

# Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA

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We have combined techniques of genetics and histochemistry to identify genes required for the nucleocytoplasmic export of mRNA in the budding yeast *Saccharomyces cerevisiae*. We adapted in situ hybridization using a digoxigenin-labeled oligo(dT)<sub>50</sub> probe to localize poly(A)<sup>+</sup> RNA in fixed yeast cells and used yeast strains carrying the *rna1-1* mutation to develop an assay. The *rna1-1* mutation is the only previously described mutation that causes defects in mRNA export. As visualized with this RNA localization assay, *rna1-1* strains accumulated poly(A)<sup>+</sup> RNA at the nuclear periphery at the nonpermissive temperature. This was in contrast to the RNA localization pattern of wild-type cells or *rna1-1* cells grown at permissive temperature. Wild-type cells showed bright uniform cytoplasmic staining with little detectable RNA in the nuclei. We used this RNA localization assay to screen a bank of temperature-sensitive yeast strains for mutants with inducible defects in mRNA trafficking. Strains identified in this manner are designated RAT mutants for ribonucleic acid trafficking. The *rat1-1* allele conferred temperature-sensitive accumulation of poly(A)<sup>+</sup> RNA in one to several intranuclear spots that appear to lie at the nuclear periphery. RNA processing was unaffected in *rat1-1* strains, except for an inducible defect in trimming the 5' end of the 5.8S rRNA. The wild-type *RAT1* gene was cloned by complementation; it encodes an essential 116-kD protein with regions of homology to the protein encoded by *SEP1* (also known as *DST2*, *XRN1*, *KEM1*, and *RAR5*). Sep1p is a nucleic acid binding protein, a 5' → 3' exonuclease, and catalyzes DNA strand transfer reactions in vitro. We discuss the possible significance of the Rat1p/Sep1p homology for RNA trafficking. We also discuss the potential of this RNA localization assay to identify genes involved in nuclear structure and RNA metabolism.

[Key Words: mRNA export; yeast; in situ hybridization; *RAT1*; *RNA1*; *SEP1*]

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mRNA trafficking, the process by which mRNA molecules are moved from their sites of transcription in the nucleus to their sites of translation in the cytoplasm, is central to the expression of eukaryotic genes (for review, see Schroder et al. 1987; Agutter 1991; Maquat 1991). mRNA trafficking can be thought of as occurring in three stages: movement of message from the sites of transcription to the nuclear pore, translocation of the mRNA through the pore, and movement to the correct location for translation.

In the first stage, mRNA probably becomes a substrate for the machinery that executes these movements when RNA processing has been completed to yield capped, spliced, and polyadenylated transcripts. Experiments by Spector and colleagues (Huang and Spector 1991) showed that nascent transcripts and splicing factors colocalize to "speckles" in mammalian nuclei. This result and others

(Bannerjee 1980; Leff et al. 1986; Beyer and Osheim 1988; Humphrey and Proudfoot 1988; LeMaire and Thummel 1990) indicate that processing events can occur cotranscriptionally. This also implies that pre-mRNA molecules are processed in the same or adjacent nuclear compartments as those in which they are synthesized. In the context of the assay described here, it is important to stress that polyadenylation of mRNA molecules occurs cotranscriptionally and probably before the initiation of transcript movement toward the nuclear periphery.

Single species of mRNA are observed to move from their site of transcription along a "track" within the nucleus to the nuclear periphery (Lawrence et al. 1989; Huang and Spector 1991; Xing and Lawrence 1991). This movement likely occurs in association with the nucleoskeleton or those elements of the nucleoskeleton identified as the nuclear matrix (for review, see Verheijen et al. 1988). In fact, 70% of nuclear RNA remains associated with the matrix (He et al. 1990).

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The structural features that distinguish pre-mRNA from other RNAs play important roles in mRNA trafficking. For example, the nuclear envelope contains receptors for poly(A) (Schroder et al. 1987); the monomethyl cap structure of mRNA efficiently signals mRNA export, and hypermethylated caps [found normally in small nuclear RNA (snRNA)] signal nuclear retention (Hamm and Mattaj 1990). Other studies suggest that introns of pre-mRNA serve as nuclear retention signals and prevent export until splicing has been completed (Legrain and Rosbash 1989; Hamm and Mattaj 1990). Determinants for RNA trafficking could lie within promoter elements that specify transcription complex formation, in the machinery that carries out the various reactions involved in RNA processing and in RNA sequences or structures not yet identified.

In the second stage of mRNA trafficking, mRNA molecules are moved through the nuclear pore complexes (NPCs), the only known channels in the nuclear envelope for the exchange of macromolecules. Movement of messenger ribonucleoprotein (mRNP) through the NPC was first visualized in *Chironomus* salivary glands by Stevens and Swift (1966). More recently, RNA-coated gold particles injected into *Xenopus* oocyte nuclei were observed by electron microscopy to exit the nucleus through NPCs. Import of proteins into the nucleus occurs through the same pores (Dworetzky and Feldherr 1988). A number of NPC proteins and their genes have been identified (Davis and Blobel 1986; Wozniak et al. 1989; Davis and Fink 1990; Nehrbass et al. 1990). Whether these proteins play an active role in mRNA export is unknown; however, functional NPCs are certainly required for mRNA export.

In the third stage of mRNA trafficking, mRNA is distributed in the cytoplasm in the appropriate context for translation. This process likely requires elements of the cytoskeleton. mRNA is translated by ribosomes in association with the actin cytoskeleton (for review, see Hesketh and Pryme 1991). Particular species of mRNA molecules are sorted such that they show specific patterns of localization in the cytoplasm (for review, see Gottlieb 1990). Specific localization can have profound effects on gene expression, establishing the importance of cytoplasmic events in mRNA trafficking.

For the experiments described in this paper we used the budding yeast *Saccharomyces cerevisiae*. Because the structure of mRNA, the pathway of pre-mRNA processing (Butler and Platt 1988; Ruby and Abelson 1991), and the structure of the NPC (Allen and Douglas 1989) are similar in *S. cerevisiae* and in metazoans, mRNA trafficking is likely to occur by similar mechanisms and by using similar structures in all eukaryotes. Recent experiments indicate that *S. cerevisiae* has a nucleoskeleton that may be composed of intermediate filaments (Cardenas et al. 1990; Mirzayan et al. 1992). One discernible difference between yeast and metazoans is that few yeast genes contain introns; therefore, efficient export of most yeast mRNAs cannot require splicing.

The only mutants known to be defective in nucleocytoplasmic transport of RNA carry conditional mutations

in the *RNA1* gene of *S. cerevisiae* (Shiokawa and Pogo 1974). The *RNA1* protein is cytoplasmic (Hopper et al. 1990) and is essential for mitotic growth. *RNA1* bears little resemblance to previously identified genes (Atkinson et al. 1985). The *rna1-1* mutant also has defects in tRNA and rRNA processing (Hopper et al. 1978); however, mRNA processing appears to be unaffected (Rosbash et al. 1981). The biochemical basis of this defect is not understood; however, the subcellular location of the *RNA1* protein is consistent with a block in trafficking occurring at, or soon after, transit through the pore.

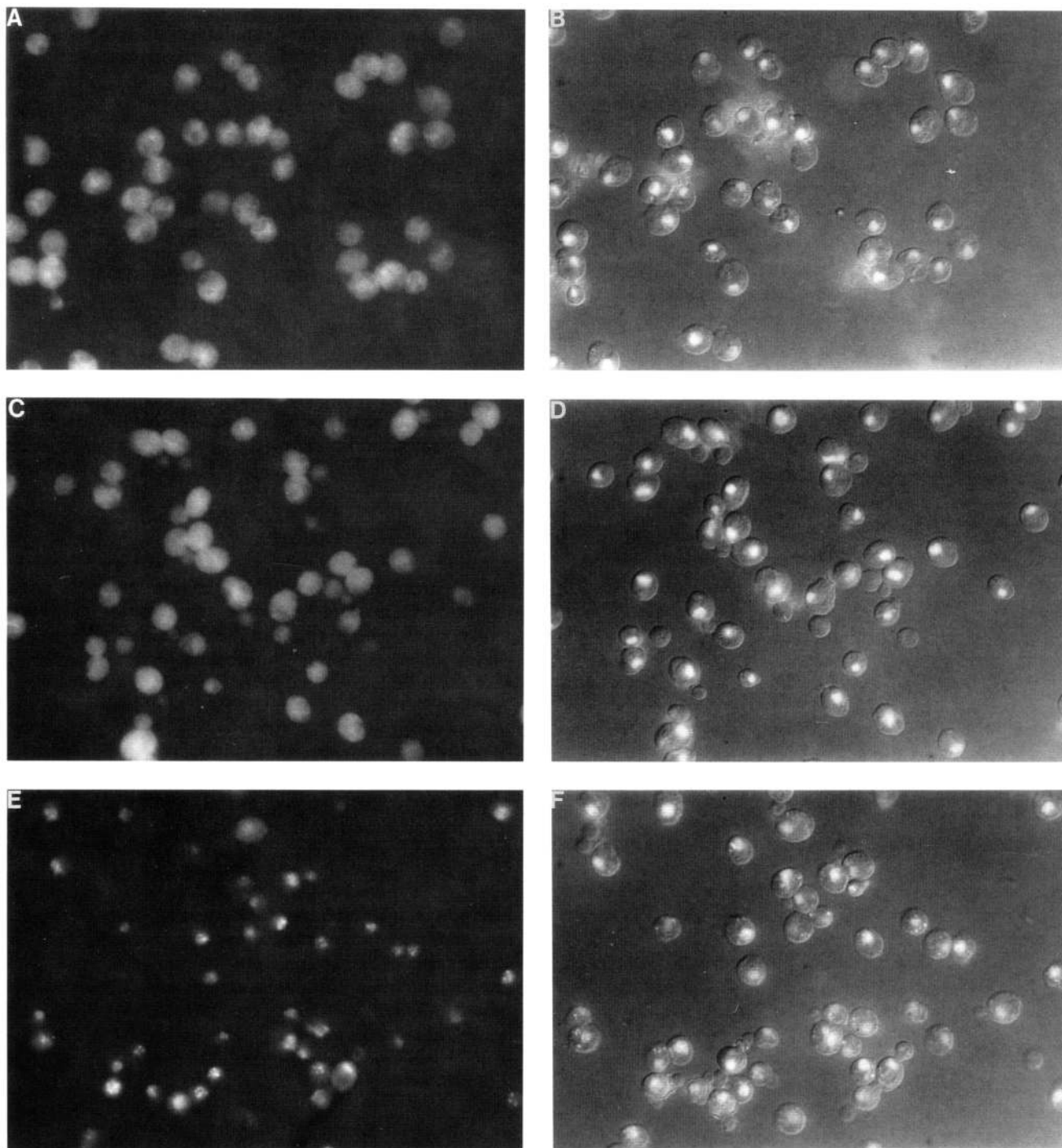
We adapted in situ hybridization techniques to localize poly(A)<sup>+</sup> RNA in fixed yeast cells. This mRNA localization assay confirmed the mRNA trafficking defects of *rna1-1* strains. We constructed a bank of 600 temperature-sensitive yeast strains and screened them using this assay. We identified seven alleles in six complementation groups and call these RAT mutations for ribonucleic acid trafficking. Here, we describe the in situ RNA localization assay and report the cloning, sequencing, and preliminary characterization of the *RAT1* gene. We discuss the use of the mRNA localization assay to identify other yeast genes important for mRNA trafficking.

## Results

### *mRNA localization assay*

We developed an assay that would permit us to visualize the location of mRNA molecules within yeast cells based on the assumption that conditional mutants with defects in mRNA trafficking would display aberrant patterns of mRNA accumulation. Furthermore, we anticipated that mutants defective for different steps in the mRNA trafficking pathway might manifest discernibly different patterns of mRNA accumulation. To visualize mRNA trafficking defects, we adapted in situ hybridization for the localization of poly(A)<sup>+</sup> mRNA in yeast cells, using a digoxigenin-labeled oligo(dT)<sub>50</sub> probe. The sites at which this probe became bound were visualized by immunofluorescence. The nuclear region of the cells was localized by staining the DNA with DAPI (4',6-diamidino-2-phenylindole-dihydrochloride).

The mRNA localization assay was developed and optimized by use of cells carrying the *rna1-1* allele as a positive control. The *rna1-1* allele causes dramatic defects in mRNA trafficking at the nonpermissive temperature of 37°C (Shiokawa and Pogo 1974). Figure 1 shows three fields of yeast cells that were carried through the localization assay and visualized by FITC fluorescence (left column), or combined DAPI fluorescence and differential interference contrast microscopy (DIC) (right column). Figure 1, A and B, shows a field of wild-type diploid cells, strain FY23X86 (Table 1). Wild-type strains generally had uniform staining throughout the cytoplasm, and the nuclei often appeared as dark holes, suggesting that yeast nuclei contain relatively little poly(A)<sup>+</sup> RNA. Figure 1, C and D, shows a field of *rna1-1/rna1-1* cells (strain 2b × 3b) grown at their permissive temperature of 23°C. The staining pattern was very sim-



**Figure 1.** Localization of mRNA in wild-type and *rna1-1* cells. mRNA was localized in fixed yeast cells by use of the in situ mRNA localization assay. Cells were grown continuously at 23°C, or shifted to 37°C for 1 hr. (A) Wild-type diploid cells (strain FY23X86) shifted to 37°C for 1 hr and visualized by FITC fluorescence. (B) The same field of cells as in A but visualized by DAPI fluorescence and simultaneous DIC. (C) Strain 2b × 3b cells (*rna1-1/rna1-1*) grown at 23°C and visualized by FITC fluorescence. (D) The same field of cells as in C but visualized by DAPI fluorescence and simultaneous DIC microscopy. (E) Strain 2b × 3b cells shifted to 37°C for 1 hr and visualized by FITC fluorescence. (F) The same field of cells as in E but visualized by DAPI fluorescence and simultaneous DIC microscopy.

ilar to that observed in wild-type cells. The *rna1-1/rna1-1* cells grown at 37°C for 1 hr (Fig. 1E,F) had very different patterns of RNA localization. All of the poly(A)<sup>+</sup> RNA appeared to be localized in the nuclear

region of the cells, and the cytoplasm was devoid of signal (E). These results are consistent with the known inducible defect in mRNA trafficking of *rna1-1* strains (Shiokawa and Pogo 1974).

**Table 1.** Yeast strains

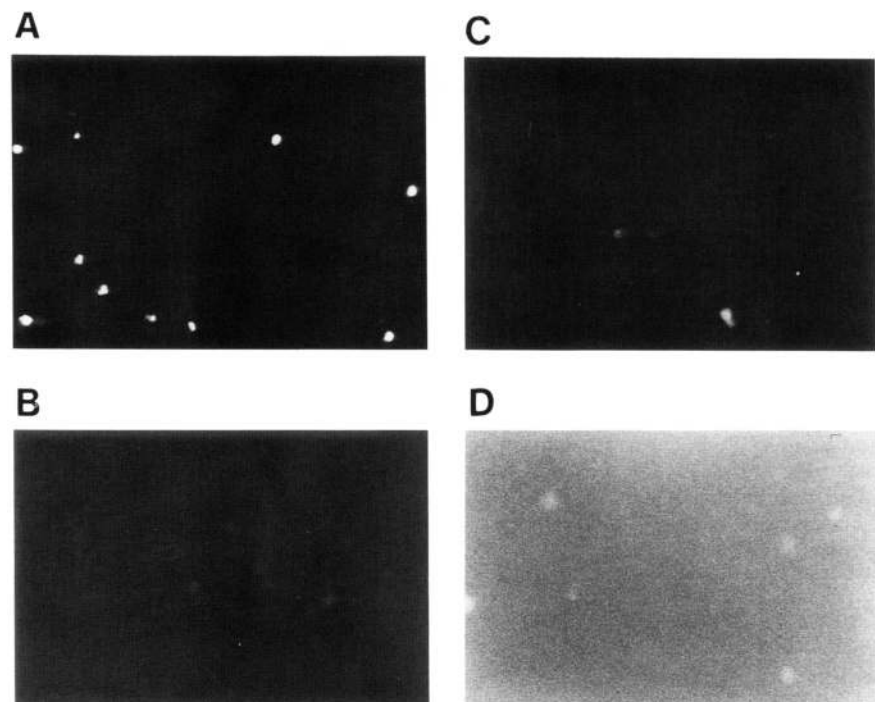
Strain	Genotype	Plasmid
FY23	<b>a</b> <i>ura3-52 trp1Δ63 leu2Δ1</i>	p6A
FY86	<b>α</b> <i>ura3-52 his3Δ200 leu2Δ1</i>	
FY23×86	<b>a/α</b> <i>ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63 his3Δ200</i>	
2b×3b	<b>a/α</b> <i>ura3-52/ura3-52 leu2-3,112/leu2-3,112 ade2-1/ade2-1 tyr1 his7 Gal<sup>-</sup> rna1-1/rna1-1</i>	
DA1-1	<b>α</b> <i>ura3-52 leu2Δ1 trp1Δ63 rat1-1</i>	
DA1-2	<b>a</b> <i>ura3-52 leu2Δ1 trp1Δ63 rat1-1</i>	
DA1×t1	<b>a/α</b> <i>ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63 his3Δ200 rat1-1/rat1-1</i>	
O251	<b>α</b> <i>his1-315 ade2</i>	
RS561	<b>a</b> <i>can1-1 leu2-3,112 his4-514 TMP1::LEU2</i>	

A number of experiments were performed to demonstrate that this assay detected poly(A)<sup>+</sup> mRNA. Figure 2 shows four fields of cells carrying the *rna1-1* allele, grown at 37°C for 1 hr, and submitted to various modifications of the assay and visualized only by FITC fluorescence. The hybridization step contained either a 1000-fold molar excess of an unlabeled, nonspecific oligonucleotide (Fig. 2A) or a 1000-fold molar excess of unlabeled oligo(dT)<sub>50</sub> (Fig. 2B). The ability of the homologous but not the heterologous oligonucleotide to compete the strong nuclear signal demonstrated the specificity of the assay. Figure 2C shows cells incubated in RNase T<sub>2</sub> for 2 hr at 37°C. This pretreatment with an adenosine-specific ribonuclease abolished the signal, presumably by hydrolyzing sequences capable of binding to the probe. A similar treatment with RNase A (which is specific for pyrimidines) failed to diminish the signal (data not shown). Cells that were incubated with a FITC-conjugated Fab fragment of unrelated specificity are shown in Figure 2D. Little labeling of the cells was observed, thus confirming the specificity of the antibody.

To confirm that the assay detected RNA polymerase II transcripts, we generated a double mutant that carries both the *rna1-1* allele and a temperature sensitive allele, *rpb1-1*, affecting the largest subunit of RNA polymerase II (Nonet et al. 1987). At 37°C, *rpb1-1* strains cease mRNA synthesis rapidly. Following growth at nonpermissive temperature, cells were processed under standard conditions. No hybridization of the oligo(dT)<sub>50</sub> probe was detected in the double mutant strain after a 1 hr shift to 37°C. The cells appear to have no poly(A) sequences in their nuclei and very little in the cytoplasm, as measured with the RNA localization assay. This result clearly demonstrates that the assay detects RNA polymerase II-derived poly(A)<sup>+</sup> RNA.

#### Screen for mRNA trafficking mutants

A bank of temperature-sensitive mutants was constructed to identify new mutants defective for mRNA trafficking. This bank was made from strains FY23 and FY86 by UV mutagenesis. These strains are isogenic at



**Figure 2.** The mRNA localization assay detects poly(A)<sup>+</sup> mRNA. mRNA was localized in a *rna1-1* strain following a shift to 37°C for 1 hr. All four panels show the FITC fluorescence of *rna1-1* cells treated in slightly different manners. (A) Cells hybridized to digoxigenin-tagged oligo(dT)<sub>50</sub> in the presence of a 1000-fold molar excess of an unlabeled and unrelated oligonucleotide. (B) Cells hybridized to digoxigenin-oligo(dT)<sub>50</sub> in the presence of 1000-fold molar excess of nondigoxigenated oligo(dT)<sub>50</sub>. (C) Cells treated with 100 U/ml of RNase T<sub>2</sub>. (D) Cells hybridized with digoxigenin-oligo(dT)<sub>50</sub> and then with a FITC-labeled Fab fragment from an antibody that is not directed against digoxigenin.

all loci, except *MAT*, *HIS3*, and *TRP1*. The RNA localization assay was used to screen 600 temperature-sensitive strains from this bank of mutant strains. Initially, pools containing cells from five mutant strains were grown to log phase and shifted to 37°C for 2 hr. Cells with aberrant mRNA localization patterns were very easy to identify in these pools, and individual members of positive pools were subsequently examined. Three general patterns of aberrant mRNA localization in the mutant strains were observed: accumulation of mRNA throughout the nucleus, accumulation of mRNA coincident with the DAPI-staining (DNA) portion of the nucleus, or discrete spots of accumulation of mRNA in the nucleus. These spots varied in number, size, and location in different mutant strains.

These aberrant localization patterns failed to cosegregate with temperature sensitivity or failed to segregate as a single lesion in half of the mutant strains that showed aberrant mRNA localization at the nonpermissive temperature. Seven alleles were identified that segregated 2 : 2 in a cross with a wild-type strain for both temperature sensitivity and the mRNA localization phenotype. In addition, with all seven of these strains, both temperature sensitivity and the RNA localization phenotypes were recessive and cosegregated in a cross with wild-type strains. With respect to the temperature-sensitive phenotype, these seven mutations fell into six complementation groups called RAT mutants.

Some strains displayed aberrant mRNA localization to a limited extent even at the permissive temperature. The *rat1-1* allele was chosen for further characterization because *rat1-1* cells displayed a wild-type phenotype at the permissive temperature and because 90% or more of the cells displayed an aberrant localization phenotype after 3 hr at the nonpermissive temperature.

#### *mRNA localization phenotype of rat1-1 strains*

Strains carrying the *rat1-1* allele have a normal distribution of mRNA at 23°C as shown in Figure 3, A and B. As early as 1 hr after a temperature shift to 37°C, 30–40% of the cells displayed a striking accumulation of mRNA in the nucleus and a concomitant decrease in cytoplasmic mRNA. By 3 hr, nearly all of the cells displayed this phenotype, though in varying intensities. The pattern of accumulation consisted of one to several large spots that surround and are distinct from the DAPI-staining region of the nucleus. This can be seen clearly in Figure 3, C and D, which shows *DAt1* × *t1* cells, a diploid homozygous for *rat1-1*, shifted to 37°C for 3 hr. Homozygous diploids were used for the experiments shown here because they are somewhat larger, but haploids were indistinguishable from the diploids in their RNA phenotype (data not shown). By 5 hr after the temperature shift, the intensity of the nuclear RNA signal had decreased and many cells were completely dark. This could indicate that in these strains transcription or polyadenylation eventually ceased and the nuclear poly(A)<sup>+</sup> RNA eventually turned over. It is also possible that poly(A)<sup>+</sup> RNA continued to be transported, but at a much reduced rate.

Despite the clonal nature of these cultures, cells carrying the *rat1-1* allele displayed the mRNA phenotype in an asynchronous manner. This led us to investigate whether appearance of the RNA localization phenotype would occur at a particular stage of the cell cycle. We examined this by cell cycle synchronization of a culture of strain *DAt1-2* (*MATa rat1-1*). At 30-min intervals following release of the culture from  $\alpha$ -mating factor arrest, cells were shifted to 37°C for 1 hr, fixed, and analyzed. At no time in the cell cycle did this strain show an increased fraction of cells displaying the RNA phenotype, as compared with a parallel asynchronous culture (data not shown).

To show that *rat1-1* strains have defects in the trafficking of RNA polymerase II-derived RNA, haploid strains that have both the *rat1-1* allele and the RNA polymerase II large subunit temperature-sensitive allele, *rpb1-1* (Nonet et al. 1987), were constructed. The *rpb1-1* allele blocked accumulation of poly(A)<sup>+</sup> RNA in the nucleus in the double mutant strain (Fig. 3E), showing that *rat1-1* strains have defects in the trafficking of mRNA.

#### *Growth characteristics of rat1-1 strains*

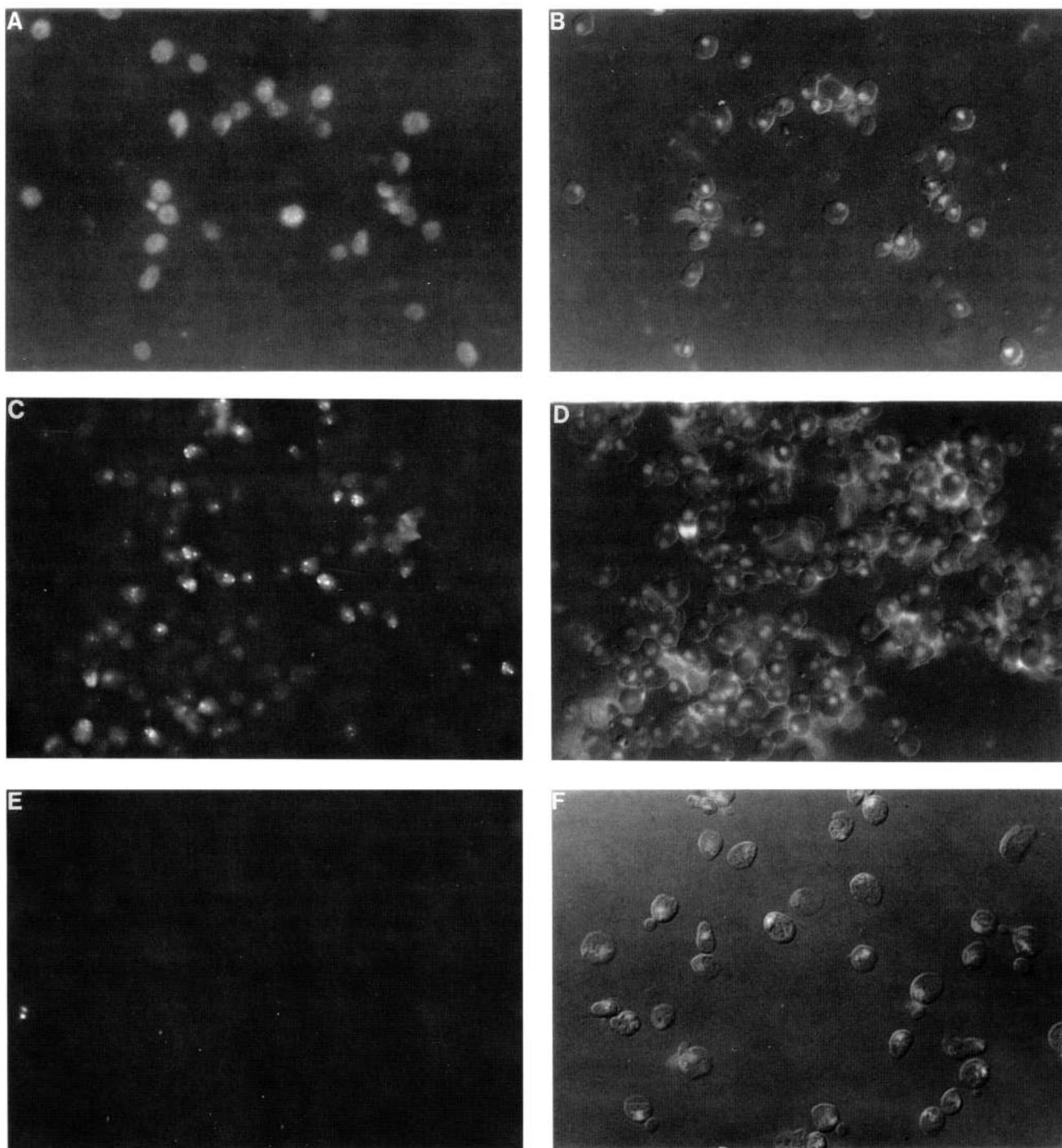
The growth rate of a *rat1-1* strain (*DAt1-1*) at 23°C and 37°C on rich media was compared with that of the parental wild-type strain (FY23) grown at 23°C on rich media (data not shown). At 23°C, both wild-type and *rat1-1* strains grew at a similar rate, doubling about every 3 hr. When shifted to 37°C, the *rat1-1* cells ceased division abruptly within 3 hr. This is the time at which the percentage of *rat1-1* cells showing nuclear accumulation of poly(A)<sup>+</sup> RNA was maximal. At the standard yeast growth temperature (30°C), *rat1-1* cells were barely able to form colonies. *rat1-1* cells remained viable for an extended time at 37°C. Even after 5 days at 37°C, 25% of the cells were still able to form colonies when returned to 23°C.

#### *RAT1 cloning, sequencing, and analysis*

The temperature sensitivity of a *rat1-1* strain (*DAt1-1*) was used to clone the *RAT1* gene by complementation. A library of yeast genomic DNA on a single-copy, *LEU2*-marked plasmid vector was transformed into strain *DAt1-1*. Cells were selected containing DNA that complemented the temperature sensitivity and leucine auxotrophy of *DAt1-1*. Plasmids were retrieved from 10 clones and amplified in *Escherichia coli*. Restriction endonuclease mapping indicated that all 10 shared restriction endonuclease digestion fragments and fell into two groups. The restriction maps of representative complementing clones pRAT1-2 and pRAT1-10 are shown in Figure 4. The clones overlap by ~5.5 kb. Subclones containing the indicated 4.8-kb *EcoRI* fragment of pRAT1-2 complemented both the temperature sensitivity and the aberrant RNA localization phenotype of the *rat1-1* strain.

We sequenced this 4.8-kb fragment containing the *RAT1* gene; the sequence of the *RAT1* gene is shown in

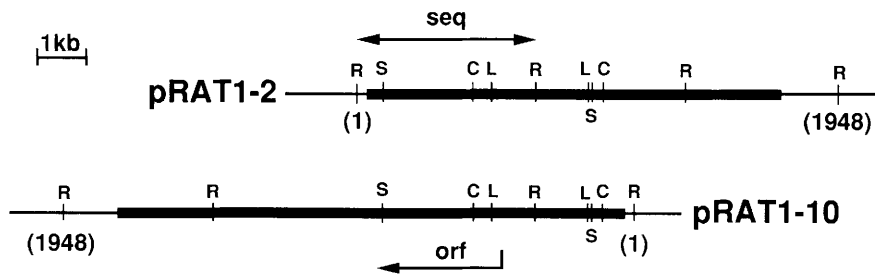
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**Figure 3.** *rat1-1* strains have inducible defects in mRNA trafficking. The mRNA localization assay was performed on *rat1-1* homozygous diploids that were grown continuously at 23°C or shifted to 37°C for 3 hr. (A) FITC fluorescence of strain DAT1 × t1 grown at 23°C. (B) The same field of cells as in A except visualized by DAPI fluorescence and simultaneous low-illumination DIC microscopy. (C) DAT1 × t1 cells grown at 37°C for 3 hr and visualized by FITC fluorescence. (D) The same field as C but visualized by DAPI fluorescence and simultaneous low-illumination DIC microscopy. The assay was also performed on *rat1-1/rpb1-1* strains. (E) FITC fluorescence of a *rat1-1/rpb1-1* double mutant shifted to 37°C for 2 hr. (F) The same field as E but visualized by DAPI fluorescence and simultaneous low-illumination DIC microscopy.

Figure 5. The sequence contains a large open reading frame (ORF) with the potential to encode a 1006-amino-acid protein of 116 kD. The putative initiator methionine codon is in a context for efficient translation initi-

ation in yeast (Donahue and Cigan 1990), and the codon bias is consistent with that seen in yeast proteins (Anderson and Kurland 1990). Except for a number of TATA boxes (boxed in Fig. 5) lying 5' of the initiator



sequence marked seq was subcloned from pRAT1-2 and sequenced. The position of the ORF found in the sequence is indicated as orf. Restriction endonuclease sites: (R) *EcoRI*; (S) *SacI*; (C) *SacII*; (L) *SalI*.

**Figure 4.** Restriction map of the inserts from the overlapping clones pRAT1-2 and pRAT1-10. The clones are displayed such that the overlapping sequences of the genomic inserts are aligned. The thin line represents vector sequences, and the numbers in parentheses are the positions of the two *EcoRI* sites in the parental vector p366 (P. Hieter, pers. comm.). The thick line represents genomic insert sequences. The

ATG, there are no obvious sequences that may play a role in transcription of the *RAT1* gene (Verdier 1990). The 3' end of the putative coding region has sequences important for splicing. There are sequences that match perfectly both the 5' splice site consensus sequence and the branchpoint TACTAAC (Green 1986). However, the first 3' splice site consensus sequence (CAG) is 95 nucleotides 3' of the branchpoint sequence. In most yeast genes that contain introns, the 3' splice site is usually located closer than this to the branchpoint sequence. A ribonuclease protection analysis with a probe that spanned the potential 5' splice site (nucleotide 2854) indicated that the *RAT1* mRNA was not spliced (data not shown). The sequences involved in 3'-end formation of mRNA in yeast are not well understood. The sequences TAG...TAGT or TATGT...TTT are believed to be involved in 3'-end formation (Zaret and Sherman 1982). These sequences have been observed in the 3' regions of many yeast genes and are found in the 3' end of *RAT1* (boxed).

The *RAT1* ORF does not contain known RNA-binding domains (Keene and Query 1991) nor does Rat1p contain sequence elements known from studies of other proteins to be important for specific functions (Devereux et al. 1984). Comparison of the *RAT1* ORF with itself by use of the REPEAT program (Devereux et al. 1984) revealed a cluster of small repeats in the hydrophilic carboxyl terminus. Two copies of the sequence SRYD, two copies of NNNY, and two copies of YSGN are found in the last 45 amino acids of the ORF.

Rat1p has homology to regions of Sep1p (Tishkoff et al. 1991). The *SEP1* gene is known alternatively as *DST2* (Dykstra et al. 1991), *KEM1* (Kim et al. 1990), *XRN1* (Larimer and Stevens 1990), and *RAR5* (Kipling et al. 1991). The Sep1p protein has DNA strand exchange activity (Dykstra et al. 1991; Tishkoff et al. 1991) and 5' → 3' exonuclease activity on both RNA (Larimer and Stevens 1990) and DNA (A. Johnson, pers. comm.). The best match of the Rat1p and Sep1p protein-coding sequences is shown in Figure 6. One block of homology is located near the amino termini of both proteins and is composed of seven short stretches of homology. These homologous sequences are separated from another cluster of homologous sequences by a region predicted in both proteins to be  $\alpha$ -helical (Chou and Fasman 1978; Garnier et al. 1978). The significance of these homologies is unknown

because the role that these sequences play in Sep1p activities is not known. The largest stretch of homology (amino acids 99–125 of Rat1p) contains many basic residues. This portion of Rat1p could be functioning as a nuclear localization signal or as part of a nucleic acid-binding domain. This sequence is followed immediately by a potential phosphorylation site in both Rat1p and Sep1p.

#### *RAT1* disruption and mapping

The requirement of *RAT1* for mitotic growth on glucose was determined by the standard method of gene replacement and plasmid rescue (Guthrie and Fink 1991). Nearly the entire *RAT1* ORF was removed and replaced with the *LEU2* gene. The resulting construct was integrated at one of the *RAT1* loci in a diploid formed by mating FY23 with FY86. The fidelity of the integration was confirmed by Southern analysis (data not shown). The resulting strain was transformed with pRAT1-18, a single-copy plasmid that contains the *RAT1* and *URA3* genes. Following sporulation, tetrads were dissected and allowed to germinate on rich media at 23°C. Cells were then replica-plated to plates either containing 5-fluoroorotic acid (5-FOA) or lacking leucine. Growth on 5-FOA selects against yeast cells that carry the *URA3* gene present on pRAT1-18 (Boeke et al. 1987). Growth on plates lacking leucine selects for cells that carry the *LEU2* gene insertion at the *RAT1* locus. All progeny that inherited the *LEU2* marker were unable to grow without the plasmid-borne copy of *RAT1*, indicating that *RAT1* is essential for mitotic growth of *S. cerevisiae*.

The chromosomal location of the *RAT1* sequence was determined by hybridizing labeled *RAT1* DNA to an ordered set of  $\lambda$  clones that covers most of the yeast genome (L. Riles and M.V. Olson, unpubl.). The *RAT1* sequence recognized two overlapping  $\lambda$  clones that map to a position ~15 cM from the centromere on the right arm of chromosome XV. This physical position of the cloned sequences agrees with the genetic mapping of the *rat1-1* allele, as shown in Table 2. Analysis of the segregation of *rat1-1* against *trp1 $\Delta$ 63* in a genetic cross indicated that the two genes were unlinked. *TRP1* is on chromosome IV. The percentage of second-division segregation of *rat1-1* from *trp1 $\Delta$ 63* was much lower than expected for non-centromere-linked genes and confirms a map posi-

1 ATTTTACTCGATTTTTATAGTATACTGAAACAAGTAGTTGTAAAAATCAATTCACCAAAAAAGCTTTCCCATCTCTGTATGAAAACTACGAAAAAGTTTACTATCA  
106 CATAATATCGGAAGTACGTTTTTAAGGCACCTGCAATGTATTATATACTTTGAACATTTGGAGGATATATACCTTACTTTCTCATCAGGTTTATATATTTGAGCGT  
AvaI (211) XbaI (242)  
211 CTCGGGAATGATAAGTCGGGTAAACGAGATCTAGATAAGATTTTCAGCTATGGCAATAAAAAACCAACCTCATCGATGAACCTATTACAACATAAAGACATC  
316 CCGTAATAATTTGTGAGACAGGTTCTCAAGGAAGTGCTACAGCCAAGTTTCGTAAATATA ATG GGT GTT CCG TCA TTT TTC AGA TGG CTA TCT  
Met Gly Val Pro Ser Phe Phe Arg Trp Leu Ser  
Sall(460)  
409 CGA AAA TAT CCA AAG ATC ATA TCC CCA GTA TTG GAA GAG CAA CCT CAG ATA GTC GAC GGT GTC ATA TTG CCG TTG GAT  
12 Arg Lys Tyr Pro Lys Ile Ile Ser Pro Val Leu Glu Glu Gln Pro Gln Ile Val Asp Gly Val Ile Leu Pro Leu Asp  
487 TAT TCC GCC TCA AAT CCA AAC GGT GAG TTA GAC AAC TTG TAT TTG GAT ATG AAT GGT ATT GTT CAT CCA TGT TCG CAC  
38 Tyr Ser Ala Ser Asn Pro Asn Gly Glu Leu Asp Asn Leu Tyr Leu Asp Met Asn Gly Ile Val His Pro Cys Ser His  
565 CCA GAA AAC AAA CCG CCT CCA GAG ACT GAA GAT Met Ala Val Asp CAA ATG CTT TTG GCA GTT TTT GAA TAT ACC AAT CGT GTG TTA AAT  
64 Pro Glu Asn Lys Pro Pro Pro Glu Thr Glu Asp Glu Met Leu Leu Ala Val Phe Glu Tyr Thr Asn Arg Val Leu Asn  
643 ATG GCT AGA CCA CGC AAG GTA CTT GTT ATG GCT GTT GAT GGT GTT GCT CCT CGT GCC AAA ATG AAC CAG CAG AGA GCG  
90 Met Ala Arg Pro Arg Lys Val Leu Val Met Ala Val Asp Gly Val Ala Pro Arg Ala Lys Met Asn Gln Gln Arg Ala  
721 CGT AGA TTT AGG AGT GCT AGG GAT GCT CAG ATT GAA AAC GAA GCC ARG GAA GAA ATT ATG AGA CAG CGA GAA GAA GTT  
116 Arg Arg Phe Arg Ser Ala Arg Asp Ala Gln Ile Glu Asn Glu Ala Arg Glu Ile Met Arg Gln Arg Glu Glu Val  
799 GGT GAG ATA ATC GAT GAT GCC GTC AGG AAC AAG AAA ACT TGG GAT TCA AAT GCG ATC ACT CCA GGC ACC CCA TTT ATG  
142 Gly Glu Ile Ile Asp Asp Ala Val Arg Asn Lys Lys Thr Trp Asp Ser Asn Ala Ile Thr Pro Gly Thr Pro Phe Met  
SacII (887) PstI (946)  
877 GAT AAG CTT GCC GCG GCC CTC CGT TAT TGG ACA GCA TTT AAA CTG GCC ACT GAC CCT GGT TGG AAA AAC CTG CAG GTG  
168 Asp Lys Leu Ala Ala Ala Leu Arg Tyr Trp Thr Ala Phe Lys Leu Ala Thr Asp Pro Gly Trp Lys Asn Leu Gln Val  
955 ATT ATC AGT GAC GCC ACC GTC CCA GGT GAA GGT GAG CAC AAA ATA ATG AAT TTC ATT AGG TCT CAA AGA GCT GAT CCT  
194 Ile Ile Ser Asp Ala Thr Val Pro Gly Glu Gly His Lys Ile Met Asn Phe Ile Arg Ser Gln Arg Ala Asp Pro  
1033 GAA TAT AAT CCC AAC ACA ACG CAC TGT ATA TAC GGT TTG GAT GCT TTA ATT TTT TTG GCG CTT GCC ACT CAG GAA  
220 Glu Tyr Asn Pro Asn Thr Thr His Cys Ile Tyr Gly Leu Asp Ala Asp Leu Ile Phe Leu Gly Leu Ala Thr His Glu  
1111 CCA CAT TTT AAA ATT CTA AGA GAA GAT TTT TTT GCT CAA GAC AAT CCG AAA AGA AAT AAC TTA AAA GAT ACT ATA AAT  
246 Pro His Phe Lys Ile Leu Arg Glu Asp Val Phe Ala Gln Asp Asn Arg Lys Arg Asn Asn Leu Lys Asp Thr Ile Asn  
1189 ATG ACT GAA GAA GAA AAA CAA TTT CTC CAA AAA CAA AAT TCC GAA CAA CCT TTC TTG TGG CTG CAC ATA AAT GTT TTG  
272 Met Thr Glu Glu Glu Lys Gln Phe Leu Gln Lys Gln Asn Ser Glu Gln Pro Phe Leu Trp Leu His Ile Asn Val Leu  
1267 AGG GAA TAC TTG TCC GCT GAG TTA TGG GTT CCG GGT CTT CCA TTT ACA TTT GAT TTA GAA AGG GCT ATC GAC GAT TGG  
298 Arg Glu Tyr Leu Ser Ala Glu Leu Trp Val Pro Gly Leu Pro Phe Thr Phe Asp Leu Arg Ala Ile Asp Asp Trp  
1345 GTT TTC ATG TGC TTT TTC TGT GGT AAT GAC TTT TTA CCA CAT TTA CCA TGC TTA GAT GTC AGA GAA AAT AGT ATT GAT  
324 Val Phe Met Cys Phe Phe Cys Gly Asn Asp Phe Leu Pro His Leu Pro Cys Leu Asp Val Arg Glu Asn Ser Ile Asp  
ScaI (1446)  
1423 ATT CTC TTG GAT ATT TGG AAG GTA GTA CTA CCG AAA TTG AAA ACG TAC ATG ACC TGC GAC GGT GTC TTG AAC CTC CCT  
350 Ile Leu Leu Asp Ile Trp Lys Val Val Leu Pro Lys Leu Lys Thr Tyr Met Thr Cys Asp Gly Val Leu Asn Leu Met Pro  
1501 TCT GTT GAA ACG CTA TTG CAA CAC CTT GGC TCC CGT GAG GGG GAT ATC TTC AAA ACT AGA CAT ATT CAA GAA GCC AGA  
376 Ser Val Glu Thr Leu Leu Gln His Leu Gly Ser Arg Glu Gly Asp Ile Phe Lys Thr Arg His Ile Gln Glu Ala Arg  
1579 AAG AAA GAA GCT TTT GAA AGA AGG AAA GCG CAG AAA AAT ATG TCA AAA GGT CAA GAC AGG CAT CCT ACA GTA GCT ACG  
402 Lys Lys Glu Ala Phe Glu Arg Arg Lys Ala Gln Lys Asn Met Ser Lys Gly Gln Asp Arg His Pro Thr Val Ala Thr  
1657 GAG CAA CTG CAA ATG TAT GAC ACA CAA GAA AAT TTA GCA AAA GGT TCA TGG AAC TTT ACC ACC AGT GAT ATG GTA AGG  
428 Glu Gln Leu Gln Met Tyr Asp Thr Gln Gly Asn Leu Ala Lys Gly Ser Trp Asn Leu Thr Thr Ser Asp Met Val Arg  
1735 TTG AAA AAG GAA CTG ATG CTT GCG AAC GAG GGG AAC GAA GCA ATC GCC AAA GTT AAG CAA CAG AGT GAT AAG AAC  
454 Leu Lys Lys Glu Leu Met Leu Ala Asn Glu Gly Asn Glu Glu Ala Ile Ala Lys Val Lys Gln Gln Ser Asp Lys Asn  
1813 AAT GAA CTG ATG AAA GAT ATC AGC AAG GAA GAA ATT GAT GAT GCT GTT AGT AAG GCT AAC AAA ACT AAT TTT AAC CTT  
480 Asn Glu Leu Met Lys Asp Ile Ser Lys Glu Glu Ile Asp Asp Ala Val Ser Lys Ala Asn Lys Thr Asn Phe Asn Leu  
1891 GCA GAA GTT ATG AAA CAG AAA ATA ATA AAT AAG AAA CAC CGT CTT GAA AAG GAT AAT GAA GAG GAA GAA ATT GCT AAG  
506 Ala Glu Val Met Lys Gln Lys Ile Ile Asn Lys Lys His Arg Leu Glu Lys Asp Asn Glu Glu Glu Ile Ala Lys  
1969 GAT AGC AAA AAA GTT AAG ACA GAG AAA GCG GAA TCA GAG TGC GAT CTC GAT GCA GAG ATC AAA GAT GAT ATT GTG GCT  
532 Asp Ser Lys Lys Val Lys Thr Glu Lys Ala Glu Ser Glu Cys Asp Leu Asp Ala Glu Ile Lys Asp Glu Ile Val Ala  
2047 GAC GTA AAC GAC AGG GAA AAC TCT GAA ACT ACA GAA GTA TCA AGA GAT TCA CCA GTC CAC AGC ACA GTG AAT GTC AGC  
558 Asp Val Asn Asp Arg Glu Asn Ser Glu Thr Thr Glu Val Ser Arg Asp Ser Pro Val His Ser Thr Val Asn Val Ser  
BstEII (2182)  
2125 GAA GGT CCC AAA AAT GGG GTC TTT GAT ACA GAT GAA TTT GTC AAA CTA TTC GAA CCT GGT TAC CAC GAA AGA TAT TAC  
584 Glu Gly Pro Lys Asn Gly Val Phe Asp Thr Asp Glu Phe Val Lys Leu Phe Glu Pro Gly Tyr His Glu Arg Tyr Trp  
2203 ACA GCT AAA TTT CAC GTT ACT CCT CAA GAT ATT GAA CAG TTA AGA AAA GAT ATG GTA AAA TGC TAT ATC GAA GGG GTC  
610 Thr Ala Lys Phe His Val Thr Pro Gln Asp Ile Glu Gln Leu Arg Lys Asp Met Val Lys Cys Tyr Ile Glu Gly Val  
2281 GCC TGG GTA CTA ATG TAT TAC TAT CAA GGT TGT GCC TCT TGG AAT TGG TTC TAT CCG TAC CAT TAT CCT CTT GCA  
636 Ala Trp Val Leu Met Tyr Tyr Tyr Gln Gly Cys Ala Ser Trp Asn Trp Phe Tyr Pro Tyr His Tyr Ala Pro Leu Ala  
NcoI (2367)  
2359 ACA GAT TTC CAT GGG TTT TCT CAC TTG GAG ATA AAG TTC GAA GAA GGT ACT CCA TTT TTA CCT TAC GAA CAA TTG ATG  
662 Trp Asp Phe His Gly Phe Ser His Leu Glu Ile Lys Phe Glu Gly Thr Pro Phe Leu Pro Tyr Glu Gln Leu Met  
2437 AGT GTT TTA CCA GCT GCA TCT GGC CAT GCT TTG CCA AAA ATT TTC CGG TCT TTG ATG TCT GAA CCT GAC TCT GAA ATC  
688 Ser Val Leu Pro Ala Ala Ser Gly His Ala Leu Pro Lys Ile Phe Arg Ser Leu Met Ser Glu Pro Asp Ser Glu Ile  
2515 ATT GAT TTT TAT CCT GAA GAA TTT CCC ATA GAT ATG AAT GGT AAG AAG ATG TCC TGG CAA GGC ATA GCG TTG CTA CCA  
714 Ile Asp Phe Tyr Pro Glu Glu Phe Pro Ile Asp Met Asn Gly Lys Lys Met Ser Trp Gln Gly Ile Ala Leu Leu Pro  
2593 TTC ATC GAT CAG GAT AGA CTG TTA ACA GCT GTT CGT GCA CAA TAT CCT TTA CTT TCT GAT GCT GAG AGG GCA AGA AAT  
740 Phe Ile Asp Gln Asp Arg Leu Thr Ala Val Arg Ala Gln Tyr Pro Leu Leu Ser Asp Ala Glu Arg Ala Arg Asn  
2671 ATT CGC GGA GAA CCT GTT TTA TTA ATA AGT AAC AAG AAT GCT AAT TAT GAG AGG TTT TCG AAG AAA TTA TAC TCA AAG  
766 Ile Arg Gly Glu Pro Val Leu Leu Ile Ser Asn Lys Asn Ala Asn Tyr Glu Arg Phe Ser Lys Lys Leu Tyr Ser Lys  
2749 GAA AAC AAC AAC AAT AAT GTT GTT GTG AAG TTT CAA CAC TTC AAG AGT GGT TTA AGT GGT ATT GTA TCA AAG GAC GTT  
792 Glu Asn Asn Asn Asn Val Val Val Lys Phe Gln His Phe Lys Ser Gly Leu Ser Gly Ile Val Ser Lys Asp Val  
2827 GAA GGT TTC GAA TTA AAT GGG AAA ATT GTA TGT CCG ATT CAA GGT GGG TCA CTG CCA AAT TTA TCT ACC ACC TTG ATT  
818 Glu Gly Phe Glu Leu Asn Gly Lys Ile Val Cys Pro Ile Gln Gly Gly Ser Leu Pro Asn Leu Ser Thr Thr Leu Ile  
2905 TTA AAG ATG TCC TAT AGA TTA ATT CCG TTG CCA TCG AGA AAT AAG TCT ATT ATA CTG AAC GGG TTC ATT CCG TCA GAA  
844 Leu Lys Met Ser Tyr Arg Leu Pro Leu Pro Ser Arg Lys Ser Ile Ile Leu Asn Gly Phe Ile Pro Ser Glu  
NdeI (2996)  
2983 CCG TTA CTA ACA GCA TAT GAC CTT GAT TCC ATA ATG TAC AAG TAT AAT AAC CAA AAT TAC TCC CGT CGC TGG AAC TTT  
870 Pro Val Leu Thr Ala Tyr Asp Leu Asp Ser Ile Met Tyr Lys Tyr Asn Asn Gln Asn Tyr Ser Arg Arg Trp Asn Phe  
3061 GGT AAT GAT TTG AAG CAA AAT ATT GTT CCA GTA GGA CCA AAG GGT ATT ACT CAG TAC AAA CCA AGA ACT GGT GGC TAT  
896 Gly Asn Asp Lys Gln Asn Ile Val Pro Val Gly Pro Lys Gly Ile Thr Gln Tyr Lys Pro Arg Thr Gly Tyr  
SacI (3160)  
3139 CGA GCA TTC TTT TAT TTT GCT GAG CTC AGT AGA AAT AAT GTC CAA CCC GCC CAC AAC TAT GGT AGG AAT AGC TAC AAC  
922 Arg Ala Phe Phe Tyr Phe Ala Glu Leu Ser Arg Asn Asn Val Gln Pro Ala His Asn Tyr Gly Arg Asn Ser Tyr Asn  
3217 AGT CAA CCC GGC TTC AAC AAT AGC AGG TAT GAT GGT GGA AAC AAT AAT TAC AGA CAA AAT TCA AAT TAC AGG AAC AAT  
948 Ser Gln Pro Gly Phe Asn Asn Ser Arg Tyr Asp Gly Gly Asn Asn Tyr Arg Gln Asn Ser Asn Tyr Arg Asn Asn  
3295 AAC TAT TCC GGA AAT AGA AAT AGT GGA CAG TAC AGC GGC AAC AGC TAC TCT CGG AAT AAC AAG CAA AGT CCG TAT GAC  
974 Asn Tyr Ser Gly Asn Arg Asn Ser Gly Gln Tyr Ser Gly Asn Ser Tyr Ser Arg Asn Asn Lys Gln Ser Arg Tyr Asp  
3373 AAT TCA AGA GCA AAT AGG CGT TAG  
1000 Asn Ser Arg Ala Asn Arg Arg \*\*\*  
3397 AACTACTCGTGCACATTTTATTTATGGTAAATTTACGTTTTCGCAAAATTTATAAAATCTTCATACAATTAATAAAAAAGCTCTTTAAGATAATAGTGTCTGCTC  
3502 TTACAACGACGACAGTAATTTTATAACAAAAATGGAACATATAAAGTAAAGAGAAAACTCTGTAAGCAATGAATATATGAAATATACGTCCTCTGTAA  
3607 ATAGCGAAATCCCTGTGAAGCCAATAGACCGAACTCTATTTTGATTTATACGTTCTGATTTTTCTCTA

Figure 5. (See facing page for legend.)



RAT1	1	MGVPSFFRWLSRKYPKIISVPLEEQPIQVDGVILPLDYSASNPGELDNL	50
SEP1	1	MGIPKFFRYISERWPMIL.....QLIEGTQIP.....EFDNL	32
RAT1	51	YLDMNGIVHPCSHPENKPPPE..TEDEMLLAVFEYTNRVLNMRPRKVLV	98
SEP1	33	YLDMNSILHNCTHGNDVDTKRLTEEEVFAKICTYIDHLFQTIKPKKIFY	82
RAT1	99	MAVDGVAPRAKMNQQRARRFRSARDAQIENEAREEIMRQREVEGEIIDA	148
SEP1	83	MAIDGVAPRAKMNQQRARRFRRTAMDA.....EKALKKAIENG....DE	121
RAT1	149	VRNKKTWDSNAITPGTFPMKLAALRYWTAFKPLATDPGWKNLQVVISDA	198
SEP1	122	IPKGEFFDSNSITPGTEFMAKLTKNLQYFIHDKISNDSKWRVQIIFSGH	171
RAT1	199	TVPGEGEHKIMNFIRSQRADPEYNPNTHHCYGLDADLIFLGLATHEPHF	248
SEP1	172	EVPGEGEHKIMNFIHRLKSQKDFNQNRHCHYGLDADLIMLGLSTHGPHF	221
RAT1	249	KILREDVFAQDNRRKNRLKDTINMTEEEKQFLQKQNSEQPFPLWLHINVL	298
SEP1	222	ALLREEV.....TFGRNNEKKSLEHQN....FYLLHLSLLR	254
RAT1	299	EYLSAELW..VPLPPTFDLERAIDDDVVMCFGCGNDFLPHLPCL....	
SEP1	255	EYMELEFKEIADEMDFEYNFERILDDFLLVMFVIGNDFLNLPLD....	
RAT1		.....EQLRKMVKCYIEGVAWVIMYYQGC	646
SEP1		.....EKVR.DLAKDYVEGLQWVLYYYRGC	552
RAT1	647	ASWNWFYPHYAPLATDFHGFHSLEIKFEEGTPFLPYEQLMVLPAAAGH	696
SEP1	553	PSWSWYYPHYAPRISDLAKGLDQDIEFDLSKFFTPFQQLMAVLPERSKN	602
RAT1	697	ALPKIFRSLMSEPDSEIIDFYPEEFPIDMNGKMM.....	1006
SEP1	603	LIPPAFRPLMYDQSEIHDYPAEYQVLDKNGKTA.....	1528

**Figure 6.** Optimal alignment of homologous sequences of Rat1p and Sep1p. Amino acid positions are indicated at the left and right of each strand. Vertical bars indicate identity, while colons indicate conservative substitutions. The series of periods represent either gaps introduced in the Sep1p sequence for optimal alignment or, where periods appear at the same positions in both sequences, nonhomologous sequences that separate the two homologous regions of the proteins.

tion for *rat1-1* at 15 cM from a centromere. In addition, the *rat1-1* allele was found to be linked genetically to an *ade2* allele that maps to the right arm of chromosome XV (Mortimer et al. 1989). Furthermore, the *rat1-1* allele was found to be tightly linked to the *TMP1/CDC21* locus, as determined by segregation patterns in a cross with a strain bearing a *LEU2* insertion adjacent to the *TMP1* locus (Ord et al. 1988). Examination of 55 tetrads from this cross indicated that the *rat1-1* allele is 30 cM from the *LEU2* insertion at *TMP1*. These genetic analyses confirm the identity of the cloned sequence as encoding the wild-type form of the gene that is defective in strains bearing the *rat1-1* allele and confirms the physical position of the *RAT1* sequence.

**Figure 5.** Sequence of the *RAT1* gene. The sequence of the coding strand of the putative *RAT1* gene is shown. The protein sequence of the large ORF is shown directly below the DNA sequence. Positions in both the DNA and protein sequences are indicated at the left of the sequences. At the 5' end are potential TATA sequences (boxed). The box at position 2854 of the DNA sequence is a potential 5' donor splice site sequence. The next boxed sequence is a possible branchpoint sequence for a potential intron of the mRNA. The next two underlined sequences are potential but unlikely 3' acceptor splice sites, whereas the next boxed sequence could serve as an efficient 3' splice site. The three boxed sequences at the very 3' end of the sequence comprise a potential polyadenylation signal.

**Table 2.** Meiotic segregation of *rat1-1*

Test allele	PD	NPD	T	Percent	
				SDS	Distance
<i>trp1Δ1</i>	15	18	15	31%	~15 cM <sup>a</sup>
<i>ade2</i>	10	4	12	—	linked
<i>TMP1::LEU2</i>	22	0	33	—	~30 cM

(PD) Parental ditype; (NPD) nonparental ditype; (T) tetratype; (SDS) second division segregation.

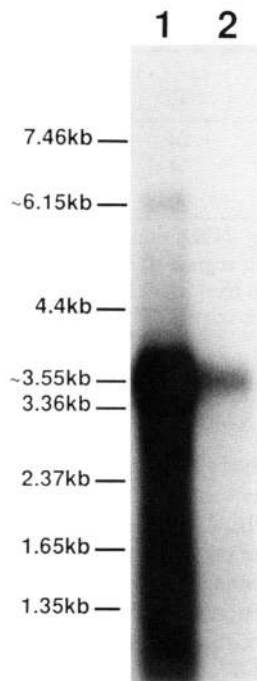
<sup>a</sup>Distance reported is from the centromere of chromosome XV as calculated from percent SDS between *rat1-1* and *trp1Δ63*.

### Expression of the *RAT1* gene

Northern blot analysis of poly(A)<sup>+</sup> mRNA indicated that the *RAT1* gene was transcribed into an abundant message of ~3.5 kb (Fig. 7). The abundance of this message was increased dramatically by the overexpression of the *RAT1* genomic locus on a high copy vector (plasmid p6A), and an additional mRNA of 6.2 kb was also detected. By overexposure of the autoradiogram (data not shown), this larger RNA was also detectable when *RAT1* was not overexpressed.

### RNA processing defects in *rat1-1* strains

The only previously identified mutant with mRNA trafficking defects, *rna1-1*, has pleiotropic defects in both tRNA and rRNA processing (Hopper et al. 1978) but not in mRNA splicing (Rosbash et al. 1981). It is possible that mRNA trafficking is integrated with steps of RNA processing. We therefore examined *rat1-1* strains for RNA processing defects. We followed an approach similar to that of Hopper et al. (1978) to examine the fidelity of tRNA and small rRNA processing. Wild-type, *rna1-1*, and *rat1-1* strains were pulse-labeled for 1 hr with <sup>32</sup>P<sub>4</sub>, either before or after a shift to 37°C. RNA extracted from these cells was analyzed by electrophoresis on a 3–15% acrylamide/urea gradient gel (Fig. 8A). The major species of small RNA molecules detectable in this manner are identified along the left side of Figure 8A. Only mature forms of these RNA molecules were visible in wild-type strains grown either continuously at 23°C (lane 1), or shifted to 37°C for 2 (lane 2) or 3 hr (lane 3). Lane 4 shows an *rna1-1* strain grown for 1 hr at 37°C. The accumulation of precursor tRNA molecules is quite evident, and accumulation of 5.8S rRNA was reduced relative to the other species of RNA detectable in this experiment. In comparison, the *rat1-1* strain grown at 23°C (lane 5) or shifted to 37°C for 2 (lane 6) or 3 hr (lane 7), showed no apparent accumulation of tRNA precursors. However,



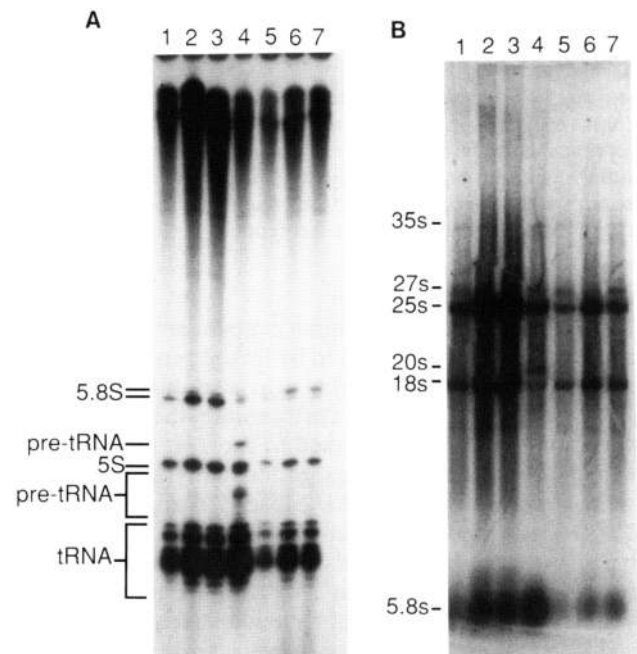
**Figure 7.** Northern analysis of *RAT1* mRNA. Poly(A)<sup>+</sup> RNA was separated on a 1% agarose/formaldehyde gel. The positions of size markers are indicated at left. (Lane 1) RNA isolated from strain FY23 carrying plasmid p6A; (lane 2) RNA (10  $\mu$ g) from strain FY23. The probe was a 605-bp *HincII* fragment from pRAT1 = 18.

processing of the 5.8S rRNA was clearly affected in a temperature-sensitive manner. The 5.8S rRNA in yeast is found in two forms, one shorter at its 5' end by 7 nucleotides (L. Lindahl, pers. comm.). The manner in which these two forms of the 5.8S rRNA are produced during processing of the large ribosomal gene transcript and the functional importance of the two forms are unknown. In wild-type or *rat1-1* cells at 23°C, the smaller form predominated. Upon shift of *rat1-1* cells to 37°C, this pattern inverted and the larger form accumulated. This is not a general secondary defect resulting from mRNA export defects because *rna1-1* cells have a normal distribution of the two forms of 5.8S rRNA.

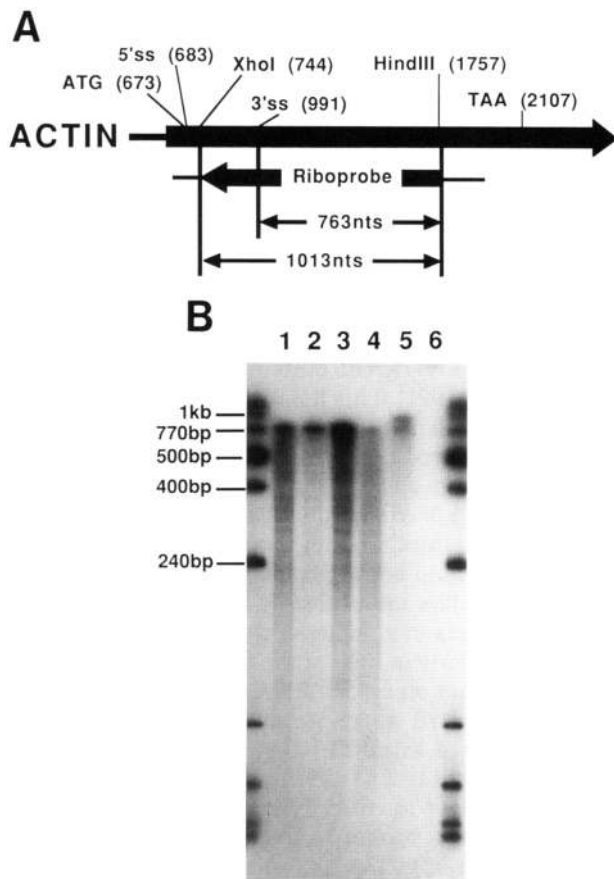
The same RNA samples were used to examine processing of the large rRNA molecules by electrophoresis on a 1% agarose/TBE gel (Fig. 8B). No obvious defects in large rRNA processing were detectable in the *rat1-1* strain, indicating that the defects in 5.8S rRNA processing were not the result of problems in its excision from a larger precursor.

We also examined whether *rat1-1* strains had splicing defects. In *S. cerevisiae*, mutations such as *prp2-1* that block splicing at a step after initiation of splicing complex formation prevent export of intron-containing mRNAs (Legrain and Rosbash 1989). Splicing of actin pre-mRNA was examined by RNase protection analysis. RNA was isolated from wild-type, *prp2*, and *rat1-1* strains. The construct from which the probe was synthesized is shown in Figure 9A. The probe spans the 3'

splice site. Hybridization to actin pre-mRNA should protect 1013 nucleotides of the probe, whereas hybridization to spliced actin mRNA should protect 763 nucleotides. The results are shown in Figure 9B. Heat shock treatment is also known to block splicing. Heat shock treatment of a wild-type strain is shown in lane 2. This treatment resulted in a slight but detectable accumulation of precursor; in many strains, this treatment produces a more dramatic effect on splicing (Yost and Lindquist 1991). Perhaps the FY23 and FY86 strains are more heat resistant than the strains used in those studies. In a *prp2* mutant strain shifted to 37°C for 1 hr, actin pre-mRNA accumulated, as expected (Last et al. 1984). RNA from a *rat1-1* strain grown at 23°C (lane 3), or shifted to 37°C for 2 hr (lane 4), appeared normal with respect to the splicing of actin pre-mRNA. Unspliced actin pre-mRNA could not be detected, even with much longer exposures of this gel (data not shown). Interestingly, there was much less total actin message in *rat1-1* cells shifted to 37°C. We do not know the cause of this reduction in actin mRNA levels, but it would not be surprising if mRNA trafficking mutants had secondary defects in other cellular processes that influence transcript abundance.



**Figure 8.** *rat1-1* strains have no tRNA processing defects but do have a 5.8S rRNA processing defect. RNA labeled with <sup>32</sup>P<sub>4</sub> was isolated from various strains, as described above, and analyzed in a 3–15% acrylamide/50% urea gel (A) or in a 1% agarose/TBE gel (B). The positions at which various species of RNA and their precursors migrated are indicated at left. (Lanes 1–3) FY23; (lanes 4) 2b × 3b (*rna1-1*); (lanes 5–7) DA1-1 (*rat1-1*). (Lanes 1,5) Cells grown at 23°C; (lanes 4) cells shifted to 37°C for 1 hr; (lanes 2,6) cells shifted to 37°C for 2 hr; (lanes 3,7) cells shifted to 37°C for 3 hr.



**Figure 9.** *rat1-1* strains have no actin pre-mRNA splicing defects. Total RNA was isolated from yeast strains and hybridized to an actin specific riboprobe. (A) Structure of the actin riboprobe. The top line represents the actin gene, with the pre-mRNA sequences indicated by the thick line and noncoding sequences indicated by the thin line. The positions of restriction sites, the start and stop codons, as well as the 5' and 3' splice sites, are shown at the top. The structure of the riboprobe is shown below. The thick line shows the complementary sequence; the thin line shows flanking vector sequences. The double-headed arrows indicate the expected sizes of probe molecules protected by the spliced or unspliced products. (B) Probe was hybridized to various RNAs, and the protected probe fragments were examined on a 5% acrylamide/50% urea gel. The sizes of the DNA markers are shown at left. (Lane 1) FY23 (wild-type) grown at 23°C, (lane 2) FY23 grown at 42°C for 1 hr; (lane 3) DA1-1 (*rat1-1*) grown at 23°C; (lane 4) DA1-1 grown at 37°C for 3 hr; (lane 5) ts368 (*prp2*) grown at 37°C for 1 hr; (lane 6) no RNA.

## Discussion

The adaptation of in situ hybridization to localize poly(A)<sup>+</sup> RNA in *S. cerevisiae* has permitted us to identify seven recessive conditional mutants in six complementation groups that showed intranuclear accumulation of poly(A)<sup>+</sup> RNA at the nonpermissive temperature of 37°C. Because we failed to identify each complementation group more than once, further screening should

identify many more genes. The efficient execution of mRNA trafficking could require the integrity of a large number of gene products, some of which may be involved only indirectly.

*What types of mutant genes might be identified using the in situ hybridization assay?*

Efficient and accurate mRNA trafficking likely depends on the integrity of the nucleoskeleton. The nuclear matrix, a meshwork of thick polymorphic fibers apparently underlaid by a meshwork of intermediate-sized core filaments (Jackson and Cook 1988; He et al. 1990), probably consists of elements of this nucleoskeleton. Actively transcribed chromatin is associated with the matrix (Robinson et al. 1982; Ross et al. 1982; Ciejek et al. 1983; Hentzen et al. 1984; Small et al. 1985; Thorburn et al. 1988), and it appears to be the substratum upon which heterogeneous nuclear RNA (hnRNA) processing occurs (Zeitlin et al. 1987, 1989). mRNA and pre-mRNA are tightly bound to structures within the eukaryotic nucleus (Zeitlin et al. 1987, 1989) and move along "tracks" from sites of synthesis in the nuclear interior to the periphery (Lawrence et al. 1989; Huang and Spector 1991; Xing and Lawrence 1991), possibly in association with fibers of the nucleoskeleton. Also likely to affect mRNA trafficking are mutations altering structural components of the nucleoskeleton, gene products involved in the organization and stabilization of this structure, proteins that attach mRNA to this nucleoskeleton, and proteins that mediate movement of RNA along the nucleoskeleton. The screen we have developed should be capable of identifying mutations in such genes.

Our screen may also identify gene products involved in transcription, 3'-end formation, splicing, or the obligatory coupling of these processes to subsequent trafficking steps. The ability to visualize Epstein-Barr virus (EBV) RNA in a track extending from the site of synthesis, where the EBV genome is integrated, to the nuclear envelope depends on ongoing transcription. When transcription is inhibited, EBV RNA made prior to inhibition of transcription, but not yet exported from the nucleus, can be detected only near its site of synthesis and no longer in a track extending to the nuclear periphery (Lawrence et al. 1989). RNA processing may also be coupled to trafficking. Experiments conducted by Birnstiel and colleagues indicate that histone mRNA trafficking is linked to 3'-end formation (Eckner et al. 1991), and several lines of investigation indicate a link between splicing and mRNA export (Chang and Sharp 1989; Felber et al. 1989; Legrain and Rosbash 1989).

Proteins that recognize structural elements of mRNA may play roles in mRNA trafficking. A nuclear protein that binds mRNA caps has been described (Ohno et al. 1990). The other obvious structural element of mRNA that may be a determinant for mRNA trafficking is the poly(A) tail. Receptors for poly(A) have been found in rat liver nuclear envelopes (Schroder et al. 1987). It is also possible that hnRNP proteins play an essential role in

mRNA trafficking and their genes might be identified by using this assay (Dreyfuss 1986).

The NPC is a complex structure estimated to be made from 100–200 distinct polypeptides in metazoan cells, with an overall mass of ~124 mD (Reichelt et al. 1990). The structures of the yeast and metazoan NPCs are similar (Allen and Douglas 1989). Although they have been studied intensively for many years, only a few nuclear pore proteins have been identified. Immunological approaches have been limited by the existence of dominant epitopes on a few NPC proteins. Components of the NPC, proteins that anchor and distribute the pore in the nuclear envelope, and proteins that mediate its attachment to the nucleolar or cytoskeleton constitute another group of gene products that we hope to identify with the assay.

Because the screen was developed by using a yeast strain mutant for Rna1p, a cytoplasmic protein, it should be possible to identify genes encoding other cytoplasmic proteins required for mRNA export. The biochemical basis for the Rna1p defect is not known but may be the result of a loss of attachment of the cytoskeleton to the nuclear pore, an inability to dissociate mRNA from the pore on its cytoplasmic side, or perhaps a loss in the ability of mRNA to associate with the cytoskeleton. Whether the integrity of the cytoskeleton is required for the export of mRNA is unknown, but it will be possible to address this with the methodology described in this paper.

#### *Possible and actual phenotypes of mutants with defects in mRNA trafficking*

In our in situ hybridization screen, we observed three general classes of aberrant mRNA localization: (1) poly(A)<sup>+</sup> RNA localized throughout the nucleus, indicating possible loss of attachment of RNA to nuclear structures; (2) poly(A)<sup>+</sup> RNA localized coincident with the DAPI-staining chromatin, possibly indicating defects early in the trafficking pathway; and (3) RNA localized to the nuclear periphery, indicating defects at later stages of intranuclear trafficking. *rat1-1* strains had from one to several large nuclear spots of poly(A)<sup>+</sup> RNA not coincident with the chromatin. This pattern contrasts with that seen in several other RAT mutant strains which showed different numbers and sizes of spots of peripheral accumulation of poly(A)<sup>+</sup> RNA. Because the fixation conditions used in our experiments were harsh, it would be unwise to assume that these localization patterns are identical to the actual patterns of mRNA accumulation in vivo. Answering these questions will require milder fixation conditions and more sophisticated imaging technology.

Some of the mutants identified by our screen displayed interesting pleiotropic defects. For example, three mutants in two complementation groups showed clustering of nuclear pores in the nuclear envelope and one of these strains had difficulty progressing through karyokinesis (D.C. Amberg, C. Copeland, M. Snyder, and C.N. Cole, in prep.). Another mutant had defects in segregating the

daughter nucleus to the bud, implicating defects in the nuclear envelope or the cytoskeleton (D.C. Amberg and C.N. Cole, unpubl.). Characterization of these mutants and the genes that encode the defective proteins is in progress and should shed more light on the mechanism of mRNA trafficking in yeast.

#### *The RAT1 gene and possible functions of the RAT1 protein*

The *RAT1* gene encodes a protein of 116 kD that bears strong resemblance (Fig. 6) to portions of the *SEP1/DST2/KEM1/XRN1/RAR5* protein (for review, see Kearsy and Kipling 1991). This homology may give clues to the function of Rat1p. Sep1p is a 5' → 3' exoribonuclease (Larimer and Stevens 1990). This may explain why *rat1-1* strains accumulate a longer form of 5.8S rRNA. Because Rat1p has a 5' → 3' exoribonuclease activity (M. Kenna, A. Stevens, M. McCammon, and M. Douglas, pers. comm.), Rat1 could be responsible for trimming the 5' ends of 5.8S rRNA molecules. It is difficult to understand how defects in a nuclease would affect RNA trafficking, particularly in light of the fact that Sep1p shows very little activity on capped RNA (Stevens and Maupin 1987). Furthermore, there is no indication that 5.8S rRNA is involved in mRNA trafficking. It seems unlikely that a defect in 5.8S rRNA processing would cause a secondary defect in mRNA export unless the longer form of 5.8S rRNA cannot be transported, resulting in a general block in RNA transport. Perhaps the exoribonuclease activity of Rat1p exists to degrade mRNA molecules that become damaged.

Two research groups identified Sep1p as having DNA strand exchange activity (Dykstra et al. 1991; Tishkoff et al. 1991). It is interesting that such an activity has also been ascribed to a 120-kD yeast protein (Halbrook and McEntee 1989), which may be Rat1p. This activity could be required for separation of newly transcribed mRNA from DNA after or during transcription. Activities from *Drosophila* and HeLa cells have been identified that stimulate transcription elongation in vitro by facilitating transcript displacement (Kane 1988; Sluder et al. 1988). Alternatively, Rat1p could be involved in the transfer of mRNA molecules from one RNA component of the trafficking apparatus to another.

The *RAT1* gene has also been cloned in other laboratories. *RAT1* was identified as a temperature-sensitive suppressor of a mutation in the promoter of a tRNA gene (G. Di Segni, E. McConaughy, R. Shapiro, T. Aldrich, and B. Hall, pers. comm.; these investigators have named this gene *TAP1*). Overexpression of a mutant form of transcription factor TFIID lacking its amino-terminal domain in the presence of wild-type TFIID causes very slow growth of some yeast strains (Zhou et al. 1991). Selection for yeast genes whose overexpression would negate this *trans*-dominant effect led to the isolation of a genomic fragment containing the *RAT1* gene and an additional reading frame (M. Schmidt, pers. comm.). These results suggest the possible involvement of Rat1p in general transcription as well as in mRNA trafficking. Fi-

nally, the laboratories of M. Douglas and A. Stevens have cloned *RAT1* (and named it *HKE1*) and demonstrated that the Hke1p has a 5' → 3' exoribonuclease activity (M. Kenna, A. Stevens, M. McCammon, and M. Douglas, pers. comm.).

There are three broad possibilities for the function of Rat1p. It may be primarily involved in transcription. Trafficking defects observed may be secondary owing to the failure to produce an RNA encoding an essential component of the trafficking apparatus. This seems unlikely because *rat1-1* cells showed a rapid induction of the RNA trafficking defect after a shift to 37°C. Furthermore, inhibition of polymerase II transcription by the *rpb1-1* allele completely blocked the accumulation of nuclear poly(A)<sup>+</sup> RNA in a *rat1-1/rpb1-1* double mutant (Fig. 3E). We cannot be visualizing nuclear mRNA synthesized before the shift to 37°C, because the nuclei of *rat1-1* cells grown at 23°C had little detectable poly(A)<sup>+</sup> RNA (Fig. 3A). This is not surprising because mRNA exits the yeast nucleus within 2 min after synthesis (Groner and Phillips 1975). Taken together, these data show that mRNA export defects in *rat1-1* cells precede defects in mRNA synthesis.

The second possibility is that Rat1p is involved primarily in the trafficking of mRNA at steps downstream from transcription. Overexpression of Rat1p or the alteration of its activities by mutation could then suppress defects in polymerase II or III transcription by increasing the efficiency of rate-limiting steps in trafficking of RNA polymerase II and III transcripts. This is unlikely because cells carrying another allele of *RAT1* (*tap1-1*) do not appear to accumulate poly(A)<sup>+</sup> RNA in their nuclei at non-permissive temperature; these cells appeared to have little poly(A)<sup>+</sup> RNA either in their cytoplasm or nuclei (D.C. Amberg, T. Aldrich, G. DiSigni, B. Hall, and C.N. Cole, unpubl.).

The possibility we favor is that *RAT1* performs essential functions in both transcription and RNA trafficking. This could reflect Rat1p having two distinct and biochemically separable functions: one in transcription, as indicated by the ability of *RAT1* to suppress transcription defects; and one in mRNA trafficking, as indicated by our mRNA localization experiments in *rat1-1* cells. Perhaps after performing its role in transcription, Rat1p stays associated with the RNA, either targeting it for trafficking or performing a necessary biochemical activity for mRNA export. Alternatively, Rat1p could integrate transcription with the earliest nuclear trafficking events, thereby obligatorily linking these two processes. If so, Rat1p could be involved in monitoring the trafficking competence of the nucleus, enabling the cell to avoid producing RNAs when transport to the cytoplasm is not possible. The homology between Rat1p and Sep1p suggests that *RAT1* may encode a strand transfer or transcript displacement factor that facilitates transcription by removing transcripts from the DNA template; this would qualify as the first step in mRNA trafficking. Our current hypothesis is that Rat1p associates with mRNA during transcription, possibly catalyzing transcript displacement, and stays associated with the message as it

moves to the nuclear periphery, playing an essential role or roles along this pathway. The peripheral accumulation of mRNA in *rat1-1* mutants may indicate that Rat1p is involved in late as well as early steps of intranuclear RNA trafficking.

The identification and study of genes that give aberrant mRNA localization phenotypes when mutated is a powerful approach not only in elucidating the biochemical pathway of mRNA trafficking in eukaryotes but also in identifying the structures and enzymatic activities required for this process. We are screening additional conditional mutants of *S. cerevisiae* and have identified several more complementation groups that affect mRNA trafficking (A.L. Goldstein, L. Gorsch, D.C. Amberg, and C.N. Cole, unpubl.). This suggests that mRNA trafficking is complex and relies on a large number of gene products for its efficient execution.

## Materials and methods

### *Yeast strains, media, and genetic methods*

The yeast strains used in this work are listed in Table 1. Standard yeast genetic methods were used for phenotype analysis, strain crosses, dominance/recessive tests, and tetrad dissection (Rose et al. 1989). Rich media (YPD), synthetic complete media (SC), and sporulation media have been described (Rose et al. 1989).

### *Terminal transferase labeling of (dT)<sub>50</sub>*

An oligonucleotide containing 50 residues of deoxythymidine was synthesized on a Millipore DNA synthesizer and purified on a NENSORB cartridge (Dupont) according to the directions of the manufacturer. The oligo(dT)<sub>50</sub> was labeled at its 3' end with digoxigenin-11-dUTP (Sigma) by use of terminal deoxynucleotidyl transferase (GIBCO BRL). The following were contained in 100 μl of the reaction buffer recommended by the enzyme supplier: 0.5 nmoles of (dT)<sub>50</sub>, 25 nmoles of digoxigenin-11-dUTP, 150 units of terminal deoxynucleotidyl transferase, and 1 μmole of dTTP. After the reaction was allowed to proceed for 15 min at 37°C, the labeled (dT)<sub>50</sub> was ethanol-precipitated and dissolved in 0.5 ml of H<sub>2</sub>O. To monitor the number of digoxigenin-11-dUTP molecules added to each (dT)<sub>50</sub>, a portion of the reaction products were subsequently labeled with [ $\gamma$ -<sup>32</sup>P]ATP by using polynucleotide kinase (New England Biolabs). Gel electrophoresis on an 8% acrylamide/7 M urea gel indicated that these reaction conditions resulted in a reduced mobility of the oligonucleotide in the gel consistent with the addition of 1–4 digoxigenated nucleotides to each (dT)<sub>50</sub> oligonucleotide.

### *In situ mRNA localization assay, microscopy, and photography*

Yeast strains were grown to log phase at 23°C (~2 × 10<sup>7</sup> to 8 × 10<sup>7</sup> cells/ml) in YPD and either remained at 23°C or were shifted to 37°C for the indicated times. Cells (in 5 ml) were collected by centrifugation, resuspended in 5 ml of 0.1 M KPO<sub>4</sub> (pH 6.5)/4% formaldehyde, incubated for 90 min at 23°C on a rotator, washed twice with 0.1 M KPO<sub>4</sub> (pH 6.5), and washed once with wash buffer (0.1 M KPO<sub>4</sub> (pH 6.5), 1.2 M sorbitol). The pellet was resuspended in 1 ml of wash buffer and stored at 4°C

for up to 18 hr. Cells in 1 ml of wash buffer were treated with 300  $\mu$ g of 100T Zymolyase (ICN Biochemicals) for 30–45 min at 23°C, followed by one wash with wash buffer. The pellet was resuspended in 0.2 ml of wash buffer, and cells were adhered to the wells of Teflon-faced slides (Cel-Line Associates) that had been pretreated with an aqueous solution containing 0.1% polylysine [m.w. > 300,000 (Sigma Chemicals)]. Nonadhered cells were removed by aspiration, the slides were plunged into methanol at  $-70^{\circ}\text{C}$  for 6 min, followed by acetone at  $-70^{\circ}\text{C}$  for 30 sec, and dried avoiding condensation. The cells were pre-equilibrated in freshly made 0.1 M triethanolamine (pH 8.0) for 2 min at 23°C, followed by blocking in the above buffer containing 0.25% acetic anhydride for 10 min at 23°C. Cells were incubated for 5 min in  $2\times$  SSC ( $1\times$  SSC = 0.15 M NaCl, 0.015 M sodium citrate), and incubated in prehybridization buffer (50% formamide, 10% dextran sulfate,  $4\times$  SSC, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll-400, 125  $\mu$ g of tRNA/ml, 500  $\mu$ g of denatured sonicated salmon sperm DNA/ml) at 37°C for 1 hr in a humid chamber. The hybridization was carried out in the above prehybridization buffer containing 500 pg of digoxigenin-labeled (dT)<sub>50</sub>/ml for 12–18 hr at 37°C in a humid chamber. Cells were washed for 1 hr in  $2\times$  SSC at 23°C, for 1 hr in  $1\times$  SSC at 23°C, 30 min in  $0.5\times$  SSC at 37°C, and 30 min in  $0.5\times$  SSC at 23°C. The cells were equilibrated in 0.1 M Tris (pH 9), 0.15 M NaCl (ABW1, antibody wash 1) for 5 min at 23°C, and blocked in ABW1 containing 5% heat-inactivated fetal calf serum (FCS) and 0.3% Triton X-100 for 1 hr at 23°C. The cells were incubated in fluoresceinated, anti-digoxigenin Fab fragment (Boehringer Mannheim) in ABW1 containing 5% FCS and 0.3% Triton X-100 for 4 hr at 23°C. Antibody was then removed by washing with ABW1 for 10 min at 23°C, ABW1 for 30 min at 23°C, and in ABW2 (0.1 M Tris at pH 9.5, 0.1 M NaCl, 50 mM MgCl<sub>2</sub>) for 10 min at 23°C, followed by washing with ABW2 for 30 min at 23°C. The nuclei were counterstained with DAPI (10  $\mu$ g/ml in ABW2) for 5 min at 23°C. Cells were washed twice with ABW2, and the slides were mounted under 90% glycerol/1 $\times$  PBS containing 1 mg of *p*-phenylenediamine/ml, and stored at  $-20^{\circ}\text{C}$  (1 $\times$  PBS contains 0.04 M K<sub>2</sub>HPO<sub>4</sub>, 0.01 M KH<sub>2</sub>PO<sub>4</sub>, and 0.15 M NaCl).

Samples were examined on a Zeiss Axiophot microscope equipped with a 100 $\times$  Plan-neofluor objective (1.3 numerical aperture) and photographed using Kodak T-Max 100 or Ilford HP5 (ASA 400) film.

#### Generation and screening of temperature-sensitive mutants

Yeast strains FY23 or FY86 (provided by F. Winston, Harvard Medical School, Cambridge, MA) were plated for single cells on YPD and inverted on a UV transilluminator (Fotodine, 300 nm) for 12 sec. This duration of exposure to UV resulted in the death of 50% of the cells. The remaining living cells were allowed to grow into colonies at 23°C, replica-plated to YPD plates, and incubated at 37°C for 2 days. Putative temperature-sensitive mutants were purified and retested for temperature sensitivity.

Mutants were screened in pools of five by diluting approximately equal portions of each culture into YPD at a total density of  $2\times 10^7$  to  $4\times 10^7$  cells/ml. These pooled cultures were allowed to grow into log phase and were then shifted to 37°C for 2 hr. At this time, the cells were prepared for the *in situ* mRNA localization assay by ensuring that the cultures remained at 37°C until the addition of formaldehyde. When pools with members that showed aberrant mRNA localization phenotypes were identified, the individual members were rescreened by using the *in situ* mRNA localization assay to identify the mutant with the trafficking defect.

#### DNA manipulations

A genomic library of *S. cerevisiae* DNA (*Sau*3A partial digestion products) cloned into a *LEU2*–*CEN* plasmid (F. Spencer and P. Hieter, unpubl.), was transformed by electroporation into yeast strain DAt1-1, carrying the *rat1-1* allele. *LEU2*<sup>+</sup>/37°C<sup>+</sup> transformants were identified and purified. Plasmids were retrieved from 10 such transformants (Rose et al. 1989) and analyzed by digestion with several restriction endonucleases.

A 4.8-kb *Eco*RI fragment of pRAT1-2 was subcloned into the *Eco*RI site of YCp50 (Rose et al. 1987) to create pRAT1-18, which complemented the temperature-sensitive defect when transformed into *rat1-1* strains. This 4.8-kb fragment was randomly fragmented by sonication, and the ends were made blunt by using the Klenow fragment of *E. coli* DNA polymerase I, followed by T4 DNA polymerase. Fragments of 300–500 bp were isolated from a 1.0% agarose gel with GeneClean (Bio101, La Jolla, CA) and cloned into M13 mp18 (New England Biolabs). Approximately 100 inserts were sequenced (Bankier et al. 1987) with the Sequenase 2.0 kit (U.S. Biochemical). The sequence of gaps in the derived sequence were determined by the use of specific oligonucleotides to sequence each strand across the gaps (Wallace et al. 1981). The sequence was assembled with the fragment assembly programs of the Genetics Computer Group (Madison, WI) (Devereux et al. 1984). DNA and protein sequence data bases were searched with FASTA, and sequence alignments were performed with Best-Fit, both programs of the GCG (Devereux et al. 1984). ORF identification and analysis were performed on a Macintosh computer using DNA Inspector software (Textco).

Genomic yeast DNA was prepared (Rose et al. 1989) and Southern analysis was performed (Southern 1975) on Nytran membranes (Schleicher & Schuell) following modifications outlined in the manufacturer's instructions.

All DNA radioactive probes were labeled with the oligonucleotide labeling technique (Feinberg and Vogelstein 1983) with a Prime-It reagent kit (Stratagene Cloning Systems).

#### RNA analyses

Poly(A)<sup>+</sup> RNA was prepared from yeast cells by standard techniques (Ausubel et al. 1988). Cells were grown to log phase ( $2\times 10^7$  cells/ml) at 23°C. Cultures were then shifted to 37°C or 42°C for 2 hr and chilled on ice for 2 hr before harvesting. Poly(A)<sup>+</sup> RNA was separated from total RNA by use of two sequential, oligo(dT)–cellulose affinity chromatography, batch purifications. Northern analyses were performed by separating 5  $\mu$ g [poly(A)<sup>+</sup>] or 20  $\mu$ g (total) of RNA on a 1% agarose/0.66 M formaldehyde gel, transferring the RNA to Schleicher & Schuell Nytran membranes, and fixing the RNA to the membrane by UV cross-linking. Filters were prehybridized for 2 hr at 42°C in 50% deionized formamide,  $5\times$  SSC, 50 mM NaPO<sub>4</sub> (pH 6.5), 50  $\mu$ g/ml of heparin, and 0.5% SDS. Filters were hybridized overnight at 42°C in prehybridization buffer containing  $\sim 1\times 10^6$  cpm/ml of <sup>32</sup>P-labeled DNA probe (Stratagene Cloning Systems Prime-It DNA labeling kit). The filters were washed twice at room temperature for 15 min each in  $2\times$  SSC/0.2% SDS, followed by two washes at 52°C for 15 min each in  $0.2\times$  SSC/0.2% SDS, followed by autoradiography.

To examine tRNA and rRNA processing defects, yeast cells (DAt1-1, FY23, or 2b  $\times$  3b) were labeled with <sup>32</sup>P-labeled inorganic phosphate (Guthrie and Fink 1991). Cells were grown to log phase ( $2\times 10^7$  cells/ml), pulse-labeled with 0.14 mCi (9000 Ci/mole) of H<sub>3</sub><sup>32</sup>PO<sub>4</sub>/ml for 1 hr, either before or after a temperature shift to 37°C, and total RNA was then isolated (Schmitt et al. 1990). Analysis of the labeled RNA was per-

formed as described previously (Hopper et al. 1978). Of each RNA sample,  $\frac{1}{100}$  was denatured and fractionated by electrophoresis in a gel containing 50% urea and a gradient of 3–15% acrylamide. After drying, the gel was autoradiographed.

RNase protection analyses were performed as described (Zinn et al. 1983) with either total RNA or poly(A)<sup>+</sup> RNA selected on oligo(dT)-cellulose. The RNase protection assay for actin mRNA was performed by using labeled antisense RNA prepared by *in vitro* transcription of pGEM-4.Actin (gift of J. Phillips, Dartmouth Medical School, Hanover, NH), which contains the *Bam*HI–*Hind*III fragment of the yeast actin gene cloned into pGEM-4 (Stratagene Cloning Systems). When linearized with *Xho*I, transcription with T7 RNA polymerase generated a 1-kb antisense probe. The probe to detect *RAT1* mRNA was prepared from pRAT1-22, which contains the 547-bp *Hinc*II–*Sac*I fragment from the 3' end of the *RAT1* gene cloned between the *Hinc*II–*Sac*I sites of pGEM-2 (Stratagene Cloning Systems.). When linearized with *Hind*III and transcribed with SP6 RNA polymerase, a 600-nucleotide *RAT1* antisense RNA was produced.

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### Noted added in proof

Sequence data described in this paper have been submitted to the EMBL/GenBank data libraries under accession no. M95626.

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## Isolation and characterization of RAT1: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA.

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