Rat1p and Xrn1p Are Functionally Interchangeable Exoribonucleases That Are Restricted to and Required in the Nucleus and Cytoplasm, Respectively

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***XRN1* encodes an abundant cytoplasmic exoribonuclease, Xrn1p, responsible for mRNA turnover in yeast. A screen for bypass suppressors of the inviability of *xrn1 ski2* double mutants identified dominant alleles of *RAT1*, encoding an exoribonuclease homologous with Xrn1p. These *RAT1* alleles restored *XRN1*-like functions, including cytoplasmic RNA turnover, wild-type sensitivity to the microtubule-destabilizing drug benomyl, and sporulation. The mutations were localized to a region of the *RAT1* gene encoding a putative bipartite nuclear localization sequence (NLS). Fusions to green fluorescent protein were used to demonstrate that wild-type Rat1p is localized to the nucleus and that the mutant alleles result in mislocalization of Rat1p to the cytoplasm. Conversely, targeting Xrn1p to the nucleus by the addition of the simian virus 40 large-T-antigen NLS resulted in complementation of the temperature sensitivity of a *rat1-1* strain. These results indicate that Xrn1p and Rat1p are functionally interchangeable exoribonucleases that function in and are restricted to the cytoplasm and nucleus, respectively. It is likely that the higher eukaryotic homologs of these proteins will function similarly in the cytoplasm and nucleus.**

In the yeast *Saccharomyces cerevisiae*, the 5'→3' exoribonuclease I, Xrn1p, encoded by the *XRN1* gene, is the primary cytoplasmic RNase responsible for mRNA degradation (30). Xrn1p acts at the last step in mRNA turnover, after deadenylation and decapping (reviewed in reference 4). Consequently, *xrn1* mutants accumulate many different mRNA species as deadenylated and decapped RNAs, displaying the highest mRNA levels relative to the wild type for short-lived transcripts (9, 18). Mutants deficient in *XRN1* also accumulate decapped intermediates of the deadenylation-independent nonsense-mediated decay pathway (34). Significantly, the decapped RNAs that accumulate in *xrn1* mutants persist in polysomes (6, 15), suggesting that in the absence of Xrn1p exonuclease, transcripts targeted for degradation by decapping continue to be translated. Xrn1p degrades other classes of RNAs as well, including internal transcribed spacer 1 (ITS1) (39), a byproduct of rRNA processing. Thus, Xrn1p has broad substrate specificity.

Xrn1p was first purified from yeast extracts in a search for RNA turnover factors based on its exoribonuclease activity (38). Subsequently, the enzyme has been purified from biochemical fractionations as a DNA strand exchange activity referred to as Sep1p (13, 28) and as a DNA G4 tetraplex-specific nuclease referred to as Kem1p (32). The gene has also been identified in various genetic screens. It was identified as *KEM1*, mutation of which enhances the karyogamy defect in *kar1* mutants (26). *kem1* mutants were shown to be hypersensitive to benomyl, a microtubule-destabilizing drug, and it has subsequently been suggested that Xrn1p may physically interact with microtubules (19). *XRN1* was also identified as *RAR5* in a screen for mutants that destabilized plasmids containing defective ARS elements (27). In addition, *xrn1* mutants display a superkiller phenotype, and the superkiller mutation *ski1-1* is

most probably allelic to *XRN1* (21). Although the myriad putative activities of Xrn1p would suggest a multifunctional enzyme that acts both in the nucleus and in the cytoplasm, these results can also be rationalized by a model in which gene regulation is globally altered by an inability to remove transcripts in a timely fashion from the translation machinery in the cytoplasm. This model is supported by the finding that Xrn1p is a cytoplasmic protein (17).

The *RAT1* gene encodes a second 5'→3' exoribonuclease of *S. cerevisiae* (24), which shares considerable homology with Xrn1p (2). The *RAT1* gene product, also identified as exonuclease II (Exo II) (40), will be referred to as Rat1p for clarity. Biochemically the two enzymes are very similar. Both require a 5'-terminal phosphate for activity and degrade single-stranded RNA to yield mononucleotides (36, 40). However, in vivo the enzymes have quite different roles: *RAT1* is essential (2, 12, 24) and *rat1-1* mutants are not rescued by high-copy-number expression of *XRN1*, whereas *XRN1* is nonessential, but high-copy-number expression of *RAT1* partially restores the growth and RNA turnover defects of *xrn1* mutants (36). The *rat1-1* allele was identified in a screen for factors involved in the export of poly(A) mRNA from the nucleus (2), although this phenotype was not observed for another allele of *RAT1* (2). The gene was also identified as *TAPI*, mutations in which activated polymerase III (Pol III) transcription of a mutated tRNA gene (1); however, this activation was dependent on the sequence context of the tRNA gene (11), suggesting that *RAT1* was not a general factor in Pol III transcription. *RAT1* was identified as *HKE1* in a reverse genetic approach based on the sequence of the purified exonuclease (24). Most recently, the *rsf11* allele of *RAT1* was identified as synthetically lethal with a deletion of *SWI4* (44). The double mutant could be rescued by high-copy-number expression of *CLN1*, *CLN2*, or *RME1*; however, *rsf11*-specific suppressors were not reported. Although these results suggest a nuclear role for *RAT1*, this has not been clearly demonstrated. In addition, it has been suggested that the residual and presumably cytoplasmic 5' exoribonuclease

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TABLE 1. Strains used in this study

Strain	Genotype	Plasmid	Source or reference
AJY354	<i>MATa ade2 ade3 leu2 lys2-801 ura3-52 xrn1Δ ski2-14</i>	pAJ76	This study
AJY415	<i>MATα ade2 ade3 leu2 lys2-801 ski2::LEU2 xrn1Δ ura3-52</i>	pRDK322	This study
AJY604	<i>MATa ura3 leu2 ade2 trp1 xrn1Δ rat1-1</i>		This study
AJY880	<i>MATα ade2 ade3 leu2 lys2-801 ski2::LEU2 xrn1Δ ura3-52 RAT1(K537E)</i>		This study
AJY881	<i>MATα ade2 ade3 leu2 lys2-801 ski2::LEU2 xrn1Δ ura3-52 RAT1(L520P)</i>		This study
CH1305	<i>MATa ade2 ade3 leu2 lys2-801 ura3-52</i>		29
CH1402	<i>MATα ade2 ade3 ura3-52 leu2</i>		29
DAT1-17	<i>MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 rat1-1</i>		2
RDKY1974	<i>MATa ade2 ade3 leu2 lys2-801 ura3-52</i>		21
RDKY1977	<i>MATa ade2 ade3 leu2 lys2-801 ura3-52 xrn1Δ</i>		21
RDKY1978	<i>MATα ade2 ade3 ura3-52 leu2 xrn1::URA3</i>		21
RDKY2053	<i>MATa ade2 ade3 leu2 lys2-801 ura3-52 xrn1Δ ski2-14</i>	pRDK297	21
RDKY2065	<i>MATa his4 lys2 xrn1::URA3 leu2::hisG</i>		21

active on decapped mRNAs observed in cells from which *xrn1* has been deleted may be due to *RAT1* (33).

Xrn1p and Rat1p homologs are found in other eukaryotes as well. Based on sequence alignments, the various homologs can be divided into Xrn1p-like and Rat1p-like proteins, suggesting that the two protein subfamilies have separate and conserved functions throughout eukaryotes. Exo II from *Schizosaccharomyces pombe* and EXOp from the mouse are homologs of Xrn1p and have been shown biochemically to be exonucleases (23, 41a). Dhp1 from *S. pombe* (41) and Dhml from the mouse (37) are homologs of Rat1p. In addition, a human homolog of *RAT1* has been cloned, and the expressed protein contains exonuclease activity (13a). Comparison of Xrn1p and Rat1p protein sequences with the complete sequence databases from the prokaryotes *Haemophilus influenzae* and *Methanococcus jannaschii* (by using the FASTA alignment algorithm, Baylor College of Medicine) revealed no significant similarity to prokaryotic gene products, suggesting that the Xrn1p protein family is unique to eukaryotes. This specificity to eukaryotic organisms may reflect basic differences in pathways of mRNA turnover between prokaryotic and eukaryotic cells. mRNA turnover in prokaryotes generally requires the activities of endonucleases and 3'→5' exoribonucleases (5). In contrast, mRNA degradation in yeast generally requires 5'→3' exoribonucleases (4, 9, 10). Intermediates for mRNA decay in higher eukaryotes have generally eluded identification, precluding the delineation of a general decay pathway. However, the presence of a functional Xrn1p homolog in the mouse suggests that the 5' degradation pathway is used.

A screen for mutations synthetically lethal with an *xrn1* mutation previously identified mutations in the antiviral genes *SKI2*, a putative RNA helicase, and *SKI3* (21) and in the essential gene *NMD3* (17a). The synthetic lethality with mutations in the *SKI2* and *SKI3* genes was independent of L-A, the major double-stranded RNA virus of yeast, and suggested a general role for *SKI2* and *SKI3* in control of translation of deadenylated transcripts (21). Ski2p may confer specificity for translation of poly(A)-containing mRNAs (21, 35, 45) or may act in a 3' RNA degradation pathway. This report presents the results of a screen for mutations that restore viability to a strain with deletions of *XRN1* and *SKI2*. It was envisioned that this screen could reveal at least two classes of mutations: those that activated additional transacting RNA turnover or translational control factors and those in critical Xrn1p or Ski2p RNA substrates that altered the RNA's susceptibility to alternative degradation pathways or its ability to be translated. However, the bypass suppressors identified in this screen were exclusively

dominant alleles of *RAT1* that resulted in mislocalization of Rat1p to the cytoplasm. In separate experiments, targeting Xrn1p to the nucleus rescued *rat1-1* mutants. These results provide compelling evidence that Rat1p and Xrn1p are functionally equivalent proteins localized to the nucleus and cytoplasm, respectively, and that the essential role of *RAT1* is in the nucleus. It is likely that the two subfamilies of Xrn1p homologs found throughout eukaryotes exist for separate cytoplasmic and nuclear functions.

MATERIALS AND METHODS

Strains and genetic methods. Yeast strains used in this study are listed in Table 1. Rich yeast extract-peptone-dextrose (YPD) medium, 5-fluoroorotic acid (5FOA), dropout media, and standard yeast manipulations were as described elsewhere (22). Low-adenine plates for colony-sectoring assay were prepared as described elsewhere (29). Yeast transformations were carried out as previously described (14), except that cells to be transformed were grown as light lawns on plates. Benomyl sensitivity was assayed as described previously (26) by using YPD plates containing 15 µg of benomyl/ml.

Screen for bypass suppressors. Three independent mutageneses were performed. Typically, an overnight culture of strain AJY415 was grown in YPD medium. The cells were collected by centrifugation, washed with sterile water, and resuspended in water at a density of 6×10^7 /ml. The cells were irradiated with UV light to give 25% survival and plated at a density of 2×10^5 surviving cells/plate on YPD plates, which were then incubated at 37°C. Fifty-seven potential temperature-resistant colonies were picked, and 21 of these were rescored as temperature resistant and resistant to 5FOA, indicating that suppression was not dependent on the plasmid-borne *xrn1-10^s* allele. PCR analysis of genomic DNA prepared from cells grown on 5FOA confirmed that none of the mutants retained an intact *XRN1* coding sequence (data not shown), indicating that the suppressors did not arise from conversion of the genomic *xrn1Δ* allele to the wild type. All mutants were mated with strain RKY2053 to test for dominance. In all cases, the diploids gave rise to sectorial colonies on YPD plates, indicating loss of the *ADE3* plasmid and demonstrating that all the suppressor mutations were dominant. The diploids from these crosses were sporulated, and the resulting tetrads were dissected. Fourteen of the mutants yielded 2:2 segregation for sectorial/solid red, indicating that the suppressors resulted from mutations at single loci. Two others gave aberrant *ade2*-dependent color development and were not analyzed further. Five diploids either failed to sporulate or yielded a high frequency of inviable spores.

Spore clones of the appropriate mating type from the 14 crosses with RKY2053 that yielded single loci were mated with strain DAT1-17, bearing the temperature sensitivity *rat1-1* allele (2). Diploids were sporulated, and tetrads were dissected. The unmarked *xrn1Δ* allele was scored by PCR. In all crosses, the suppressing mutation, scored as viable *xrn1Δ ski2::LEU2* spore clones, segregated away from the *rat1-1* allele, scored by temperature sensitivity, indicating that the suppressing mutations were tightly linked to *rat1-1*.

Allele rescue and mapping. Plasmid pAJ202, bearing *URA3*, was digested with *XbaI* and *SstI* restriction enzymes to remove sequences from -134 to 2785 of *RAT1*. The vector fragment was then transformed into 12 of the original suppressor strains, 9 of which had been identified as *RAT1* alleles (see above) and three of which had not been determined to be alleles of *RAT1* due to poor sporulation after mating with RKY2053. DNA was prepared from the *Ura⁺* colonies and was transformed into *Escherichia coli*. Plasmids propagated in *E. coli* were then transformed into yeast strain AJY354 to test for suppression of

TABLE 2. Plasmids used in this study

Plasmids	Relevant markers	Source or reference
pAJ60	2 μ m <i>LEU2 ADE3</i>	36a
pAJ76	2 μ m <i>LEU2 ADE3 XRN1</i>	This study
pAJ202	CEN <i>URA3 RAT1(wt)</i>	This study
pAJ203	CEN <i>LEU2 RAT1(wt)</i>	This study
pAJ206	CEN <i>URA3 RAT1(K537E)</i>	This study
pAJ209	CEN <i>URA3 RAT1(L520P)</i>	This study
pAJ226	CEN <i>URA3 RAT1(wt)GFP</i>	This study
pAJ228	CEN <i>LEU2 RAT1(NLSΔ)</i>	This study
pAJ229	CEN <i>LEU2 RAT1(KKK$\rightarrow$AAA)</i>	This study
pAJ230	CEN <i>URA3 RAT1(K537E)GFP</i>	This study
pAJ231	CEN <i>URA3 RAT1(L520P)GFP</i>	This study
pAJ232	CEN <i>URA3 RAT1(NLSΔ)GFP</i>	This study
pAJ233	CEN <i>LEU2 RAT1(KKK$\rightarrow$AAA)GFP</i>	This study
pAJ237	2 μ m <i>URA3 XRN1-NLS-GFP</i>	This study
pAJ240	CEN <i>LEU2 RAT1(K537E)</i>	This study
pAJ241	CEN <i>LEU2 RAT1(L520P)</i>	This study
pAJ351	CEN <i>LEU2 RAT1(K535N)</i>	This study
pAJ352	CEN <i>LEU2 RAT1(Δ492-529)</i>	This study
pAJ353	CEN <i>LEU2 RAT1(H518Y)</i>	This study
p2-26/8-3	2 μ m <i>SKI2</i> genomic clone	This study
pRAT1-10	<i>RAT1</i> genomic clone	2
pRAT1-18	<i>RAT1</i> subclone	12
pTD150	<i>GFP m2</i> allele	8
pRDK318	2 μ m <i>URA3 XRN1-HA</i> epitope	
pRDK242	2 μ m <i>URA3 XRN1</i>	20
pRDK252	CEN <i>URA3 XRN1</i>	42
pRDK297	2 μ m <i>URA3 ADE3 XRN1</i>	21
pRDK322	CEN <i>URA3 xrn1-10⁸</i>	21
pRRP590	<i>MFA2</i>	R. Parker

xrn1 ski2 by a colony-sectoring assay. All rescued plasmids allowed sectoring on Ura dropout low-adenine plates, whereas the wild-type *RAT1* allele did not, confirming that all the rescued suppressing mutations were within, or immediately 5' of, the *RAT1* coding sequence. From these analyses, 17 of the original suppressor mutations were identified as *RAT1* mutations.

To localize the mutations in the gap-rescued *RAT1* alleles, plasmid pAJ203 was digested with *XbaI* and *SalI*, *SalI* and *SacII*, *SacII* and *NcoI*, and *NcoI* and *SstI* to yield gaps in the *RAT1* sequence from nucleotide -134 to 85, 85 to 512, 512 to 1992, and 1992 to 2785, respectively. The gapped plasmids were then transformed into the 12 Ura⁺ isolates in the AJY354 background, described above, and Leu⁺ transformants were scored for growth on 5FOA, indicative of the conversion of the transforming vector by the *RAT1* dominant alleles, allowing loss of the *URA3* vector. In all cases, the highest frequency of 5FOA resistance resulted from plasmids gapped by *SacII* and *NcoI*. The *RAT1* alleles in pAJ202 were sequenced from nucleotide 512 to 1992 by dye-terminated dideoxy sequencing (Molecular Biology Core Facility, University of Texas at Austin), and all contained single nucleotide changes. PCR was used to amplify the *RAT1* locus from the remaining five *RAT1* mutants that had not been rescued to plasmids and from one mutant which had not been characterized previously due to poor spore viability. Oligonucleotides AJO131 (5'-TCTTGCCAGTAAAGCTCTC ATG) and AJO150 (5'-CGCTATGCTTGGCAGG) were used to amplify *RAT1* from nucleotide 476 to 2208. The amplified products were reamplified with oligonucleotides AJO132 (5'-GGGCTATCGACGATTGGG) and AJO150. The products were gel purified, and the region from nucleotide 1450 to 1800 was sequenced by dye-terminated sequencing (BioTech Core, Palo Alto, Calif.).

Plasmids. See Table 2 for a compilation of plasmids used in this work. All nucleotide numbering is relative to the translation start, defined as +1 of the respective gene. pAJ76 is a 2 μ m-based *ADE3 XRN1* plasmid. A 5.8-kb *SKI2*-containing *XbaI* fragment from the *SKI2* genomic clone, p2-26/8-3, was blunt ended with T4 DNA polymerase and ligated into the *SmaI* site of pAJ60. pAJ202 was constructed by replacing the 448-bp *PvuII* fragment of pRS416 with the *RAT1*-containing fragment of pRAT1-18, which had been digested with *EcoRI* and filled in with Klenow fragment. pAJ96 was made by replacing the 445-bp *PvuII* fragment of pRS315 with the ~9-kbp *RAT1*-containing *AatII* fragment of pRAT1-10, which had been blunt ended with T4 DNA polymerase. pAJ203 was derived from pAJ96 by removing a 2-kbp *XbaI*-to-*NheI* fragment upstream of and including the *XbaI* site at position -1542 of *RAT1*. pAJ96 was partially digested with *XbaI* and fully digested with *NheI*. The appropriate fragment was gel isolated and religated, yielding a unique *XbaI* site at position -134 of *RAT1*. pAJ240 and pAJ241 contain the K537E and L520P *RAT1* mutants subcloned into pAJ203.

NLS and green fluorescent protein (GFP) constructs. pAJ228 was derived from pAJ203 and contains a mutated *RAT1* gene that expresses Rat1p from which the nuclear localization signal (NLS) has been deleted. Oligonucleotide primers AJO131 (5'-CGATCACTCCAGGACCC) and AJO140 (5'-GATTCC GCTTCTCTGTATTATTATTCTGTTTC), reading antiparallel and encoding the NLS deletion, were used to amplify *RAT1* from nucleotide 476 to 1628 and to delete the nucleotide sequence of *RAT1* from 1546 to 1611, AAGAAACA CCGTCTTGAAAAGGATAATGAAGAGGAAGAAATTGCTAAGGATAG CAAAAAGTTAAG, encoding KKHRLEKDNEEEIIAKDSKKVK. Separately, oligonucleotides AJO139 (5'-ACAGAGAAAGCGGAATC) and AJO150 were used to amplify a fragment of *RAT1* from nucleotide 1612 to 2208. The two products, which overlapped by 17 nucleotides, were mixed and reamplified with outside primers only. The resulting product was digested with *ScaI* and *NcoI* to replace the corresponding fragment in pAJ203. pAJ229, prepared similarly, expresses Rat1p in which the three lysines of the second basic cluster of the NLS have been changed to alanines, changing the sequence KKVK (amino acids 534 to 537) to AAVA. Oligonucleotide primer AJO139 and the mutagenic primer AJO141 (GATTCGCTTCTCTGTGCGCAACTGCTGCGTATCCTTAGC AATTTC), reading antiparallel, were used to amplify *RAT1* from nucleotide 476 to 1628. Separately, oligonucleotides AJO139 and AJO150 were used to amplify a fragment of *RAT1* from nucleotide 1612 to 2208. The two products, which overlapped by 17 nucleotides, were mixed and reamplified with outside primers only. The resulting product was digested with *ScaI* and *NcoI* to replace the corresponding fragment in pAJ203.

RAT1-GFP gene fusions were prepared by amplifying the m2 mutant GFP with enhanced fluorescence from plasmid pTD150 by using the oligonucleotide primers AJO143 (5'-CGCTCCGAGAGCTCATGAGTAAAGGAGAA) and AJO142 (5'-CGGGCTAGCTCCGGATTATTTGTATAGTTTATC). The resulting fragment, which contained the GFP gene flanked by *BspEI* sites, was cleaved with *BspEI* and ligated into the *BspEI* site at nucleotide 2926 of *RAT1* in plasmids pAJ202, pAJ206, pAJ209, pAJ228, and pAJ229 to yield plasmids pAJ226, pAJ230, pAJ231, pAJ232, and pAJ233, respectively. The correct orientation of the GFP insert in these plasmids was determined by restriction digests. The remaining *RAT1* mutants, Δ 492-529, H518Y, and K535N, were gap rescued by using plasmids pAJ228 and pAJ232 digested with *SalI* and *SstI*. The correct mutants were identified by the replacement of the Δ NLS mutant in pAJ228 and pAJ232.

pAJ237 contained the *XRN1*-GFP fusion containing the simian virus 40 (SV40) large-T-antigen NLS. It was made by amplifying m2 mutant GFP from plasmid pTD150 with the oligonucleotide primers AJO158 (5'-CTGGGCTAGC CAAAGAAGAAGAGAAAGGTCGACATGATGATAAAGGAGAAG) and AJO142, described above (nucleotides encoding the NLS PKKKRKV are underlined). The resulting DNA fragment was digested with *NheI* and ligated into *NheI*-digested pRDK318. pRDK318 is a *URA3* 2 μ m shuttle vector derived from pRDK242 containing the *XRN1* gene in which an oligonucleotide encoding the hemagglutinin (HA) epitope and introducing a unique *NheI* site was inserted into the *EcoRI* site at nucleotide 3894 of *XRN1*. The construction of pRDK318 will be described elsewhere. Xrn1-HA expressed from pRDK318 is indistinguishable from the wild type in vivo. The *XRN1* mutant, *xrn1*(E178G), was made by in vitro random PCR mutagenesis of *XRN1* on a plasmid and was identified by its lack of complementation of *xrn1 ski2* lethality. A more extensive description of this allele will be presented elsewhere. The *xrn1*(E178G) mutant was subcloned on a 4.5-kb *SstI* and *HindIII* restriction fragment into pAJ237 to make an *xrn1*(E178G) SV40 NLS-GFP fusion.

Northern blot analysis. Ten-milliliter cultures were grown in dropout medium to a density of approximately 10⁷ cells/ml. RNA was prepared, and 10 μ g of total RNA was fractionated by electrophoresis through formaldehyde-agarose gels (1.25% agarose) as described elsewhere (22). RNA was transferred to a Zeta-Probe membrane (Bio-Rad) by vacuum transfer in 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Radiolabeled probes for *CYH2* and *MFA2* were prepared by random priming (High Prime; Boehringer Mannheim), by using as templates a *CYH2* fragment (nucleotide 34 to 934) generated by PCR amplification of the genomic *CYH2* locus with the oligonucleotides AJO119 (5'-AGAGGTCACGTCTCAGG) and AJO120 (5'-ACAACACCACAGCA GC) and the *MFA2*-containing *EcoRI* fragment from pRP590. A radiolabeled probe specific for ITS1 was prepared by phosphorylating oligonucleotide AJO130 (5'-TCTTGCCAGTAAAGCTCTCATG). Blotting was carried out according to the manufacturer's instructions for ZetaProbe (Bio-Rad). Blots were imaged and quantitated on a Molecular Dynamics PhosphorImager.

Microscopy. Fresh overnight cultures of cells grown in appropriate selective media were diluted to approximately 10⁷ cells/ml and cultured for 4 h, after which 4',6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 0.5 μ g/ml. The cultures were grown for an additional 30 min and then photographed with a Zeiss Axiophot microscope fitted with a 100 $\times$ objective and using Kodak T-Max 400 film. All cultures in the *rat1-1* background (DAT1-17) were grown at 26°C.

Western blotting. Anti-Rat1p antiserum was a generous gift of Mark MacCammon and was affinity purified against a TrpE::Rat1 fusion protein expressed in *E. coli* from plasmid pTT3 as described previously (1). Yeast total soluble protein extracts were prepared and protein concentrations were determined as previously described (20). Proteins were separated on a sodium dodecyl sulfate-8% polyacrylamide gel and transferred to nitrocellulose membranes, and

FIG. 1. Western blot analysis of Rat1p. Ten micrograms of total soluble protein from the strains indicated was separated on a sodium dodecyl sulfate–8% polyacrylamide gel. Affinity-purified anti-Rat1p polyclonal antiserum was used as the primary antibody. Strains: for the wild type (WT), AJY415; for K537E, AJY880; and for L520P, AJY881.

Western blotting was carried out with a Protoblot kit (Promega) with affinity-purified anti-Rat1p (1:500 dilution) as the primary antibody.

RESULTS

A screen of UV-mutagenized cells was carried out to identify mutations that rescued the inviability of *xrn1 ski2* mutants. Twenty-one such bypass suppressors were identified at a frequency of approximately 5×10^{-7} . All were dominant mutations, and 18 were characterized by linkage analysis or direct sequencing of genomic PCR products as alleles of *RAT1*, encoding an exoribonuclease that shows considerable homology with Xrn1p. The remaining three mutants were not further characterized due to aberrant tetrad analysis. Since *RAT1* is a high-copy-number suppressor of *xrn1* (36), a potentially trivial explanation for bypass suppression by the *RAT1* dominant alleles was that they allowed overexpression of *RAT1*. However, Western blot analysis showed that the dominant alleles did not significantly overexpress Rat1p (Fig. 1 and data not shown).

The 18 *RAT1* mutants comprised five distinct mutations: H518Y, L520P, K535N, K537E, and $\Delta 492-529$. The H518Y mutation, in which histidine at position 518 is changed to tyrosine, was represented by one isolate and resulted from a C-to-T transition at nucleotide 1552. L520P was represented by three isolates and resulted from a T-to-C transition mutation of nucleotide 1559. The K535N mutant was found once and resulted from an A-to-T transversion mutation at nucleotide 1605. Nine isolates of K537E were identified, and all contained an A-to-G transition mutation of nucleotide 1609. Lastly, the $\Delta 492-529$ mutation was a small in-frame deletion of 114 nucleotides between and including one of two repeated dodecamer DNA sequences, AGGAAGAAATTG, which occur at nucleotides 1463 to 1474 and 1577 to 1588. The abundant mutants, K537E and L520P, were most extensively characterized. When reintroduced on single-copy plasmids into an *xrn1 ski2* double mutant, the L520P and K537E *RAT1* mutants, but not the wild type, allowed the loss of an *XRN1*-bearing plasmid (Fig. 2). However, the two mutations differed in their abilities to suppress the growth defect of the double mutant; the L520P mutation showed weaker suppression than the K537E mutation. To confirm that these mutations alone were sufficient for the dominant phenotype, the sequenced regions were subcloned into an otherwise wild-type *RAT1* allele, where they again conferred a dominant phenotype (data not shown). In this plasmid shuffle assay, the mutations K535N, $\Delta 492-529$, and H518Y showed suppression similar to that of the K537E mutation (data not shown).

To test the specificity of suppression, plasmids bearing the dominant *RAT1* mutations, K537E and L520P, were trans-

WT L520P K537E

FIG. 2. Single-copy vectors containing dominant alleles of *RAT1* rescue the inviability of *xrn1 ski2* double mutants, but wild-type *RAT1* does not. Strain RDKY2053 (*xrn1 Δ ski2-14*; pRDK297) was transformed with pAJ203 (WT), pAJ240 (K537E), or pAJ241 (L520P). Transformants were patched onto 5FOA plates and incubated at 30°C for 2 days. Growth indicates viability upon loss of the *URA3 XRN1*-containing plasmid pRDK297.

formed into *xrn1 ski3* and *xrn1 nmd3-1* double mutants that also contained a plasmid bearing *XRN1* and *URA3* genes. The dominant alleles allowed loss of the *XRN1 URA3* plasmid from these cells, demonstrating suppression (data not shown). These results and the complementation of *xrn1*-specific in vivo defects (see below) show that the *RAT1* alleles are specific for the suppression of *xrn1*.

All five suppressor mutations fell within a discrete region of the *RAT1* gene predicted to be hydrophilic and encoding a short polypeptide loop not found in the *XRN1* homologs (Fig. 3a). Examination of the sequence surrounding the four *RAT1* dominant alleles revealed similarity to the consensus bipartite NLS: two basic residues, a 10-amino-acid spacer of any sequence, followed by a cluster of basic residues in which three of five are basic (Fig. 3b). The putative *RAT1* NLS differs from this consensus in that the spacer is 15 amino acids. The K537E

a

Rat1p	504	N-LAEVMKQKIIN	<u>KKHRL</u> EKDNEEEI <u>AKDSKKVK</u>	TEKAES
Dhp1p	510	NKLAASALKQLV	SNETLQNVPLEDSIASSAYED	TDSEIS
Dhm1p	477	N----TSFAS---	-----D	G--SPS
Xrn1p	452	SYSLKMDLDSINP	-----D	ETEEEF
ExoII	440	THLLRITFRDLEF	N-----D	EDEDEI

b

Consensus		10 aa	
NLS		<u>KK</u> XXXXXXXXXX <u>XKKXK</u>	
		RR	RR R
RAT1 alleles		15 aa	
Wild-type	516	<u>KK</u> HRLKEDNEEEI <u>AKD</u> <u>SKKVK</u>	537
H518Y		Y	
L520P		P	
K535N			N
K537E			E
$\Delta 492-529$	[	492 - 529 deleted	]
KKK→AAA			AA A
NLS Δ	[	516 - 537 deleted	]

c

Rat1p	383	HLGSREGDIF	KTRHIQEARKKEAFERRKAQK	NMSKGQDRHP
Dhp1p	386	AVGNQEDDIF	KRLKQEDRRNENYRRRQRE	SNQES--Y
Dhm1p	395	AVGEVEDSIF	<u>KKRKDD</u> ED-- <u>SFR</u> RRQKEK	RKRMR----
Xrn1p	341	YLSQFELLNF	EKDDIDV---	EWFNKQLENI
ExoII	340	----LELENF	EK-----	EIFKAEVSET
				KNN-----

FIG. 3. Dominant *RAT1* alleles map to a putative bipartite NLS. (A) Alignment of Rat1p with homologs. Dhp1p, Rat1p homolog from *S. pombe*; Dhm1p, Rat1p homolog from the mouse; Exo II, Xrn1p homolog from *S. pombe*. The underlined sequence is the putative NLS of Rat1p. (B) Comparison of consensus bipartite NLS with the putative NLS of Rat1p. Wild-type Rat1p and mutant sequences are shown. X represents any amino acid. The order of the three lysine residues in the second basic cluster of the consensus is variable. Square brackets indicate boundaries of deletions. (C) Putative NLSs in the mouse and *S. pombe* homologs of Rat1p lie approximately 100 amino acids amino-terminal to the putative NLS of Rat1p.

FIG. 4. Northern blot analyses of pre-CYH2 and CYH2 (a), *MFA2* transcript (b), and ITS1 (c). Ten micrograms of total RNA was separated on formaldehyde-agarose gels, transferred to nylon membranes, and probed. All cultures were grown at 30°C. The strain for L520P, K537E, K535N, Δ492-529, and H518Y, was AJY354, with pAJ209, pAJ206, pAJ351, pAJ352, and pAJ353, respectively, replacing pAJ76; strains for *XRN1* and *xrn1Δ* were RDKY1974 and RDKY1977, respectively.

mutation alters a conserved basic residue, and the Δ492-529 mutation removes the amino-terminal basic cluster. In addition, the L520P and H518Y mutations alter amino acids of the spacer region, which is not part of the consensus sequence and reportedly can be of any amino acid sequence. Examination of the amino acid sequence of two Rat1p homologs, Dhp1 from *S. pombe* and Dhml from the mouse, revealed that these two proteins also contained putative NLSs, but at a position different from that found in Rat1p (Fig. 3c). In Dhp1 the sequence KRLKQEDRRNENYRRR (amino acids 396 to 412) differs slightly from the consensus by having a spacer region of 12 amino acids. Dhml protein contains a consensus NLS, KKRKDDSDSFRRRQKEKRR (amino acids 407 to 423), in a position homologous with that of the NLS in Dhp1. The homologous position within Rat1p contains some characteristics of a bipartite NLS, but the spacer region is extremely short, containing only five amino acids. Nevertheless, it is possible that this sequence serves as a weak NLS (see Discussion).

***RAT1* dominant alleles restore *XRN1*-like function.** *Xrn1p* is an abundant cytoplasmic exoribonuclease (30) and is responsible for the majority of the deadenylation-dependent and deadenylation-independent degradation of mRNA species as well as of other RNAs, including ITS1, from rRNA processing (39). Consequently, RNA degradation was assayed in the *RAT1* mutants. The *RAT1* dominant alleles significantly reduced the levels of pre-CYH2 relative to the levels observed in the *xrn1Δ* mutant (Fig. 4a). Quantitation of the blot in Fig. 4a indicated that whereas the ratios of pre-CYH2 to CYH2 in *xrn1Δ* and the wild type were 0.7 and 0.05, respectively, the ratios in the *RAT1* mutants ranged from 0.24 to 0.29. Steady-state levels of *MFA2* transcript, which is degraded by a deadenylation-dependent pathway, also were assayed (Fig. 4b). Again, the *RAT1* mutant alleles reduced the steady-state level from that observed in the *xrn1Δ* mutant to levels similar to those observed in wild-type cells. Lastly, the *RAT1* alleles reduced the levels of ITS1 observed in *xrn1Δ* mutants to levels similar to those found in wild-type cells (Fig. 4c). Quantitation of the blot in Fig. 4c indicated that ITS1 in wild-type cells was present at approximately 2% of the level found in *xrn1Δ* mutants. The levels of ITS1 in the *RAT1* mutants ranged from 1 to 3% relative to those in the *xrn1Δ* mutant. Thus, for three

FIG. 5. Complementation of *xrn1Δ* benomyl hypersensitivity. Three microliters of each culture was spotted onto a YPD plate or a YPD plate supplemented with 15 μg of benomyl/ml and allowed to grow for 2 days at 30°C. Strains: WT, RDKY1974; *xrn1Δ*, RDKY1977; L520P, K537E, K535N, Δ492-529, and H518Y, RDKY1977 containing pAJ241, pAJ240, pAJ351, pAJ352, and pAJ353, respectively.

distinct RNA degradation reactions, the *RAT1* alleles restored *XRN1*-like cytoplasmic RNA degradation. Interestingly, the *RAT1* alleles were less efficient at deadenylation-independent degradation of pre-CYH2; however, there did not seem to be a correlation between specific alleles and efficiency of the various degradation reactions.

xrn1 mutants are hypersensitive to benomyl (26), and *Xrn1p* may interact directly with tubulin (19). Consequently, the ability of the *RAT1* dominant alleles to suppress the benomyl hypersensitivity was tested. The K537E, H518Y, and Δ492-529 mutants fully suppressed the benomyl hypersensitivity of *xrn1Δ*, whereas the L520P and K535N mutants did not (Fig. 5). In addition, the L520P and K535N mutants showed slightly slower growth on YPD (Fig. 5), and the L520P mutant showed weaker suppression in a plasmid shuffle assay (Fig. 2). Thus, there may be a correlation between suppression of benomyl hypersensitivity and suppression of the growth defect of *xrn1Δ*.

xrn1 mutants are also defective for sporulation, arresting at pachytene (43), although the underlying defect causing this arrest has not been defined. The ability of the dominant K537 mutant of *RAT1* to suppress the sporulation defect of *xrn1* mutants was tested by mating an *xrn1 RAT1(K537)* double mutant with an *xrn1* deletion mutant in the SK-1 strain background, which sporulates with high efficiency. The K537E mutant restored sporulation of a diploid homozygous for *xrn1* deletion to the levels observed for an *xrn1* heterozygote (Table 3).

Mutant Rat1p is mislocalized to the cytoplasm. To test if the putative NLS of *RAT1*, amino acids 516 to 537, is required for protein localization to the nucleus, mutations were introduced into the *RAT1* coding sequence that either eliminated the putative NLS, the resultant mutant being identified as the NLSΔ mutant, or changed the three lysines of the second basic cluster, amino acids 534 to 537, to alanines, the resultant mutant being identified as the KKK→AAA mutant (Fig. 3b). The

TABLE 3. A dominant *RAT1* mutation complements the *xrn1* sporulation defect

Relevant genotype	Cross	% Sporulation
<i>XRN1 RAT1</i>	RKY1975 × RKY2065	69
<i>xrn1::URA3 RAT1</i>		
<i>xrn1Δ RAT1</i>	RKY1978 × RKY2065	18
<i>xrn1::URA3 RAT1</i>		
<i>xrn1Δ RAT1(K537E)</i>	AJY880 × RKY2065	70
<i>xrn1::URA3 RAT1</i>		

TABLE 4. Localization and complementation by *RAT1* and *XRN1* alleles

Allele	Complementation of:		Localization ^a
	<i>xrn1</i> Δ ^b	<i>rat1-1</i> ^c	
<i>RAT1</i>	—	++	Nucleus
<i>RAT1(K537E)</i>	++	++	Whole cell and nucleus
<i>RAT1(L520P)</i>	+	++	Mostly nuclear
<i>RAT1(NLSΔ)</i>	++	++	Whole cell
<i>RAT1(KKK→AAA)</i>	++	++	Whole cell
<i>XRN1</i>	++	—	Cytoplasm ^d
<i>XRN1(NLS)</i>	++	++	Nucleus

^a Determined by fluorescence microscopy of GFP fusion proteins unless indicated otherwise.

^b Scored by colony-sectoring assay in RDKY2053 or AJY354. Scores: —, no complementation; +, weak complementation; ++, full complementation.

^c Scored by complementation of temperature sensitivity of *rat1-1*.

^d Determined previously (17).

localization of wild-type and mutant proteins was monitored by the use of GFP fusions to Rat1p and the mutant proteins. The GFP coding sequence was inserted into the *BspEI* site of *RAT1* at nucleotide 2926, deleting the C-terminal 29 amino acids of Rat1p. All *RAT1* GFP fusions were expressed on single-copy CEN vectors under the control of the wild-type *RAT1* promoter.

The various protein constructs were tested for their abilities to complement the temperature sensitivity of the *rat1-1* allele and the inviability of *xrn1 ski2*, assayed in a colony-sectoring assay. Whereas wild-type *RAT1* on a single-copy vector did not complement *xrn1 ski2* inviability, the four *rat1* mutants, NLSΔ, K537E, L520P, and KKK→AAA, all complemented both the *rat1-1* temperature sensitivity and the *xrn1 ski2* inviability (Table 4). Identical complementation results were obtained with constructs lacking GFP (Fig. 2 and data not shown), suggesting that deletion of the C-terminal 29 amino acids and the addition of GFP to Rat1p did not alter protein function.

Wild-type Rat1p fused to GFP was observed uniformly in the nucleus in nearly all cells (Fig. 6a through c), regardless of cell cycle position. Control cells expressing Rat1p without GFP did not show any significant fluorescence (data not shown). Deletion of the putative NLS (NLSΔ) caused mislocalization of the protein throughout the cell (Fig. 6j); however, the mutant protein was not clearly excluded from the nucleus. Similar results were obtained with the KKK→AAA and Δ492-529 mutations (data not shown). The K537E mutation, which alters a basic residue of the putative NLS consensus sequence, also resulted in mislocalization of the protein throughout the cell in addition to distinct fluorescence within the nucleus (Fig. 6g). The localization of the K535N and H518Y mutant proteins was similar to that of K537E (data not shown). The weaker suppressing mutation, L520P, resulted in primarily a nuclear localization (Fig. 6d). Similar localizations of wild-type and mutant Rat1p GFP fusion proteins were also observed in a diploid strain (data not shown).

These results indicate that the putative NLS is required for efficient nuclear localization of Rat1p. However, since the NLSΔ mutant complemented the *rat1-1* allele, either Rat1p can carry out its essential role in the cytoplasm in a capacity that Xrn1p cannot, or sufficient Rat1p still enters the nucleus for nuclear activity, suggesting that the protein may bear an additional NLS or can enter the nucleus by another mechanism. The first possibility was tested indirectly by targeting Xrn1p to the nucleus (see below).

FIG. 6. Localization of wild-type and mutant Rat1p. Cells expressing Rat1-GFP fusion proteins were stained with DAPI and photographed with filters for GFP fluorescence (GFP) or DAPI, or with differential image contrast (DIC). The haploid strain DAT1-17 was transformed with plasmid pAJ226 (WT), pAJ231 (L520P), pAJ230 (K537E), or pAJ232 (NLSΔ).

Xrn1p targeted to the nucleus complements *rat1-1*. Previous work indicated that the Xrn1 protein can accept deletion of and protein fusions to the C terminus of the protein (19, 25). Consequently, a construct was made that encodes Xrn1p fused to GFP, including the SV40 large-T-antigen NLS, PKKKRKV, at the fusion junction. Xrn1p-NLS-GFP rescued the inviability of *xrn1 ski2* mutants and the temperature sensitivity of *rat1-1* (Table 4). Similar results were obtained with a construct expressing intact Xrn1p bearing the SV40 large-T-antigen NLS but lacking GFP and from constructs expressed from a single-copy vector (data not shown). Microscopic examination of cells containing this plasmid revealed bright nuclear fluorescence in most cells (Fig. 7). That the *XRN1*-NLS fusion complemented

FIG. 7. Xrn1p can be targeted to the nucleus. Strain DAT1-17 expressing an Xrn1-NLS-GFP fusion protein from plasmid pAJ237 was stained with DAPI and photographed as described for Fig. 6.

FIG. 8. Xrn1(E178G) is deficient for exonuclease activity in vivo. Northern blot analyses of ITS1 and CYH2 were carried out as described for Fig. 4. Strains: *xrn1(E178G)*, 1977 with *xrn1(E178G)* on pRS315; *xrn1Δ*, 1977 with pRS315; WT, RDY1977 with *XRN1* on pRS315.

an *xrn1 ski2* double mutant suggests that sufficient Xrn1p remained in the cytoplasm to provide Xrn1p function. Since wild-type Xrn1p is cytoplasmic and does not complement *rat1-1*, even when *XRN1* is present on a high-copy-number plasmid, these results show that the SV40 large-T-antigen NLS is sufficient to direct Xrn1p to the nucleus. More importantly, the presence of Xrn1p in the nucleus, but not in the cytoplasm, complemented the temperature sensitivity of *rat1-1*, demonstrating that Rat1p has an essential nuclear function that can be carried out by Xrn1p if Xrn1p is localized to the nucleus.

To determine if the essential function of Rat1p or nuclear-targeted Xrn1p is 5' exoribonuclease or another unidentified function of these proteins, an exonuclease-deficient mutant Xrn1p was targeted to the nucleus by the SV40 NLS and tagged with GFP. Replacement of glutamate 178 with glycine in Xrn1p abolishes detectable exonuclease activity (Fig. 8), and the mutant protein is stable in vivo (data not shown). The localization of the exonuclease-deficient Xrn1p(E178G)-NLS-GFP was indistinguishable from that shown in Fig. 8 and did not rescue the temperature sensitivity of *rat1-1* (data not shown). Thus, nuclear 5' exoribonuclease is essential.

RAT1 dominant alleles simultaneously complement *xrn1Δ* and *rat1-1* temperature sensitivity. By the way in which the dominant alleles of *RAT1* were identified, it was expected that a single copy of a dominant *RAT1* allele would simultaneously complement both *xrn1* and *rat1* defects. This was tested explicitly by introducing the dominant alleles on single-copy vectors into *xrn1Δ rat1-1* double mutants. Transformants were tested for growth at 26 and 37°C. *xrn1* mutants display a slow-growth phenotype at 26 and 37°C which is rescued by a plasmid bearing wild-type *XRN1* but not by a plasmid bearing wild-type *RAT1* (Fig. 9). Similarly, the temperature sensitivity of *rat1-1* strains is rescued by a plasmid bearing *RAT1* but not by one bearing *XRN1*. However, when the *rat1-1 xrn1Δ* double mutant was transformed with a single-copy plasmid bearing either the L520P or K537E *RAT1* mutation, the transformants grew as well as the wild type at 37°C. Thus, in these cells the two exoribonucleases, Xrn1p and Rat1p, normally found in the cytoplasm and nucleus, respectively, have been replaced by a single exoribonuclease generally distributed throughout the cell.

DISCUSSION

Deletion of *XRN1*, encoding the major cytoplasmic exoribonuclease responsible for mRNA degradation in yeast, is lethal in combination with null mutations of *SKI2*, encoding a puta-

FIG. 9. Mislocalized Rat1p simultaneously provides Xrn1p and Rat1p function. The *xrn1Δ rat1-1* double mutant (AJY608) was transformed with plasmids bearing wild-type *RAT1* (pAJ202), the dominant mutations K537E (pAJ206) and L520P (pAJ209), or wild-type *XRN1* (pAJ52). Transformants were grown on Ura dropout plates at 23 or 37°C and photographed.

tive RNA helicase. A screen for bypass suppressors of *xrn1 ski2* inviability identified eighteen dominant mutants representing five mutations of *RAT1*, which encodes a 5' exonuclease that has considerable sequence homology with Xrn1p. Three of the mutations were T-to-C transitions in a repeated T sequence, and one mutation was a T-to-A transversion, typical of UV-induced lesions (31). Although the screen was highly specific for *RAT1*, it remains possible that other, weaker suppressors exist which were not identified. Candidate *xrn1-10^{ts}* allele-specific suppressors were also identified and will be characterized separately.

The *RAT1* alleles identified were dominant gain-of-function alleles. Because *RAT1* is an essential gene, the alleles must retain *RAT1* function and at the same time gain the ability to complement *xrn1* or *ski2* deficiency. The alleles were localized to a region of the *RAT1* sequence encoding a potential bipartite NLS (11). The putative NLS of *RAT1* differs from the consensus NLS in that the 10-amino-acid spacer region between the basic clusters is extended to 15 residues. However, it has been reported that small insertions or deletions within the spacer region have little effect on nuclear targeting (11). The different alleles of Rat1p displayed differing degrees of cytoplasmic mislocalization. The most extensive mislocalization, with no specific localization to the nucleus, was observed for mutations that altered more than one basic residue ($\Delta 492-529$, NLS Δ , and KKK $\rightarrow$ AAA). Mutations that affected only single basic residues of the consensus sequence resulted in cytoplasmic and nuclear localization, and the L520P mutation, which alters an amino acid of the spacer region and also is a weak suppressor, resulted in nearly wild-type nuclear localization. It is interesting that the H518Y mutation, which altered a residue other than a consensus residue, produces strong mislocalization. It is possible that this histidine actually serves as a basic residue in the NLS of Rat1p.

The finding that suppressors of *xrn1 ski2* lethality were mutations in a putative NLS of Rat1p suggested that suppression was the result of mislocalization of Rat1p to the cytoplasm, where it could substitute for Xrn1p. This was confirmed by the use of GFP fusions to wild-type Rat1p and to Rat1 mutant proteins. Whereas wild-type Rat1-GFP was found exclusively in the nucleus, deletion of the putative NLS or mutations that altered conserved lysine residues led to distribution of the mutant proteins throughout the cell.

Since wild-type Rat1p is nuclear and apparently is targeted

to the nucleus by a bipartite NLS, it was surprising that Rat1p lacking the potential NLS still complemented the *rat1-1* mutant. Although by fluorescence microscopy it was not possible to conclude that deletion of the putative NLS prevented Rat1p entry into the nucleus, it could be inferred from the results of targeting Xrn1p to the nucleus that mutations within or deletion of the NLS of Rat1p do not entirely eliminate Rat1p from the nucleus. Thus, Rat1p may enter the nucleus by an alternate mechanism. It is possible that another sequence within Rat1p serves as a weak NLS. Examination of the protein sequences of the *S. pombe* and mouse homologs of Rat1p revealed potential NLSs in a position approximately 100 amino acids closer to the N terminus than the Rat1p NLS (Fig. 3b). In the homologous position in Rat1p, a possible NLS with a spacer of 5 amino acids exists. It is possible that this sequence was the NLS of an ancestral protein and that the *S. cerevisiae* protein acquired a second NLS allowing divergence of the sequence used in the homologs. Alternatively, it is possible that Rat1p enters the nucleus by association with another protein or RNA. Indeed, during the purification of Rat1p, a 45-kDa protein was reported to copurify with Rat1p; however, the function of the 45-kDa protein is not known (40). The previous finding that high-copy-number expression of *RAT1* complemented an *xrn1* mutation suggests that import of Rat1p into the nucleus becomes saturated by high-copy-number expression, allowing sufficient protein to remain in the cytoplasm. A previous attempt at Rat1p localization relied on high-copy-number expression of *RAT1*. Results of that analysis indicated Rat1p in the nucleus and cytoplasm (24), consistent with the notion that high-copy-number expression of *RAT1* leads to cytoplasmic mislocalization of the protein.

Rat1p and Xrn1p display similar in vitro biochemical activities. However, *RAT1* is an essential gene whereas *XRN1* is not. Since Rat1p function was maintained even when Rat1p was mislocalized throughout the cell, it was possible that the essential role of Rat1p could be carried out in the cytoplasm and that Xrn1p was incapable of carrying out this reaction. To test this idea, Xrn1p was directed to the nucleus by replacing the C-terminal 229 amino acids of Xrn1p with the SV40 large-T-antigen NLS fused to GFP. Such an *XRN1*-NLS-GFP1 construct complemented the temperature sensitivity of the *rat1-1* allele and was localized to the nucleus. Thus, targeting Xrn1p to the nucleus allows Xrn1p to substitute for Rat1p, indicating that Rat1p carries out an essential function in the nucleus. This result strongly suggests that the mutations that result in the mislocalization of Rat1p complement the *rat1-1* temperature sensitivity because sufficient Rat1p remains in the nucleus. More importantly, these results indicate that Rat1p and Xrn1p are functionally interchangeable proteins that normally reside and function in the nucleus and cytoplasm, respectively.

Possible roles of Rat1p. Although the data described here define Rat1p as a nuclear protein, they do not identify the substrates of Rat1p. From in vitro studies of Rat1p and Xrn1p, these enzymes are 5' exoribonucleases that require RNA substrates containing 5' PO₄ termini (40). Numerous candidate substrates exist in the nucleus, including excised introns, rRNA intermediates, and tRNA processing products. The nucleases responsible for degradation of excised introns in the nucleus are largely unknown. However, deletion of the gene encoding the lariat debranching enzyme in yeast is not lethal but results in accumulation of excised introns (7). This would suggest that if Rat1p plays a role in intron degradation, it is not an essential cellular function. *rat1* mutants display increased levels of an extended form of 5.8S RNA (2), suggesting that one pathway for generating the 5' end of 5.8S RNA involves trimming by Rat1p (16). However, other mutants that accumulate such

extended 5.8S RNA are viable (16), suggesting that this is not an essential function of Rat1p. It is also possible that Rat1p is required for the efficient removal of the portion of RNA polymerase II transcripts downstream of the poly(A) site. During transcription, the nascent transcript is cleaved at the site at which poly(A) is added. However, the portion of the transcript 3' to the cleavage site is a separate RNA species which could be eliminated by nucleases, perhaps in conjunction with an RNase H. In this regard, *rat1-1* does not show synthetic lethality with a deletion of the RNase H domain of Rnh1 (unpublished data). Lastly, it is likely that Rat1p, like Xrn1p, will act on numerous substrates, complicating the identification of its essential role.

Implications for Xrn1p. The complementation of an *xrn1* mutant by the mislocalization of Rat1p to the cytoplasm indicates that the pleiotropy of *xrn1* mutants is due to lack of cytoplasmic mRNA degradation. Thus, the observed nuclear defects of *xrn1* mutants (27, 32, 43) are likely the indirect consequences of altered gene regulation due to defective RNA turnover in the cytoplasm. The interchangeability of Rat1p and Xrn1p also suggests that specific protein-protein interactions of these proteins with other nuclear and cytoplasmic factors are not critical for their function. In this regard it has been suggested, based partially on the hypersensitivity of *xrn1* mutants to the tubulin-destabilizing drug benomyl, that Xrn1p interacts with tubulin. Complementation of the benomyl hypersensitivity by cytoplasmic Rat1p could suggest that Rat1p is also capable of tubulin interaction. On the other hand, fluorescence of Rat1-GFP and indirect immunofluorescence of Xrn1p in *S. cerevisiae* do not show colocalization with tubulin (unpublished data).

The various *RAT1* mutations identified here all restored cytoplasmic RNA turnover to similar degrees, even though localization studies indicated that the amount of Rat1p in the cytoplasm varied widely among the mutants. It is possible that Xrn1p and Rat1p are expressed in large excess over what is typically required in the cell and that significantly lower enzyme levels are sufficient for wild-type rates of most RNA degradation reactions. However, allele specificity was observed for suppression of benomyl hypersensitivity. This may reflect some other function of these proteins or the fact that unidentified RNA degradation or processing reactions are limiting in certain alleles, resulting indirectly in altered benomyl sensitivity.

Homologs of the *XRN1* and *RAT1* genes have been identified from mouse cells (3, 37), and a *RAT1* homolog has been identified from human cells (13a). The mouse Xrn1p homolog and the human Rat1p homolog display exonuclease activity (3, 13a), and the mouse *XRN1* and *RAT1* homologs complement the respective mutants in yeast (3, 37). Sequence analysis reveals that the Rat1p homologs contain putative bipartite NLSs, whereas the Xrn1p homologs do not. Thus, it is likely that within this family of eukaryotic exoribonucleases, the Xrn1 subfamily exists in the cytoplasm, where these proteins are responsible for mRNA turnover, whereas the proteins of the Rat1 subfamily are in the nucleus, where they function in some as yet unknown but essential role in RNA degradation.

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