left arm of chromosome XI. This physical position of the cloned DNA agreed with genetic mapping data (see below).

Integration of an Auxotrophic Marker Adjacent to RAT2 and Genetic Mapping of RAT2

A 3.3-kb fragment containing part of the RAT2 gene was isolated after di-

gestion of pCH1 and ligated into the polylinker region of Kpn-digested YlPlac211 to create pCH7. This plasmid was linearized by digestion with ClaI, which cleaves within RAT2 sequences, and transformed by elec-

troproportion into wild-type strain FY86 and into YCC282 carrying the rat2-1 allele. Cells were plated and grown on SC-URA plates for 4 d. Colonies were selected. Southern analysis was used to show that the fragment had inte-

grated into the RAT2 locus (data not shown). The resulting strain was transformed with plasmid pCH3 which contains the complete RAT2 gene and the URA3 gene in YCplac33. After sporulation, tetrads were dis-

sected and allowed to germinate on rich media at 23°C. Cells were then replica plated to SC-HIS plates containing 5-fluoroorotic acid (5-FOA) and the plates were incubated at both 23°C and 37°C.

Electron Microscopy

Electron microscopy was performed by a modification of published proce-

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dure (Byers and Goetsch, 1975). Cells were cultured at 23°C in YPD to a density of 0.2-2 × 10^6 cells/ml harvested by filtration, fixed for 2 h at room temperature in 3% (vol/vol) glutaraldehyde containing 0.1% tannic acid and 0.1 M sodium cacodylate, pH 6.8. Fixed cells were washed twice in 50 mM KH2PO4/K2HPO4 pH 7.5, and then digested with 125 mg/ml Zymolyase 1010 (ICN Immunchemoicals, Irvine, CA) in 50 mM KH2PO4/K2HPO4 pH 7.5, for 40 min at 30°C. After two washes in 0.1 M sodium cacodylate, pH 6.8, the spheroplasted cells were treated with 2% (vol/vol) KOAc, pH 6.8, for 1 h on ice. Samples were washed and stored overnight at 4°C in 0.1 M sodium cacodylate, pH 6.8. Samples were washed three times in ddH2O and treated for 1 h with 2% uranyl acetate in ddH2O. After two washes in ddH2O, cells were embedded in 2% agar, dehydrated through graded ethanol, and embedded in Spurr’s medium. Thin sections were cut, stained with uranyl acetate and Reynolds’ lead citrate, and examined on a JEOL 100CX electron microscope at 60 kV accelerating voltage.

**Epitope Tagging and Immunolocalization**

Site-directed mutagenesis was used to create a unique site near the COOH terminus of the RAT2 gene where the sequence encoding three copies of the epitope recognized by the 9E10 anti-locus monoclonal antibody (EQKLISEEEDLN) was inserted in frame with the R2 coding region. A 6.1-kb SacI-Sal fragment of pCH4 including RAT2 was inserted into the multicloning site of pUC19. The resulting plasmid pCH9 was used as the template for the site-directed mutagenesis using a kit obtained from Clonetech Laboratories, Inc. (Palo Alto, CA). A 28-bp oligonucleotide (CTTATTAGAGACGATGATGAGTTACG) containing a single nucleotide mutation was used to direct the insertion to a site 24 nucleotides from the start of the coding region of the RAT2 gene, yielding a plasmid (pCH9) with a unique XbaI site at that position. Digestion of the plasmid pKK-1 (obtained from G. Fink, Whitehead Institute, Cambridge, MA) with XbaI yielded a 120-bp cassette containing a triplicate of the epitope recognized by the 9E10 monoclonal antibody. This was gel purified on a 0.8% agarose gel and ligated into pCH9 which had been digested with XbaI, yielding pCH10. Insertion and orientation were confirmed by restriction endonuclease digestion patterns. The resulting plasmid (pCH13) was transformed into strain CHY104, carrying the rat2A allele, yielding strain CHY107. Cells were grown to early log phase and fixed with 1/10 volume of 37% formaldehyde for 1 h. Cells were then washed three times with 1.2 M sorbitol; 50 mM K2HPO4, pH 7.5 (solution A), resuspended into 1 ml of solution A containing 30 mg of 100T Zymolyase (Seikagaku, Rockville, MD), and incubated at room temperature for 10-60 min until spheroplasts were formed. Spheroplasts were washed once with solution A and adhered to polylisine-coated 12-well slides. Samples were washed sequentially with PBS + 0.1% BSA (solution B), solution B + 0.1% NP-40, and solution B, with a 5-min incubation per wash. Cells were incubated with 1:1,000 mouse IgG monoclonal anti-locus antibody in solution B overnight at 4°C. Cells were then washed with solution B, solution B plus 0.1% NP-40, and solution B, each for 5 min. Cells were incubated with secondary antibody (FITC-conjugated goat anti-mouse IgG) in solution B for 2 h at room temperature and then washed with solution B, solution B plus 0.1% NP-40, and containing 10 mg of DAPI per ml, and solution B, each for 5 min. Cells were mounted with mounting solution (70% glycerol/30% PBS containing 1 mg p-phenylenediamine per ml). Hybridoma cell clones producing the 9E10 antibody were a generous gift of Drs. J. Zhu and J. M. Bishop (University of California, San Francisco, CA).

To examine the co-localization of Rat2p and nuclear pore complexes, cells were doubly stained with both the anti-myc epitope monoclonal antibody and with the R1 anti-nucleoporin monoclonal antibody. The anti-myc antibody is an IgG while the R1 antibody is an IgM. Secondary antibodies, horse-anti-mouse IgG conjugated to FITC and goat-anti-mouse IgM coupled to Texas red (both from Vector Laboratories) were used at 1:500 and 1:650 dilution, respectively. Control experiments indicated that there was no detectable signal in the Texas red channel when both primary antibodies and the FITC-conjugated secondary antibody were used; similarly, there was no detectable signal in the FITC channel when both primary antibodies and the Texas red-conjugated secondary antibody were used (data not shown).

**Analysis of Pre-rRNA Processing by Pulse Labeling**

All strains to be used that were rat2-1 were URA3+ by transformation with YCP1a33 (Gietz and Sugino, 1988), which contains the URA3 gene. Cells were grown to early to mid-log phase (A600 of 0.3 to 0.5) in SC-Ura. 1.6 ml of cells were labeled with 160 mCi of [5-3H]uridine (New England Nuclear, Boston, MA; 22 Ci/mmol) for 10 min. 30 s before the end of the labeling period, cells were collected by microcentrifugation for 20 s, the supernatant was aspirated, and the cells were quickly frozen in a dry ice/ethanol bath. Total RNA was extracted by the method of Torlakoff and Mattaj (1987). Briefly, to the pelleted cells were added 50 ml of 4 M guanidinium thiocyanate, 50 ml of phenol, 5 mg of RNAse, and 100 ml of glass beads. Tubes were vortexed in a cold room. Then 300 ml of phenol, 30 ml of 4 M guanidinium thiocyanate were added and the mixture incubated at 65°C for 5 min. 300 ml of chloroform and 160 ml of 3 M sodium acetate were added. The mixture was centrifuged for 2 min in a microcentrifuge and the upper layer was transferred to a new tube. Extraction was repeated twice with phenol and chloroform. RNA was precipitated with ethanol and resuspended in RNase-free water. Samples were electrophoresed on a 2.5% agarose gel containing a 1.2% agarose/6M formaldehyde gel. The RNA was lifted onto a Northern blot and hybridized to a digoxigenin-labeled (poly(dT)20) probe. An FITC-tagged anti-digoxigenin antibody was used to permit visualization of sites to which the RNA probe hybridized. Therefore, the different sites to which the RNA probe hybridized could be visualized on the same Northern blot.

**Synthetic Lethality**

Diploids containing both the rat2-1 and rat3-1 alleles were obtained by mating strains CCY282 and OLY101 with selection for diploids on SD + Leu plates. Diploids were transformed with a URA3-marked plasmid containing the wild-type RAT2 gene (pCH3). Diploids containing the rat2-1 and the rat2-1/ura3-19 alleles were obtained by mating strains CCY282 and OLY101 and were transformed with plG4, a CEN plasmid containing the wild-type RAT2/Ura3-1 plasmid under control of its own promoter. Diploids were sporulated and viable spores allowed to germinate after tetrad dissection. Viable spores were tested for their ability to grow in the absence of the plasmid-borne RAT2 or URA3 gene by plating on media containing 5-FOA at 23°C, as well as for their ability to grow on YPD at 37°C. Of the 20, 4 spore tetrads analyzed following sporulation of the strain heterozygous for rat2-1 and rat3-1/ura3-19, the ratio of those yielding 2, 3, or 4 haploids able to grow on 5-FOA plates was 3:13:4. From the tetrads yielding two haploids able to grow on 5-FOA plates, two of the strains able to grow were not temperature sensitive. From the tetrads yielding three haploids able to grow on 5-FOA plates, two of the strains were temperature sensitive and one was not. Of the tetrads yielding four haploids able to grow on 5-FOA plates, all four were temperature sensitive. Only five four-spore tetrads were obtained from the diploid heterozygous for rat2-1 and rat3-1/ura3-19. All four yielded three viable haploids when plated on 5-FOA.

**Results**

**Nuclear Accumulation of Poly(A)+ RNA Occurs in Cells Carrying Mutant Alleles of RAT2 Following a Shift to the Nonpermissive Temperature**

A collection 1,200 S. cerevisiae temperature-sensitive mutants was screened to identify strains which accumulated poly(A)+ RNA in their nuclei. Growing cells were shifted to the nonpermissive temperature (37°C) for 2 h and examined by in situ hybridization with a digoxigenin-labeled oligo(dT)20 probe. An FITC-tagged anti-digoxigenin antibody was used to permit visualization of sites to which the oligo-dT20 hybridized. Based on the initial screening, approximately 30 strains were selected for further study. These strains fall into at least 10 complementation groups. We described previously the identification and characterization of RAT2/XRN2/HKE1 (Amberg et al., 1992), encoding an exoribonuclease, and RAT7/NUP159 (Gorsch et al., 1995) and RAT3/NUP133 (Li et al., 1995), encoding nucleoporins. Among the strains identified, six fell into the RAT2 complementation group. The rat2-1 and rat2-2 alleles were used for the analyses presented below.

The RNA localization patterns observed in a wild-type (FY23) and a rat2-1 strain (CCY282) are shown in Fig. 1. The top row of photographs shows the distribution of RNA localization patterns observed in a wild-type (FY23) and a rat2-1 strain (CCY282) are shown in Fig. 1. The top row of photographs shows the distribution of
Figure 1. Accumulation of poly(A)$^+$ RNA in the nuclei of rat2-1 and rat2Δ cells following a shift to 37°C. Wild-type (FY23) (A–E), rat2-1 (CCY282) (F–J), and rat2Δ cells (CHY104) (K–O) were grown to early log phase in YPD at 23°C. Aliquots either held at 23°C (A, B, F, G, K, and L) or shifted to 37°C (C, D, E, H, I, J, M, N, and O) for 4 h were fixed and analyzed by an in situ hybridization assay using a digoxigenin-tagged oligo(dT)$_{50}$ probe. Sites where probe had hybridized were visualized by using a FITC-conjugated anti-digoxigenin antibody. (A) WT, 23°C, FITC; (B) same field of cells as in A, stained with DAPI; (C) WT, 37°C, FITC; (D) same field of cells as in C, stained with DAPI; (E) same field of cells as in C, stained with DAPI and simultaneously viewed by low illumination DIC microscopy; (F) rat2-1, 23°C, FITC; (G) same field of cells as in F, stained with DAPI; (H) rat2-1, 37°C, FITC; (I) same field of cells as in H, stained with DAPI; (J) same field of cells as in H, stained with DAPI and simultaneously viewed by low illumination DIC microscopy; (K), rat2Δ, 23°C, FITC; (L) same field of cells as in K, stained with DAPI; (M) rat2Δ, 37°C, FITC; (N) same field of cells as in M, stained with DAPI; (O) same field of cells as in M, stained with DAPI and simultaneously viewed by low illumination DIC microscopy.

poly(A)$^+$ RNA in wild-type cells grown continuously at 23°C (Fig. 1 A) or shifted to 37°C for 4 h (Fig. 1 C). Under both conditions, RNA was distributed throughout the cell. In contrast, poly(A)$^+$ RNA accumulated in approximately 15–20% of the nuclei of rat2-1 cells grown at 23°C (Fig. 1 F). All cells also showed staining for cytoplasmic poly(A)$^+$ RNA, indicating that RNA export was occurring even though some nuclear accumulation of poly(A)$^+$ RNA was detectable. When the rat2-1 culture was shifted to 37°C (Fig. 1 H), the percentage of cells showing nuclear accumulation of poly(A)$^+$ RNA increased gradually and reached a plateau of ~70–80% after 3–4 h. The signal over the cytoplasmic region was reduced, consistent with turnover of cytoplasmic RNA and reduced export from the nucleus. Even when shifted to 37°C for periods up to 12 h, the percentage of cells accumulating poly(A)$^+$ RNA in their nuclei did not increase further (data not shown).

Mutation of the RAT2 Gene Does Not Affect Nuclear Protein Import

To determine whether mutation of the RAT2 gene also caused defects in nuclear protein import, we examined the subcellular distribution of a fusion protein containing the nuclear localization signal (NLS) of the yeast histone H2B protein fused to E. coli β-galactosidase (Moreland et al., 1987) and expressed from the GAL1 promoter. We used a yeast strain carrying the nup1-106 allele as a positive control since these cells are known to be defective for nuclear protein import (Bogerd et al., 1994). Cultures growing exponentially in YP-raffinose media were either maintained at 23°C or shifted to 36°C. We used 36°C as a non-permissive temperature because the GAL1 promoter is more efficiently induced at 36°C than at 37°C (Gorsch, L. C., and C. N. Cole, unpublished results). Following incubation at 36°C for 2.5 h, the synthesis of the NLS-bearing reporter protein was induced by addition of galactose. Incubation was continued an additional 1.5 h before cells were fixed and processed for indirect immunofluorescence.

In wild-type cells, the fusion protein was found solely in the nucleus, regardless of whether cells were grown at 23°C (Fig. 2 A) or shifted to 36°C (Fig. 2 C). In nup1-106 cells, fusion protein was found primarily in the nucleus in the culture maintained at 23°C (Fig. 2 F), but there was dramatic accumulation of the NLS-lacZ reporter protein in the cytoplasm of nup1-106 cells shifted to 36°C for 4 h (Fig. 2 H). In rat2-1 cells maintained at 23°C (Fig. 2 K) or shifted to 36°C for 4 h (Fig. 2 M), the fusion protein was
Figure 2. Normal import of a nuclear protein in a rat2 mutant strain. Wild-type (FY23) (A-E), nup1 (LDY97) (F-J), and rat2-1 cells (CCY282) (K-O) were either incubated continuously at 23°C (A, B, F, G, K, and L) or shifted to 36°C (C, D, E, H, I, J, M, N, and O) for 2.5 h before addition of galactose (to 2% final concentration) to induce expression of the reporter protein consisting of the histone H2B nuclear localization signal fused to E. coli β-galactosidase. After addition of galactose, incubation was continued for an additional 1.5 h before cells were fixed and processed for indirect immunofluorescence. (A) WT, 23°C, FITC; (B) same field of cells as in A, stained with DAPI; (C) WT, 36°C, FITC; (D) same field of cells as in C, stained with DAPI; (E) same field of cells as in C, viewed by DIC microscopy; (F) nup1, 23°C, FITC; (G) same field of cells as in F, stained with DAPI; (H) nup1, 36°C, FITC; (I) same field of cells as in H, stained with DAPI; (J) same field of cells as in H, viewed by DIC microscopy; (K) rat2-1, 23°C, FITC; (L) same field of cells as in K, stained with DAPI; (M) rat2-1, 36°C, FITC; (N) same field of cells as in M, stained with DAPI; (O) same field of cells as in M, viewed by DIC microscopy.

detected solely in the nucleus. Production of the fusion occurred only during the last 90 minutes of incubation at the non-permissive temperature. We conclude that the rat2-1 mutation has little, if any, effect on nuclear protein import.

Genetic Analysis of the rat2-1 Mutation

We determined that defects in a single gene, RAT2, accounted for all three phenotypes observed: temperature-sensitivity, nuclear accumulation of poly(A)^+ RNA and nuclear pore complex clustering (see below). Haploid cells bearing the rat2-1 allele were mated with wild-type cells, and diploids were selected, sporulated and subjected to tetrad analysis. For each allele, at least 40 tetrads were examined and found to show 2:2 segregation of temperaturesensitivity clearly implicating a single gene as the cause. Ten tetrads, including five from the third backcross were examined for co-segregation of temperature sensitivity and the RNA export phenotypes. In all cases, co-segregation and 2:2 segregation was observed. Analyses of heterozygous diploids indicated that the phenotypes of temperature sensitivity and nuclear poly(A)^+ RNA accumulation were recessive.

Cloning and Mapping of RAT2

The RAT2 gene was cloned by complementation of the temperature-sensitive growth defect caused by the rat2-1
Figure 3. The RAT2 gene. (A) Schematic representation of the region of yeast chromosome XI which includes the RAT2 gene. Key restriction endonuclease cleavage sites are shown. The arrows show three putative ORFs in this region. Also shown is the location of the DraI fragment common to all complementing plasmids and used to probe a blot of electrophoretically separated yeast chromosomes and a set of ordered cosmids and lambda phages. (B) Strategy for the RAT2 disruption. The region of the gene deleted and replaced with HIS3 to make the RAT2::HIS3 null mutation is indicated. 86% of the RAT2 ORF (from amino acids 86-977) was deleted by digestion with NcoI and PstI, followed by insertion of a DNA fragment containing the HIS3 gene. (C) The predicted 1,037-amino acid sequence of Rat2p. These sequence data are available from Genbank/EMBL/DDBJ under accession number Z28057.

mutation. All four distinct plasmids obtained following transformation of the rat2-1 strain with a yeast genomic library carried on a CEN plasmid contained overlapping DNA fragments. A fragment derived from the region of overlap was used to probe a blot of electrophoretically separated yeast chromosomes. This indicated that the cloned DNA was derived from chromosome XI (data not shown). The same fragment was used to probe a blot containing DNA from cosmids and phages which contain ordered fragments of yeast DNA (Riles et al., 1993). This analysis confirmed that the cloned DNA was derived from chromosome XI and placed the cloned DNA near the MET14 and PUT3 genes. Genetic mapping experiments with a met14 strain and with a strain containing a TRP1 gene integrated at the PUT3 locus indicated that the rat2-1 mutation was located ~40 cM from PUT3 and 54 cM from MET14. Finally, we integrated a URA3 marker adjacent to the RAT2 locus in a wild-type strain and mated it with a strain carrying the rat2-1 allele. In all haploids which arose following sporulation, the temperature sensitivity conferred by the rat2-1 mutation segregated from the uracil prototrophy conferred by the URA3 gene. This indicates that the RAT2 gene, not a suppressor of the rat2-1 mutation, is contained on the yeast DNA fragment cloned by complementation.

Based on its chromosomal position, the DNA sequence from the MET14/PUT3 region of chromosome XI was ob-
tained from Drs. S. Rasmussen and D. von Wettstein (Carlsberg Laboratory, Copenhagen, Denmark) (Rasmussen, 1994). The smallest complementing clone was found to contain three new open reading frames (Fig. 3A). Each open reading frame was subcloned into YCplac33 or YCplacl11; the subclone containing the SacI to SalI DNA fragment was able to complement the rat2-1 growth defect at 37°C and the nuclear poly(A)+ RNA accumulation defect. This subclone contains a single open reading frame encoding a protein of 1,037 amino acids with a predicted size of 120 kD. The sequence of this protein is shown in Fig. 3C. Rat2p lacks obvious structural features and motifs but is predicted to have a modest potential to form coiled-coil interactions through amino acids 427–465 (Lupas et al., 1991). This open reading frame is designated YKL057C in the published sequences of yeast chromosome XI (Dujon et al., 1994).

To determine whether the RAT2 gene is essential, all of

Figure 5. NPC clustering and segregation of NPC clusters in mitotic rat2-2 cells. Diploid cells were cultured at room temperature, fixed and processed for indirect immunofluorescence microscopy with anti-nuclear pore complex antibody RL1 (a, c, and e) or anti-tubulin (f). (b and d) Corresponding Hoechst 33258 stain for DNA. (a and b) Wild-type cells. (FY23x86); (c and d) rat2-1/rat2-1 cells (DA4xt4); (e and f) rat2-2/rat2-2 cells (DA2xt2). The arrowheads indicate mitotic wild-type or rat2-2/rat2-2 cells. Bar, 5 μm.
The punctate pattern observed is characteristic of NPC
Nuclear Pore Complexes Are Clustered in Yeast Strains
Remain Clustered in Nuclei Isolated from Mutant Cells

Immunolocalization of Rat2p

The RAT2 gene was "tagged" with an epitope-coding se-
Nuclear Pore Complexes Are Clustered in Yeast Strains Bearing Mutant Alleles of the RAT2 Gene and Remain Clustered in Nuclei Isolated from Mutant Cells

The NPC plays a critical role in nucleocytoplasmic transport. Since it contains as many as 100 different polypep-
tides, we expected that some mutants identified in our screen would have defects in the structure, function, or assembly of NPCs. Therefore, we performed a series of immunofluorescence experiments on yeast strains bearing mutant alleles of rat2 using antibody RL1, which reacts with nuclear pore complex components in mammalian, amphibian, and yeast cells (Snow et al., 1987; Featherstone et al., 1988; Copeland and Snyder, 1993).

For these experiments we used diploid cells homozy-
gous for the rat2-1 or rat2-2 mutations. Immunofluores-
cence micrographs of wild-type yeast and strains homozy-
gous for the rat2-1 or rat2-2 alleles are shown in Fig. 5. In

In wild-type yeast (Fig. 5 A), antibody RL1 stained the nuclear periphery in a discontinuous or punctate manner, a pattern indicative of nuclear pore complex staining. This nuclear rim-staining completely surrounded the DAPI-stained region of the nucleus (Fig. 5 B). When rat2-1 and rat2-2 cells were stained with antibody RL1, a very different pattern was seen. Instead of a punctate pattern around the nuclear rim, the RL1 antigens were clustered in an area adjacent to rather than surrounding the nuclear DNA (Fig. 5 C). In cells carrying the rat2-1 allele the patch was usually one discrete entity and was often curved or cup-shaped. Little cytoplasmic staining was seen. In cells carrying the rat2-2 allele (data not shown), the RL1 antigens were more often grouped into two or three smaller patches. For both rat2 mutant strains, all of the cells in the population exhibited the clustering phenotype. Fig. 5 E shows the RL1 staining pattern in a rat2-2 cell in the midst of karyokinesis. It can be seen that both daughter nuclei have received some of the clustered NPCs. The location of the mitotic spindle is shown in Fig. 5 F, where an antitubulin antibody was used. Clustering of NPC antigens was not altered following a shift to the nonpermissive temperature for up to 4 h (data not shown). We also examined the segregation pattern for NPC clustering when the original strain containing the rat2-1 allele was backcrossed. The NPC clustering was also present in the wild-type diploid strain when the original strain containing the rat2-1 allele was backcrossed. The NPC clustering observed in strains carrying the rat2-2 allele was indistinguishable from that observed in the rat2-1 strain (data not shown).

Unlike the clustering of NPCs in rat2 cells, which was observed at permissive as well as restrictive temperatures, accumulation of poly(A)⁺ in the nucleus was temperature conditional. Nuclear RNA accumulation occurred in nearly all cells at the elevated temperature, but only in a small subpopulation at permissive temperature. We investigated whether the difference in temperature induction of the two phenotypes resulted from a lower threshold temperature for pore complex antigen mislocalization than for poly(A)⁺ RNA accumulation. To examine this possibility, we performed similar immunofluorescence analyses on...
cells cultured at 15°C. Clustering of the pore complex antigens was observed in *rat2-1* and *rat2-2* strains cultured continuously at 15°C. Thus, the *rat2-1* and *rat2-2* mutations caused conditional accumulation of poly(A)+ RNA in the nucleus at nonpermissive temperature (37°C) and constitutive mislocalization of nuclear pore complex antigens at all temperatures tested.

The position of NPCs in wild-type and mutant cells was also examined by electron microscopy. Fig. 6 shows the results of an experiment using diploid cells shifted to 37°C for 3.5 h. For wild-type cells and cells homozygous for the *rat1-1* mutation, NPCs were distributed around the nuclear envelope (Fig. 6, C and D) although occasional local concentrations were observed, as has been previously reported (Severs et al., 1976). In contrast, in cells homozygous for the *rat2-1* mutation, NPCs were always observed in one discrete area of the nuclear envelope, frequently in a protuberance, and the rest of the nuclear envelope profile appeared barren of NPCs (Fig. 6, A and B). When pore complexes of *rat2-1/rat2-1* cells were sectioned tangentially, the characteristic structure of a ring and central granule could be seen (Fig. 6, A and B).
ters were also observed in diploid cells homozygous for the rat2-2 mutation (data not shown). No gross differences were observed in the morphology of the nucleoplasm or the nucleolus in cells carrying mutant alleles of rat2 as compared to wild-type. The darker, nucleolar region can be observed at the lower right of the nucleus in Fig. 6 B, at the upper right in Fig. 6 C and at the lower left in Fig. 6 D.

Fig. 6 B shows a mitotic cell, with part of the spindle pole body and elongated mitotic spindle included in the plane of section. In this cell, a cluster of NPCs was located in the constricted area of the elongated nuclear envelope, possibly migrating between mother and daughter cells. The results from electron microscopy extend the immunofluorescence observations and demonstrate clearly that NPCs were clustered, often in a protuberance of the nuclear envelope. A close association between the spindle pole body and NPC clusters was often seen when the thin section included both the spindle pole body and NPCs. Thus NPCs apparently underwent assembly and insertion into the nuclear membrane in cells carrying the rat2-1 or rat2-2 alleles, but failed to become distributed around the nuclear periphery. At this low level of resolution the NPCs appeared to have normal morphology.

To begin to address the mechanism by which NPC clusters were generated, we tested whether the clusters were maintained in nuclei isolated from rat2 mutant cells. It seemed possible that aberrant connections between nuclear pore complexes and cytoskeletal elements might play a role in the clustering phenotype, and that if these putative connections were broken, pore complexes might be free to redistribute around the nucleus. Subcellular fractions enriched in nuclei were prepared from rat2-1/rat2-1 and from isogenic wild-type cells. We examined in parallel nuclei isolated from rat3-1/rat3-1 cells. Nuclei were fixed directly and processed for indirect immunofluorescence with antibody RL1. Alternatively, nuclei were incubated at 23°C or 30°C for 4–6 h before fixation to allow potential diffusion of NPCs within the nuclear envelope. In this case, clustering of NPCs was maintained in nuclei isolated from rat2-1/rat2-1 and rat3-1/rat3-1 cells (data not shown). Thus, the pore complex clusters observed in rat2 and rat3 cells do not appear able to redistribute freely around the nuclear envelope when nuclei are separated from cytoplasmic components.

Effect of Mutations of RAT2/NUP120 on Nucleolar Structure and Function

Fragmentation of the nucleolus has been reported for some yeast strains which accumulate poly(A)+ RNA in their nuclei (Kadowaki et al., 1994). To determine whether strains carrying either the rat2-1 or rat2Δ alleles showed a similar nucleolar fragmentation, we performed indirect immunofluorescence using antibodies to two nucleolar proteins, Nop1p (monoclonal antibody A66) and Nsr1p (monoclonal antibody 2.3B). Identical results were obtained with each antibody. The results using antibody 2.3B are shown in Fig. 7. In wild-type cells incubated at 23°C, a brightly staining crescent-shaped pattern was seen in many cells as well as a less brightly staining nucleoplasm. This crescent shaped region is the nucleolus, and the additional staining probably reflects the presence of some nucleolar protein in the nucleoplasm. A dramatic fragmentation of the nucleolus was seen in the rat2-1/nup159-1 strain shifted to 37°C for 4 h (Fig. 7 R); few if any cells had a crescent-shaped staining pattern while most showed from two to six bright spots of fluorescence. Fragmentation could be detected in some cells as soon as 20 min after shifting rat7-1 cells to 37°C (Gorsch, L. C., and C. N. Cole, unpublished results). Cells carrying mutant alleles of RAT2 showed a phenotype that was not completely wild-type but was considerably less abnormal than that seen in the rat7-1/nup159-1 strain (compare Figs. 7, H and M with R). At 37°C, we saw a higher level of staining for nucleolar antigen in the nucleoplasm of rat2-1 and rat2Δ cells than in wild-type cells. While one to four bright fluorescent spots were seen in some mutant nuclei (Fig. 7, H and M), other nuclei appeared very similar to those of wild-type cells.

To examine whether nucleolar function was affected by mutation of RAT2, we analyzed the incorporation of [3H]uridine into ribosomal RNA. 2.5 h after a shift to 37°C, cells carrying the rat2-1 mutation incorporated approximately 50% as much [3H]uridine into TCA-precipitable material as did wild-type cells while cells carrying the rat7-1/nup159-1 mutation incorporated less than 10% as much [3H]uridine as wild-type cells after a 30-min shift to 37°C (data not shown). Labeled RNA was analyzed by electrophoresis and fluorography (Fig. 8). The patterns of incorporation were essentially the same for wild-type and rat2-1 mutant cells; mature 18S and 25S rRNA and precu-

Figure 8. rRNA processing and export occur normally in rat2-1 and rat3-1 cells. [3H]Uridine-labeled RNA was isolated from several strains and analyzed by electrophoresis in a 1.2% agarose-formaldehyde gel. (Lane 1) WT (FY86) grown at 23°C; (lane 2) WT shifted to 37°C for 3.5 h; (lane 3) rat2-1 grown at 23°C; (lane 4) rat 2-1 shifted to 37°C for 2.5 h; (lane 5) rat2-1 shifted to 37°C for 3.5 h; (lane 6) rat3-1 grown at 23°C; (lane 7) rat3-1 shifted to 37°C for 2.5 h; (lane 8) rat3-1 shifted to 37°C for 3.5 h; (lane 9) rnal-1 grown at 23°C; (lane 10) rnal-1 shifted to 37°C for 40 min (*) indicates position of a 23 S intermediate product produced during rRNA processing and accumulating in the rnal-1 mutant strain.
isor 20S and 27S rRNAs were readily seen. Thus, synthesis and processing of rRNA appeared normal. As a control, we examined a strain which is known to have defects in rRNA processing and which contained the *rnal-1* allele (Traglia et al., 1989). While the pattern seen at 23°C in the *rnal-1* strain was indistinguishable from wild-type (compare lanes 1 and 9), precursor 35S and 32S pre-rRNAs accumulated in the *rnal-1* strain following a shift to 37°C (lane 10), aberrantly processed species accumulated (see asterisk at right of lane 10), and little 18S rRNA accumulated. We conclude that mutation of RAT2/NU120 had a minimal affect on the synthesis and processing of ribosomal RNAs even though some morphological alterations to the nucleolus were detected.

We examined a strain which is known to have defects in rRNA processing and which contained the *rnal-1* allele (Traglia et al., 1989). While the pattern seen at 23°C in the *rnal-1* strain was indistinguishable from wild-type (compare lanes 1 and 9), precursor 35S and 32S pre-rRNAs accumulated in the *rnal-1* strain following a shift to 37°C (lane 10), aberrantly processed species accumulated (see asterisk at right of lane 10), and little 18S rRNA accumulated. We conclude that mutation of RAT2/NU120 had a minimal affect on the synthesis and processing of ribosomal RNAs even though some morphological alterations to the nucleolus were detected.

**Figure 9.** Growth properties of yeast cells carrying the *rat2-1* mutation. (A) Growth curves of wild-type (FY23) and *rat2-1* cells (CCY282). Cells were grown at 23°C to early log phase. After 4 more hours growth at 23°C, aliquots of each strain were shifted to 37°C and growth curves were made by counting cells every 4 h. (B) Growth characteristics of wild-type (FY23) and *rat2-1* (CCY282) cells grown on plates containing either YPD or YPD supplemented with 1.0 M sorbitol. Overnight cultures of wild-type (FY23) and *rat2-1* cells (CCY282) cells were diluted and grown on YPD or YPD/1.0 M sorbitol plates at 23°, 30°, 34°, or 37°C. The *rat2-1* cells form colonies at 23°C but cannot do so at 37°C on YPD plates. The addition of 1.0 M sorbitol allowed the *rat2-1* cells to form colonies at 37°C. The plates were photographed after 14 d of growth.
Growth Properties of rat2 Mutant Strains and Suppression of Temperature-sensitive Growth Defects by Sorbitol

A culture of cells carrying the rat2-1 allele was examined for growth characteristics and the results are shown in Fig. 9 A. The wild-type culture (FY86) had a doubling time of approximately 2 h at 23°C. The rat2-1 strain grew more slowly at this temperature with a doubling time of almost 3.0 h. After the shift to 37°C, the growth rate of the wild-type strain was unaltered but it grew to a lower final density at 37°C than at 23°C. After the shift to 37°C, the rat2-1 mutant strain continued to grow at approximately the same rate at 37°C as it had at 23°C for the next 4 h. Doubling times for the culture increased dramatically beyond this point. The rat2-1 strain showed a doubling time of approximately 12-15 h during the latter part of the experiment, and reached a final cell density substantially below that obtained under continuous growth at 23°C. A portion of the mutant culture incubated at 37°C was removed after 28 h of incubation and tested for the presence of revertants by plating cells at both 23°C and 37°C. No colonies were formed during 7 d of growth on plates incubated at 37°C, indicating that growth of revertants did not contribute to the growth behavior observed in this experiment.

We also examined the growth properties of cells carrying the rat2-1 mutation on YPD plates incubated at 23°C, 30°C, 34°C, and 37°C (Fig. 9 B). Under these conditions, colonies formed by wild-type and mutant cells grown at 23°C were indistinguishable. Mutant colonies formed on plates incubated at 30°C and 34°C were considerably smaller than wild-type cells colonies. Very tiny colonies could be seen when plates containing rat2-1 cells were incubated at 37°C for 14 d. The inclusion of either 1.0 M sorbitol or 0.9 M NaCl in the YPD plates reduced the size of the colonies formed by wild-type cells. These supplements had little effect on mutant colony size for mutant cells incubated at 23°C and caused a slight reduction in colony size following incubation at 30°C. In contrast, these supplements enhanced the size of colonies formed at 34°C. At 37°C, these supplements permitted cells containing the rat2-1 allele to form colonies. The growth behaviors of the strain carrying a disruption of RAT2 were the same as those seen with the rat2-1 strain, in media either with and without 1.0 M sorbitol (data not shown). These results indicate that maintenance of osmotic stability overcame the deleterious effects of the mutations on growth properties.

We wished to determine how the presence of 1.0 M sorbitol affected the various mutant phenotypes seen in rat2-1 cells. Fig. 10 shows representative micrographs from an experiment where the distribution of poly(A)+ RNA was determined by in situ hybridization (Fig. 10 B) and the location and clustering of NPCs was determined by indirect immunofluorescence with an anti-Rat7p/Nup159p antibody (Fig. 10 A). Cultures were incubated overnight at 23°C in the presence of sorbitol and were subsequently shifted to 37°C for 4 h in the presence of sorbitol. The data indicate that growth in the presence of sorbitol had no effect on the clustering of NPCs (compare Fig. 10 A, panels D and E). The accumulation of poly(A)+ RNA in nuclei of rat2-1 cells was not completely suppressed by sorbitol, but the nuclear fluorescence was much weaker in the presence of 1.0 M sorbitol (compare Fig. 10 B, panels D and E), suggesting that sorbitol acted to enhance RNA export to a degree sufficient to permit continued growth, but that the suppression of the nuclear accumulation of poly(A)+ RNA was not complete. Interestingly, a modest accumulation of poly(A)+ RNA was seen in the nuclei of some wild-type cells cultured in the presence of 1.0 M sorbitol and incubated at 37°C (Fig. 10 B, panel B).

Synthetic Lethality

Two mutations are said to be synthetically lethal if yeast strains carrying either mutation can grow but strains carrying both are unable to do so (Bender and Pringle, 1991; Costigan et al., 1992). Synthetic lethality has been used to provide insight into whether two gene products interact, are part of the same macromolecular complex, or are redundant. Synthetic lethality has been observed between mutant alleles of several nucleoporins (for review see Doyle and Hurt, 1995) and has also been observed between nucleoporins and proteins that interact with nucleoporins but are themselves not components of the NPC (Belanger et al., 1994). Synthetic lethality analyses are performed by mating strains carrying mutations of interest, introducing into the heterozygous diploid a CEN URA3 plasmid containing the wild-type allele of one of the genes being tested, sporulating, dissecting tetrads, and determining whether those resulting haploids carrying mutant alleles of both genes require the presence of a wild-type allele of one of the genes for growth. Strains heterozygous for both rat2-1 and rat3-1/nup133-1 or for both rat2-1 and rat7-1/nup159-1 were sporulated, tetrads were dissected, and progeny haploid strains were analyzed. All of the 24-sporo tetrads obtained from the diploid heterozygous for rat2-1 and rat3-1/nup133-1 yielded one or more haploid strains unable to grow on 5-FOA (see Materials and Methods). From the diploids heterozygous for rat2-1 and rat7-1, only five tetrads yielding four viable haploid strains were obtained. For each, one of the four strains was unable to grow on 5-FOA. Since RAT2/NUP120 is not linked to either RAT7/NUP159 or RAT3/NUP133, this indicates that rat2-1 is synthetically lethal with both rat7-1/nup159-1 and rat3-1/nup133-1.

Discussion

In this paper, we have described the cloning and characterization of the RAT2/NUP120 gene of S. cerevisiae, which encodes a 120.4-kD protein. We refer to this protein as Rat2p/Nup120p. We believe that this protein is a nucleoporin based on the following findings: (a) epitope-tagged, Rat2p was found to be located at the nuclear rim in a punctate pattern diagnostic of NPC proteins; (b) the staining pattern for Rat2pmy c was identical to that seen with the RL1 monoclonal antibody, which is known to recognize yeast nucleoporins; (c) NPCs were clustered in the nuclear envelope of cells carrying mutant alleles of RAT2. This phenotype has been observed in strains carrying mutant alleles of several nucleoporins and has not been associated with mutations of other classes of genes. (d) Synthetic lethality was observed between the rat2-1 allele and mu-
Figure 10. Effect of 1.0 M sorbitol on NPC clustering and poly(A)$^+$ RNA distribution phenotypes in rat2-1 mutant cells. (A) IF of wild-type (FY23) (panels A–C) and rat2-1 cells (CCY282) (panels D–F) using an anti-Rat7p/Nup159p antibody and an FITC-conjugated secondary antibody. Cultures were incubated overnight at 23°C in either YPD or YPD + 1.0 M sorbitol and were shifted to 37°C for 0 h. (Panel A) WT grown on YPD and shifted to 37°C, FITC; (B) WT grown on YPD + 1.0 M sorbitol and shifted to 37°C, FITC; (C) the same field of cells as in panel B, stained with DAPI; (D) rat2-1 cells grown on YPD and shifted to 37°C, FITC; (E) rat2-1 cells grown on YPD + 1.0 M sorbitol and shifted to 37°C, FITC; (F) the same field of cells as in panel E, stained with DAPI. Rat2-1 cells grown on YPD + 1.0 M sorbitol at 37°C still display clustering of NPCs (compare panels A and B). (B) Localization of poly(A)$^+$ RNA, visualized with a FITC-conjugated anti-digoxigenin antibody. Cultures were prepared as in A. (Panel A) WT cells grown on YPD and shifted to 37°C, FITC; (B) WT cells grown on YPD + 1.0 M sorbitol and shifted to 37°C, FITC; (C) the same field of cells as in panel B, stained with DAPI; (D) rat2-1 cells grown on YPD and shifted to 37°C, FITC; (E) rat2-1 cells grown on YPD + 1.0 M sorbitol and shifted to 37°C, FITC; (F) the same field of cells as in panel E, stained with DAPI. The accumulation of poly(A)$^+$ RNA in the nuclei of the rat2-1 cells grown on YPD + 1.0 M sorbitol at 37°C was not completely suppressed, but the nuclear fluorescence was much weaker (compare panels D and E).

Tant alleles of RAT3/NUP133 and RAT7/NUP159. Rat2p/Nup120p is the fourth yeast nucleoporin identified that lacks the repeat motifs found in the majority of nucleoporins described to date. Nic96p, Rat3p/Nup133p, and Nup82p are the other three yeast nucleoporins lacking repeats.

Nucleocytoplasmic Export of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA accumulated in the nuclei of rat2-1 and rat2Δ mutant cells after a shift to 37°C. A low percentage of cells showed nuclear poly(A)$^+$ RNA accumulation at 23°C or 15°C while most cells showed this phenotype within 4 h of a shift to 37°C. The RNA accumulation phenotype of these mutant strains was much less dramatic than we saw in strains with mutant alleles of RAT7/NUP159 (Gorsch et al., 1995). In the latter case, 100% of the cells showed bright nuclear fluorescence (reflecting accumulation of poly[A]$^+$ RNA) within 30 min of a shift to 37°C.

Several explanations are possible for the gradual and slow appearance of the RNA export defect in rat2-1 and rat2Δ cells. Repeat-containing nucleoporins may be located along the entire axis of the NPC, from the nuclear basket to the cytoplasmic filaments. This would enable them to interact with substrate (either protein or RNP).
which could be passed along from one repeat-containing nucleoporin to another (Radu et al., 1995). Nucleoporins lacking repeats, such as Rat2p/Nup120p, could play a role in anchoring repeat-containing nucleoporins within the overall NPC framework. Complexes can be isolated that contain both repeat-containing and nonrepeat-containing nucleoporins (Grandi et al., 1993, 1995a,b). If such complexes were normally anchored by more than one NPC protein, loss of one of these (by disruption of the gene encoding it) might permit retention of function at 23°C, but shifting cells to 37°C could cause the complex to dissociate from NPC. By this model, Rat2p/Nup120p and some other NPC protein could work together to anchor such a complex in the pore framework. A related possibility is that the putative Rat2p/Nup120p-containing complex is absent from NPCs at all temperatures but that the mutant NPCs retain an ability to export RNA at 15° and 23°C. Such altered pores might be thermosensitive and could undergo greater loss of function following a shift of mutant cells to 37°C. Whatever changes in pore structure and function occur following a temperature shift, they do not appear to cause overall failure of all NPC functions, since no defect in import of nuclear proteins was observed in rat2-1 cells.

Alterations to Nucleolar Structure in Mutant Strains

Modest alterations in nucleolar structure were seen by indirect immunofluorescence when rat2/nup120 mutant cells were shifted to 37°C. However, the continued incorporation of [3H]uridine into rRNA and its normal processing into 18S and 25S rRNA suggests that whatever alterations occurred to nucleolar structure, nucleolar functions were not compromised. Since the final processing of 20S to 18S rRNA is thought to occur in the cytoplasm (Udem and Warner, 1973), export of ribosomal subunits must still be occurring 3.5 h following a shift of rat2-1 cells to 37°C since labeled 18S rRNA was produced and the ratio of 20S to 18S rRNA was very similar in wild-type and mutant strains shifted to 37°C. This is in contrast to the dramatic disruption of the nucleolus following shift to 37°C of strains carrying the rat7-1 allele; in that strain, synthesis and processing of rRNA ceased very rapidly following the shift.

How mutations in NPC proteins might affect nucleolar structure and function is uncertain. One possibility is that there are direct connections between NPCs and nucleolar components. The yeast nucleolus lies closely apposed to the nuclear envelope (see Fig. 6), and this arrangement

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may facilitate efficient export of ribosomal subunits. Perhaps these interactions are sensitive to disruption in strains lacking specific nucleoporins or containing altered NPC proteins. Alternatively, nucleolar alteration or fragmentation could reflect an indirect effect caused by a block to the export of ribosomal subunits. Perhaps accumulation of rRNA within the nucleus signals the nucleolus to cease production of additional ribosomal subunits. If the processing of 20S to 18S rRNA can be taken as evidence for rRNA export, rRNA export continued after a shift of rat2 mutant strains to 37°C. Although incorporation of [3H]uridine into rRNA was reduced approximately 50%, processing appeared normal (Fig. 8). Thus, the slightly altered nucleoli in rat2 mutant strains appeared to function quite well at 37°C. In contrast, fragmentation of the nucleolus occurred rapidly in cells carrying the rat7-1/nup159-1 allele (within 30 min; our unpublished results); biogenesis of ribosomes also ceased very soon following a shift of rat7-1/nup159-1 cells to 37°C.

**Nuclear Pore Complex Clustering**

We have described clustering of NPCs in yeast strains with mutations of RAT3/NUP133 (Copeland, C. S., D. C. Amberg, and C. N. Cole. 1991. *J. Cell Biol.* 115:317a; Li et al., 1995) and RAT7/NUP159 (Gorsch et al., 1995). NPC clustering has also been reported for strains with mutations of NUP116 (Wente et al., 1992; Wente and Blobel, 1993), and NUP145 (Fabre et al., 1994; Wente and Blobel, 1994; Dockendorff, T. C., A. L. Goldstein, and C. N. Cole, manuscript in preparation). We have also found NPC clustering in yeast strains carrying a disruption of the RAT9/NUP85 gene (Goldstein, A. L., and C. N. Cole, manuscript in preparation). In most of these strains, clustering was constitutive, while in strains with the nup159-1/rat7-1 allele, clustering was reversed following a shift to 37°C and the mutant Rat7p/Nup159p appeared to dissociate from the NPCs following temperature shift. In some of these mutant strains, nuclear envelope defects were also observed, with NPCs clustering together in abnormally structured regions of the nuclear envelope. In other mutant strains (e.g., rat7-1/nup159-1), no nuclear envelope abnormalities were observed, and a region of nuclear envelope could be seen surrounding each NPC in a linear cluster. Thus, in different mutant strains, NPC clustering may reflect defects in different aspects of NPC biogenesis and behavior. These mutant strains should be useful for investigating NPC biogenesis and inheritance.

It is not known whether NPCs are assembled at a single site in the nuclear envelope or at many sites. If at a single site, the NPC clustering would reflect a failure to distribute NPCs around the nuclear envelope. If inserted at many sites, the clustering would most likely reflect the aggregation of NPCs containing nucleoporins, perhaps due to exposure of protein domains that are normally hidden in wild-type NPCs but which become exposed in mutant NPCs and can interact with similar domains of other NPCs in mutant strains. Examination of isolated nuclei indicates that, once formed, the NPC clusters do not redistribute in the absence of gross connections to the cytoskeleton. Thus the presence of NPC clusters does not depend on the continuous presence of an intact cytoskeleton. Since clustering of NPCs and other abnormalities of NPC distribution and nuclear envelope morphology have been seen in strains carrying mutant alleles of several nucleoporins, it is unlikely that each of these nucleoporins plays a direct role in the distribution of NPCs. Rather, we favor the idea that the NPC clustering observed reflects a failure to distribute NPCs. Since clusters of NPCs were often seen near to the spindle pole body in rat2 (for example see Fig. 6 a) and rat3 mutant cells (Copeland, C. S., D. C. Amberg, M. Snyder, and C. N. Cole, unpublished results), we hypothesize that the spindle pole body provides the site for insertion of NPCs into the nuclear envelope. This suggests that synthetic lethality might be found between components of the spindle pole body and some nucleoporins; direct protein-protein interactions between NPC and spindle pole body components would also be expected.

**Growth Defects and Their Suppression by High Osmolarity Media**

What blocks growth of rat2 cells at nonpermissive temperature? The experimental results indicate that nuclear pore complex clustering is not sufficient to block growth and slows cell growth and division only slightly for the first several hours following a temperature shift to 37°C. The reduced growth rate at 37°C may be secondary to the RNA export defect and could result from loss of critical cellular functions encoded by genes which produce abundant and short-lived mRNAs or proteins. The increase in growth rate when cells were grown with osmotic support suggests that an essential function altered by the rat2 mutations at 37°C may relate to osmotic balance. An increase in extra cellular osmolarity results in a large number of changes in the yeast cell. These include reorganization of the actin cytoskeleton (Chowdhury et al., 1992), induction of an osmosensing signal transduction pathway (Brewster et al., 1993), alterations in gene expression including induction of some of the heat shock genes (Varela et al., 1992), and a large increase in intracellular glycerol level (Edgley and Brown, 1983; Reed et al., 1987). These effects can dramatically alter the expression of mutant phenotypes and this phenomenon has been exploited in the isolation of both osmotic-remedial and osmotic-sensitive mutations (Megnet, 1966; Bassel and Douglas, 1968; Metzenberg, 1968). The mechanisms by which a rise in extracellular osmolarity can affect the expression of mutant phenotypes are poorly understood.

Changes in intracellular glycerol concentrations in response to alterations in osmolarity could alter the properties of the intracellular environment, thereby stabilizing protein-protein interactions within the NPCs. Since high osmotic strength media suppresses the phenotypic defects of strains with a null mutation of RAT2, perhaps the large increase in intracellular glycerol which occurs in response to high osmotic strength media stabilizes protein-protein interactions, which may be weaker in NPCs lacking Rat2p. Alternatively, osmo-induced signal transduction pathways described by Gustin and colleagues (Chowdhury et al., 1992; Brewster et al., 1993) could be responsible for the expression of genes whose products reduce the cell’s requirement for Rat2p/Nup120p functions. It is also possible that greater osmotic support stabilizes the nuclear env-
lopes or interactions between the nuclear envelope and the NPCs in mutant cells. A similar enhancement of growth defects by high osmolarity media was seen in strains carrying mutant alleles of \textit{RAT7/\textit{NUP}159, RAT3/\textit{NUP}133, and NUP116}, but not in strains with mutant alleles of \textit{RAT9/\textit{NUP}85} or \textit{RAT10/\textit{NUP}145} (Heath, C. V., and C. N. Cole, unpublished results).

**Identical Phenotypes in rat2 and rat3 Mutant Strains**

The phenotypes observed in strains with mutant alleles of \textit{RAT2/\textit{NUP}120} are strikingly similar to those seen in strains with mutant alleles of \textit{RAT3/\textit{NUP}133}. In both cases, a small percentage of cells showed nuclear accumulation of poly(A)$^+$ RNA at 23°C and the percentage was much higher following a shift to 37°C. In both cases, accumulation in 100% of the cells was seen with the disruption allele but not with the originally isolated \textit{rat2}-1 or \textit{rat3}-1 alleles. With both \textit{rat2/nup120} and \textit{rat3/nup133} mutant strains, constitutive clustering of NPCs was observed. No other defects in nuclear structure were seen in either strain except for the modest alteration to nucleolar structure reported here for \textit{rat2}-1 cells. We have not yet examined the \textit{rat3}-1 strains for nucleolar defects but it showed the same modest decrease in $[^3]H$uridine incorporation and the same normal pattern of 27, 25, 20, and 18S rRNA species as seen in the \textit{rat2}-1 strain (Fig. 8). In both \textit{RAT2/\textit{NUP}120} and \textit{RAT3/\textit{NUP}133} mutant strains, growth defects were suppressed by high osmolarity. Finally, the \textit{rat2}-1 allele was synthetically lethal with the \textit{rat3}-1 allele. While some of these properties are shared with some other strains carrying mutant nucleoporin genes, no other mutant strains share all of the phenotypes shared by \textit{rat2/nup120} and \textit{rat3/nup133} mutant strains.

The \textit{RAT2/\textit{NUP}120} and \textit{RAT3/\textit{NUP}133} proteins lack any significant homology with each other or with other known proteins. Rat2p/Nup120p and Rat3p/Nup133p are acidic proteins with pI of 4.86 and 4.82, respectively. Although no significant alignment of Rat2p/Nup120p with Rat3p/Nup133p was achieved using either the FASTA program or a Pustell matrix analysis, the two have almost identical amino acid compositions. Rat2p contains 41% nonpolar residues, 35.6% polar residues, 12.5% acidic residues, and 10.9% basic residues; for Rat3p/Nup133p, the percentages are 39.9, 35.0, 13.6, and 11.5%, respectively. We do not understand the significance of this similarity nor whether these two proteins interact directly.

In summary, strains carrying mutations of the \textit{RAT2/\textit{NUP}120} gene show constitutive clustering of nuclear pore complexes that are often seen very close to the spindle pole body. When these cells are shifted to 37°C, we observed a defect in the nucleocytoplasmic export of poly(A)$^+$ RNA and some alterations in the structure of the nucleolus. However, synthesis and normal processing of $r$RNA continued, suggesting that little or no loss of nucleolar function occurred. Major questions remain concerning the actual functions of Rat2p/Nup120p. Is its primary role in insertion of NPCs or NPC subassemblies into the nuclear envelope? Is it a component of an NPC subassembly which plays a direct role in export of RNA? By what mechanism do mutations within nucleoporins affect nucleolar structure and function? Both genetic and biochemical approaches can be employed to identify proteins with which Rat2p/Nup120p interacts, and this should provide additional insight into its functions.

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