The Product of the Saccharomyces Cerevisiae RSS1 Gene, Identified as a High-Copy Suppressor of the Rat7-1 Temperature-Sensitive Allele of the RAT7/NUP159 Nucleoporin, is Required for Efficient mRNA Export

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The Product of the \textit{Saccharomyces cerevisiae} \textit{RSS1} Gene, Identified as a High-Copy Suppressor of the \textit{rat7-1} Temperature-sensitive Allele of the \textit{RAT7/NUP159} Nucleoporin, Is Required for Efficient mRNA Export

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\textit{RAT7/NUP159} was identified previously in a screen for genes whose products are important for nucleocytoplasmic export of poly(A)$^+$ RNA and encodes an essential nucleoporin. We report here the identification of \textit{RSS1} (Rat Seven Suppressor) as a high-copy extragenic suppressor of the \textit{rat7-1} temperature-sensitive allele. \textit{Rss1p} encodes a novel essential protein of 538 amino acids, which contains an extended predicted coiled-coil domain and is located both at nuclear pore complexes (NPCs) and in the cytoplasm. \textit{RSS1} is the first reported high-copy extragenic suppressor of a mutant nucleoporin. Overexpression of \textit{Rss1p} partially suppresses the defects in nucleocytoplasmic export of poly(A)$^+$ RNA, rRNA synthesis and processing, and nucleolar morphology seen in \textit{rat7-1} cells shifted to the nonpermissive temperature of 37°C and, thus, restores these processes to levels adequate for growth at a rate approximately one-half that of wild-type cells. After a shift to 37°C, the mutant \textit{Rat7-1p/Nup159-1p} is lost from the nuclear rim of \textit{rat7-1} cells and NPCs, which are clustered together in these cells grown under permissive conditions become substantially less clustered. Overexpression of \textit{Rss1p} did not result in retention of the mutant \textit{Rat7-1p/Nup159-1p} in NPCs, but it did result in partial maintenance of the NPC-clustering phenotype seen in mutant cells. Depletion of \textit{Rss1p} by placing the \textit{RSS1} open reading frame (ORF) under control of the \textit{GAL1} promoter led to cessation of growth and nuclear accumulation of poly(A)$^+$ RNA without affecting nuclear protein import or nuclear pore complex distribution, suggesting that \textit{RSS1} is directly involved in mRNA export. Because both \textit{rat7-1} cells and cells depleted for \textit{Rss1p} are defective in mRNA export, our data are consistent with both gene products playing essential roles in the process of mRNA export and suggest that \textit{Rss1p} overexpression suppresses the growth defect of \textit{rat7-1} cells at 37°C by acting to maintain mRNA export.

\section*{INTRODUCTION}

The exchange of macromolecules between the nucleus and the cytoplasm is an essential process in eukaryotic cells. After transcription and post-transcriptional RNA processing, mRNAs are transported to the cytoplasm through nuclear pore complexes where they function in protein synthesis. Initial approaches to investigate RNA export involved microinjection of RNA-coated gold particles into the nucleus of \textit{Xenopus} oocytes,
which showed that poly(A)^+ RNA, tRNA, and 5S rRNA can be exported efficiently and in a saturable manner (Dworetzky and Feldherr, 1988). Nucleocytoplasmic export of Balbiani ring granules, abundant and specific premessenger ribonucleoprotein particles (RNP) produced in the salivary glands of Chironomus tentans, has been shown by electron microscopy to migrate through the central channel of the NPC (Mehlin et al., 1992). These RNP particles change from a globular to a more linear conformation as they begin to translocate through NPCs, with the 5' end of the RNP particle moving through the NPC first (Mehlin et al., 1992). Several proteins that mediate RNA transport have been identified in yeast. One of these proteins, called Npl3p, is closely related to mammalian hnRNP proteins and is known to shuttle between the nucleus and the cytoplasm (Flach et al., 1994). Despite all these findings, the lack of an in vitro system to study RNA export has made it difficult to gain a complete understanding of this process and to identify all of the genetic products this process requires.

Nuclear pore complexes (NPCs) form the only channels between nucleus and cytoplasm and are embedded in the nuclear envelope at places where the inner and outer nuclear membranes are fused. These complex structures, with a mass of ~125 mDa in metazoan cells and 65 mDa in Saccharomyces cerevisiae, are thought to contain at least 50 different polypeptide species (for review, Rout and Wente, 1994; Davis, 1995; Doye and Hurt, 1995). From high-resolution electron microscopy image reconstruction, NPCs are thought to consist of a central spoke/ring assembly that is anchored in the nuclear envelope by transmembrane protein components of the NPC, a central transporter through which substrates move as they are actively translocated into or out of the nucleus, a basket-like structure located at the nucleoplasmic face of the NPC, and fibrils that emanate from the cytoplasmic face of the NPC (Maul, 1977; Unwin and Milligan, 1982; Richardson et al., 1988; Allen and Douglas, 1989; Akey, 1990, 1991, 1995; Akey and Radermacher, 1993; Reichelt et al., 1990; Jarnik and Aebi, 1991; Goldberg and Allen, 1992; Hinshaw et al., 1992; Ris and Malecki, 1993). NPCs possess 9-nm channels through which solutes, ions, metabolites, and proteins smaller than 50 kDa move by passive diffusion. The transport of larger macromolecules, including proteins and ribonucleoproteins, is thought to use the transporter in the central channel of the NPC and requires both energy and specific signals within the transported macromolecule (for review, Elliot et al., 1994; Fabre and Hurt, 1994; Davis, 1995; Izaurralde and Mattaj, 1995).

Approximately 20 yeast nucleoporins and a smaller number from metazoan cells have been identified by biochemical, immunochemical, and genetic approaches (for review, Doye and Hurt, 1995). On the basis of their primary sequence, they fall into three broad classes. The first class consists of proteins with multiple repeats of short motifs that include GLFG, XFXFG, or XXFG. These repeats seem to interact with a multiprotein complex that contains a karyophilic protein to be imported into the nucleus and the heterodimeric receptor that recognizes nuclear localization sequences (NLSes). This receptor consists of Srp1p and Kap95p (Görlich et al., 1995; Iovine et al., 1995; Moroianu et al., 1995; Rexach and Blobel, 1995). The second class of nucleoporins consists of a few with transmembrane domains that are thought to anchor the pore in the nuclear envelope (Wozniak et al., 1989, 1994; Wozniak and Blobel, 1992; Greber et al., 1990). The third class consists of proteins that lack peptide repeats and are not transmembrane proteins. Many NPC proteins contain potential coiled-coil domains that could provide a basis for interactions among NPC polypeptides.

Through a screen for temperature-sensitive mutants of S. cerevisiae that accumulate poly(A)^+ RNA in their nuclei after a shift to the nonpermissive temperature, we isolated mutant alleles of RAT2/NUP120, RAT3/NUP133, RAT7/NUP159, RAT9/NUP85, and NUP145 (Gorsch et al., 1995; Heath et al., 1995; Li et al., 1995; Goldstein et al., 1996; Dockendorf and Cole, unpublished results). In cells carrying the rat7-1 allele of RAT7/NUP159, poly(A)^+ RNA accumulates very rapidly in the nuclei after a shift to the nonpermissive temperature, but there is no apparent defect in nuclear protein import. rat7-1 mutant cells show clustering of NPCs at 23°C and also have defects in nucleolar function and structure at 37°C (Gorsch et al., 1995; Heath et al., 1995; Kraemer et al., 1995). When rat7-1 cells are shifted to 37°C, Rat7p is lost from NPCs, and the pores rapidly become less clustered (Gorsch et al., 1995). By immunoelectron microscopy, Rat7p/Nup159p has been localized to a position at the cytoplasmic periphery of the NPC, and it may be a component of the cytoplasmic fibrils (Kraemer et al., 1995).

We conducted a screen for high-copy suppressors of the ts growth defect of the rat7-1 strain. The RAT7/NUP159 gene and plasmids encoding the carboxyl portion of Rat7p/Nup159p were isolated repeatedly. The most frequently isolated extragenic suppressor has been named RSS1 (Rat Seven Suppressor). RSS1 encodes a novel protein of 538 amino acids, which contains an extended predicted coiled-coil domain, and is located both at NPCs and in the cytoplasm. The presence of high-copy RSS1 permitted the rat7-1 strain to grow well at 37°C as it does in the absence of the suppressor at 23°C, but it did not restore wild-type growth properties to rat7-1 cells. RSS1 partially suppressed the nucleolar defects and the RNA export block of rat7-1 cells, but the mutant Rat7p/Nup159p was still lost from nuclear pores when cells were shifted to the nonpermissive temperature. Inter-
estingly, in the presence of high-copy RSS1, NPCs remained partially clustered in rat7-1 cells shifted to the nonpermissive temperature. When the RSS1 open reading frame (ORF) was placed under control of a GALI promoter, cells were able to grow only when the GALI promoter was active. When Rss1p was depleted by transferring these cells to glucose-containing media, almost all of the cells developed a defect in the export of poly(A)⁺ RNA within 9 h but did not show a defect in nuclear protein import, suggesting that Rss1p plays a primary role in RNA export.

MATERIALS AND METHODS

Yeast Strains, Cell Culture, and Genetic Methods

Yeast strains used in this study are listed in Table 1, and the plasmids used are listed in Table 2. Strains were cultured by standard methods (Sherman, 1991), using rich (YPD) media or defined media, synthetic complete (SC), lacking the appropriate amino acids or nucleotides. Wild-type strain FY86 was obtained from Fred Winston (Harvard Medical School, Boston, MA). Genetic techniques, including matings, sporulations, and plasmid transformations with electroporation, were performed by standard methods (Rose et al., 1989; Guthrie and Fink, 1991).

For temperature-shift experiments, cells cultured in liquid media at room temperature were shifted to a 37°C water bath, and incubation was continued as indicated in the figure legends. To prepare growth curves, single colonies of wild-type, mutant, or suppressed yeast cells were inoculated into 5 ml of SC-Leu and allowed to grow overnight at room temperature. Cell density was determined with a hemocytometer. Duplicate cultures of each strain were diluted to between 1 and 2 × 10⁶ cells/ml in SC-Leu and recounted. One duplicate was incubated at 33°C and the other at 37°C. Duplicate samples were removed from each culture after various times up to 30 h, and the cell number was determined by counting.

Isolation of Suppressors and Identification of RSS1

To isolate high-copy suppressors of the rat7-1 allele, we grew cells (LGY101) to an optical density (OD) of 1.4 (mid-log phase) and transformed them with electroporation by using a yeast genomic library present in the high-copy plasmid YEp13 (Nasmyth and Tatchell, 1980). Transformants were plated on SC-Leu plates and incubated at the restrictive temperature of 37°C. Colonies that appeared contained putative high-copy suppressors. To confirm that the suppression was due to a gene present on the library plasmid and not to a secondary mutation in the strain genome, we retrieved the plasmid from individual strains that grew at 37°C and tested it for its ability to restore growth at 37°C to rat7-1 cells (LGY101). Plasmids were digested with various restriction endonucleases to identify those that contained overlapping DNA fragments. The majority of plasmids identified contained either RAT7/NUP159, fragments of RAT7/NUP159, or the region of chromosome IV containing the RSS1 gene, the subject of this report. RSS1 was cloned by retrieving the plasmid containing the library fragment with the suppressor gene from the rat7-1 mutant strain. Both ends of the library fragment were sequenced with an ABI373 automated sequencer. These identified a region of yeast chromosome IV that had been sequenced as part of the yeast genome sequencing project and contained multiple open reading frames. The nucleotide sequence of this portion of chromosome IV was determined by an Applied Biosystems 373 (Foster City, CA) automated sequencer that used fluorescent dye terminators and cycle sequencing. The sequence of both DNA strands was determined. Results of one set of sequencing reactions were used to design primers to permit extension of the sequence.

Table 1. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FY86</td>
<td>MATa ura3-52 his3Δ200 leu2Δ1</td>
<td>Wild-type; derived from S288C; (Winston et al., 1995)</td>
</tr>
<tr>
<td>LGY101</td>
<td>MATa ura3-52 his3Δ200 leu2Δ1 rat7-1ts</td>
<td>Sogregant from 3rd backcross (Gorsch et al., 1995)</td>
</tr>
<tr>
<td>VDPY102</td>
<td>MATa ura3-52 his3Δ200 leu2Δ1 rat7-1ts pVPD2: LEU2 RSS1 2μ</td>
<td>pVPD2 isolated as a high-copy suppressor of rat7-1</td>
</tr>
<tr>
<td>VDPY107</td>
<td>MATa ura3-52 his3Δ200 leu2Δ1 rat7-1ts pVPD7: LEU2 RSS1 2μ</td>
<td>pVPD7: RSS1 ORF subcloned into YEpLac1, provided by Dr. Anita Corbett, Harvard Medical School, Boston, MA</td>
</tr>
<tr>
<td>ACY1</td>
<td>MATα/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/hipsΔ200 trp1Δ63/TRP1</td>
<td>heterozygous diploid rss1 null strain</td>
</tr>
<tr>
<td>VDPY108</td>
<td>MATα/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/hipsΔ200 trp1Δ63/TRP1 RSS1/RSS1::his3</td>
<td>heterozygous diploid rss1 null strain harboring RSS1 plasmid</td>
</tr>
<tr>
<td>VDPY109</td>
<td>MATα/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/hipsΔ200 trp1Δ63/TRP1 RSS1::his3 URA3 RSS1::CEN</td>
<td>haploid rss1 null resulting from VDPY109 sporulation</td>
</tr>
<tr>
<td>VDPY111</td>
<td>MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 RSS1::his3 (pVPD9: URA3 RSS1 CEN)</td>
<td>haploid rss1 null strain harboring RSS1myc- containing plasmid</td>
</tr>
<tr>
<td>VDPY112</td>
<td>MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 RSS1::his3 (pVDP12: LEU2 RSS1myc CEN)</td>
<td>haploid rss1 null strain harboring RSS1myc in a 2μ plasmid</td>
</tr>
<tr>
<td>VDPY113</td>
<td>MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 RSS1::his3 (pVDP13: LEU2 RSS1myc 2μ)</td>
<td>haploid rss1 null strain harboring GAL1::RSS1myc- containing plasmid</td>
</tr>
<tr>
<td>VDPY114</td>
<td>MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 RSS1::his3 (pVDP14: LEU2 GAL::RSS1 CEN)</td>
<td>haploid rss1 null strain harboring GAL1::RSS1myc- containing plasmid</td>
</tr>
<tr>
<td>OLY101</td>
<td>MATα his3Δ200 leu2Δ1 ura3-52 rat3-1 nup133-1</td>
<td>Li et al., 1995</td>
</tr>
<tr>
<td>CCY282</td>
<td>MATα trp1Δ63 ura3-52 leu2Δ1 rat2-1 nup120-1</td>
<td>Heath et al., 1995</td>
</tr>
<tr>
<td>AGY401</td>
<td>MATα ura3-52 leu2Δ1 trp1Δ63 rat9-1 nup85-1</td>
<td>Goldstein et al., 1996</td>
</tr>
<tr>
<td>AGY48</td>
<td>MATα his3Δ200 leu2Δ1 ura3-52 nup145-10</td>
<td>Dockendorf and Cole, unpublished data</td>
</tr>
</tbody>
</table>
The ORF responsible for suppression of the rat7-1 mutation was identified by deleting various ORFs contained in the library plasmid, transforming the derived plasmids back into rat7-1 cells, and plating at 37°C. The RSS1 ORF and flanking regulatory regions sufficient for suppression of the rat7-1 mutation were subcloned into YepLac181 (Gietz and Sugino, 1988).

**Disruption of RSS1**

A polymerase chain reaction (PCR)-based gene deletion approach was used to delete RSS1 (Baudin et al., 1993). By PCR amplification, a cassette was generated that contained the yeast HIS3 gene flanked at its ends by 45 nucleotides identical to sequences just upstream and downstream of the RSS1 coding region. For both oligonucleotides used for PCR, the last 17 nucleotides are identical to sequences at the ends of the HIS3 selectable gene. The oligo sequences are the following: for the 5′ end, 5′-CGAAGAATGAGATTTGTGTTCGAT-GAGGTITTTTCAATCAGATACCTGCTCTCTAGATCCT-3′; and for the 3′ end, 5′-GTGCTCATATAATGGATGTTTCTAGATCTCAGATTCTA-AGGAGACATTTCGGAACGGCGCTCTGGTACCGATGCT-3′. We used plasmid pB2815 (obtained from P. Silver, Harvard Medical School, Boston, MA) containing the HIS3 gene as a template in the PCR reaction. The PCR conditions were 30 cycles with denaturation at 94°C for 1.5 min, annealing at 50°C for 2.0 min, and extension at 72°C for 2.0 min. The PCR product was transformed into a wild-type diploid strain (ACY1), homozygous for his3Δ200. The flanking upstream and downstream sequences targeted the deletion construct to the RSS1 locus such that the RSS1 ORF was replaced with HIS3 by homologous recombination. Recombinants were selected by plating the transformed cells on SC-His plates. Homologous recombination at this locus was verified by PCR and by Southern analysis.

To determine whether RSS1 is essential for vegetative growth, we transformed the strain heterozygous for RSS1 (VDPY108) with pVDP9, which contains WT RSS1 in YEpLac33 (ura¹). The transformants (VDPY109) were sporulated, the tetrads were dissected, and the four-spore tetrads were scored for auxotrophic markers and for the ability to grow on 5-FOA plates.

**GAL1::RSS1 and GAL1::RSS1myc Constructs**

A PstI-HindIII fragment containing the RSS1 ORF was subcloned into the BamHI-HindIII sites of the YCPGAL1 vector (provided by Anita Corbett, Harvard Medical School, Boston, MA). The resulting plasmid (pVDP14) was transformed into VDPY111 strain, and the plasmid-borne wild-type copy of RSS1 was eliminated by plating cells onto 5-FOA. To obtain the GAL1::RSS1myc construct, a fragment of the RSS1 ORF containing the "myc" epitope at its carboxy terminus was subcloned by similar procedures.

**In Situ Hybridization and Immunofluorescence Assays**

In situ hybridization assays for poly(A)⁺ RNA localization and indirect immunofluorescence (IF) techniques for protein localization have been described previously (Amberg et al., 1992; Copeland and Snyder, 1993). Antibody against Rat7p/Nup159p raised in guinea pigs (Gorsch et al., 1995) was used at 1:3000 dilution. FITC-conjugated goat anti-guinea pig immunoglobulin G (IgG; Vector Laboratories, Burlingame, CA) was used at 1:250 dilution. RL-1 monoclonal antibody (a gift from Larry Gerace, Scripps Research Institute, La Jolla, CA; Snow et al., 1987) was used at a dilution of 1:200. FITC-conjugated goat anti-mouse IgM (Vector Laboratories) was used at a dilution of 1:600. Monoclonal antibody 2.2B, which recognizes nucleolar Nar1p in yeast (a gift from M. Snyder, Yale University, New Haven, CT), was used at a dilution of 1:10. FITC-conjugated goat anti-mouse IgG (Vector Laboratories) was used at a dilution of 1:600.

For all fluorescence microscopy, images were obtained by using a cooled CCD (charge-coupled device) camera. Identical exposure conditions were used for all comparable images within each figure; that is, all fluorescein isothiocyanate (FITC) images within a single figure were obtained by using the same exposure conditions, as were all 4′,6-diamidino-2-phenylindole (DAPI) images, although the exposure conditions for FITC images and for DAPI images were not the same. Composites were prepared with Adobe Photoshop without altering the images.

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

RNA was extracted by the method of Tollervey (Snow et al., 1987), and the incorporation of [³²P]-uridine into rRNA and its precursors was monitored as described previously (Heath et al., 1995).

**Epitope Tagging and Immunolocalization**

Site-directed mutagenesis was used to create a unique restriction endonuclease site near the C-terminal region of the RSS1 gene where the sequence encoding three copies of the epitope recognized by the 9E10 anti-myc monoclonal antibody was inserted in-frame within the RSS1 coding region. The triple myc tag was inserted into a BamHI site created at amino acid 529 (total of 538 amino acids) by using a site-directed mutagenesis kit (Clonetech Laboratories, Palo Alto, CA). The plasmid containing the myc-tagged RSS1 (pVDP12 or pVDP13) was transformed into the strain VDPY111 carrying a disruption of RSS1 and a plasmid containing a wild-type copy of RSS1 (pVDP9). After selection on SC-Leu, the plasmid containing the wild-type RSS1 gene was eliminated by plating on 5-FOA. Cells carrying the myc-tagged RSSI allele (VDPY112, VDPY113) were subjected to indirect immunofluorescence. The primary antibody used was 9E10 mouse IgG monoclonal anti-myc from tissue culture hybridoma cells (a gift from Drs. J. Zhu and J.M. Bishop, University of California, San Francisco, CA) at a dilution of 1:1. The secondary antibody used was FITC-conjugated goat anti-mouse IgG (Vector Laboratories) at a dilution of 1:100.

To examine colocalization of Rss1pmyc and NPC antigens, we stained cells simultaneously with the RL-1 anti-nucleoporin antibody (1:200 dilution) and the 9E10 anti-myc monoclonal antibody (1:1 dilution). RL-1 is an IgM, and 9E10 is an IgG. We used FITC-conjugated goat anti-mouse IgG (1:100 dilution) to visualize the sites where Rss1pmyc was located and used Texas Red-conjugated goat anti-mouse IgM (1:600 dilution) to visualize where RL-1 antigens were located. When the FITC-conjugated secondary antibodies were left out, there was no signal from the Texas Red secondary.
antibody in the FITC channel; similarly, when the Texas Red-conjugated secondary antibodies were left out, there was no signal from the FITC-conjugated secondary antibody in the Texas Red channel.

**Western Analysis**

Cells were grown to a total OD\textsubscript{600} of 0.75. Cells were pelleted by centrifugation at 2500 rpm for 2.5 min and washed once with water. Acid-washed glass beads (200 mg) and 200 \mu l of hot sample buffer (62.5 mM Tri-cit, pH 6.8, 2% SDS, 10% glycerol, 8 M urea, 0.72 M 2-mercaptoethanol, and 0.05% bromophenol blue) were added to the cell pellets. After brief vortexing, cells were transferred to a 100°C water bath for 3 min. Cells were then lysed as follows: 10 s of vortexing and 50 s of incubation in a 100°C water bath, repeated five times during 5 min. The supernatant was then transferred to a clean Eppendorf tube, and equal volumes of cell lysate were loaded onto a 10% SDS-polyacrylamide gel. The gel was run at 200 V for ~50 min. Proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting overnight at 100 mA in a cold room. The membrane was briefly washed with a 1 \times PBS, 0.1% Tween 20 solution (solution A) and then blocked for 1 h with a 1 \times PBS, 0.1% Tween 20, 5% nontat milk solution (solution B). The membrane was then incubated with 9E10 anti-myc monoclonal antibody at a 1:10 dilution in solution B for 2 h. Four washes of 10 min each were done with solution A. The membrane was then incubated with anti-mouse antibody coupled to the enzyme (Amersham, Arlington Heights, IL) diluted 1:3000 in solution B for 1 h. Washes were performed as before (see above). Signal was developed by treating the membrane with the enhanced chemiluminescence (ECL) kit (Amersham). As a control for loading, membranes were incubated with polyclonal rabbit anti-Secl3p antibody (kindly provided by Dr. Charles Barlowe, Dartmouth Medical School, Hanover, NH) at a 1:2000 dilution and anti-rabbit antibody coupled to horseradish peroxidase (Amersham) at a 1:10,000 dilution.

**Electron Microscopy**

*S. cerevisiae* were examined by electron microscopy with the following previously described protocols: Byers and Goetsch (1975), Wright and Rine (1989), and Goldstein et al. (1996). Sections were cut on a Sorvall MT5000 (RMC, Tucson, AZ) ultramicrotome with a section thickness of 90 nm and examined on a JEOL 100CX (Peabody, MA) electron microscope at 80 kV.

**RESULTS**

**Identification of RSS1 in a Screen for High-Copy Extragenic Suppressors of the rat7-1 ts Allele**

To identify proteins that might interact with Rat7p/Nup159p, we performed a high-copy extragenic suppressor screen of the *rat7-1* ts allele. The *rat7-1* strain (LYG101) was grown to mid-log phase and transformed by electroporation with a yeast genomic library present in a 2μ-based, high-copy vector. The transformants were plated at 37°C to select for those that acquired the ability to grow at the nonpermissive temperature because of the presence of a library plasmid. More than 30,000 colonies were screened and 38 putative suppressors isolated. The results are summarized in Table 3. We isolated plasmids containing the complete RAT7/NUP159 gene several times, as well as DNA fragments encoding the C-terminal portion of the Rat7p/Nup159p. The fact that the C-terminal end of Rat7p/Nup159p can suppress the *rat7-1* ts allele suggests that this domain probably plays a critical role in the function of Rat7p/Nup159p, and further studies of this possibility are in progress. In addition to these intragenic suppressors, we isolated a number of extragenic suppressors. One of these was independently identified four times, and we call this gene *RSS1* (Rat Seven Suppressor 1). Because of the frequency with which *RSS1* was isolated, it was selected for further study.

To identify the *RSS1* gene, the plasmid containing this extragenic suppressor was retrieved from the *rat7-1* strain, and DNA sequence was obtained from both ends of the library fragment. This sequencing data positioned *RSS1* on chromosome IV between *NHP2* and *HEM3*. The suppressing plasmid contained three new ORFs in addition to the fragments of *NHP2* and *HEM3* (Figure 1A). To determine which of the ORFs corresponded to *RSS1*, we deleted each putative ORF individually, the ligated library vector was transformed into *rat7-1* cells, and cells were plated at 37°C. When ORF1 was deleted, the growth defect at 37°C of *rat7-1* cells could not be suppressed, indicating that this ORF was likely *RSS1*. To confirm, we subcloned ORF1 and flanking regulatory signals into YepLac181 (to produce pVDP7), and we found them to suppress the growth defect of *rat7-1* cells at 37°C. *RSS1* encodes a novel protein of 538 amino acids with a predicted molecular weight of 62 kDa and a pI of 9.03. The sequence of this protein is shown in Figure 1B. An extended segment of Rss1p (amino acids 124–255) is predicted (Lupas et al., 1991) to have a very high potential (p > 99.9%) to form a coiled-coil structure (Figure 1C). No other obvious structural features or motifs were found. Rss1p shows no homology with other proteins outside of its potential coiled-coil region.

To determine whether *RSS1* is an essential gene, we replaced the *RSS1* coding region with the *HIS3* gene. Haploid cells containing the disrupted copy of *RSS1*
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A

NHP2 ORF1 RSS1 ORF2 ORF3 HEM3

B

1. MRFVFDEVYN SDDPCEFEE TCSTTSSTSS QCPTPEPSGA IKLPSPTKVG TTKLVNESVV
61. ILDPALENAL RDLNQSQSLI FINEPVAAS SILVPHSTMN PPLRASHSSL LDAKNSNAT
121. APLLEAIEES QFRDGNCQVL ANQEISIR ENKREVEOR KRKEEEER KEAERKARE
181. QELLAQDKDR EERKKEAEKL KLAQQKEEEE RKLKKKKEEA KLLQKDKLG
241. KAVTNFDKIS KMFNYKIDKI AQIKDQIVLP IEDKADNVRN LLSRKHRRKN PKKQLTSNSN
301. QQLFKIQNEL TQLINDTGD SILAYHWILNF IAKAVVHAQE TERVKPESEA LLPKQLTLYL
361. LQQFPFELQEL FMARLVKCP FVGFTCEID TEGROQKMGW KRUNENKWEED NTSYERKKG
421. ILSLFAPTR LQQPQEIFIT TSHFFPIALS WHILRICNT PLNLTNTNP VIGSWMDOAA
481. AQQFLQAYGN QASKLLILIG EELTSHMAEK KYYYAARLLI LLAEAWQNNRM ESFFPESP

C

Coiled-coil probability of Rss1p

Figure 1. The S. cerevisiae RSS1 gene. (A) Schematic representation of the region of yeast chromosome IV in which RSS1 is located. Important restriction endonuclease digestion sites are shown. The arrows show the putative ORFs in this region. The two HindIII sites indicated were used to subclone RSS1. (B) Predicted amino acid sequence of Rss1p, which contains 538 amino acids. (C) Coiled-coil probability for Rss1p; the PEPCOIL program was used to search Rss1p for potential coiled-coil regions (Lupas et al., 1991).

were not able to grow in the absence of a plasmid containing the RSS1 gene, indicating that RSS1 is essential for growth. We also transformed pVDP7 (the Yeplac181 vector containing RSS1) into a strain containing a disruption of RAT7/NUP159 and containing the wild-type RAT7/NUP159 gene on a URAS3/CEN plasmid. These cells were unable to grow on 5-FOA, indicating that overexpression of RSS1 cannot bypass the need for Rat7p/Nup159p. When the RSS1 gene was cloned into a CEN plasmid, it was no longer able to suppress the rat7-1 growth defect at 37°C, indicating that overexpression of RSS1 is required for suppression.

To determine whether suppression of rat7-1 by high-copy RSS1 was specific for the rat7-1 mutant allele, we introduced pVDP7 into strains carrying the rat2-1/nup120-1, rat3-1/nup133-1, rat9-1/nup85-1, and nup145-10 ts mutant alleles. The transformants were plated both at 23 and 37°C. At 23°C, the growth of none of the strains was affected by the RSS1 suppressor. However, no growth was detected in any of the strains at the restrictive temperature in the presence of high-copy RSS1. These data indicate that RSS1 is not a general suppressor of ts alleles of yeast nucleoporins.
Partial Suppression of rat7-1 Defects by High-Copy RSS1

rat7-1 cells rapidly stop growing when shifted to the nonpermissive temperature of 37°C (Gorsch et al., 1995). To determine how well RSS1 in high copy suppressed the growth defect of rat7-1 cells at 37°C, we monitored the growth rates of wild-type and rat7-1 cells in the presence and absence of RSS1 (Figure 2). At the permissive temperature (23°C), the growth behavior of the rat7-1 was the same in the presence or absence of high-copy RSS1, but cells did not multiply as rapidly as wild-type cells. At 37°C, the presence of high-copy RSS1 permitted rat7-1 cells to grow at 37°C at a rate essentially identical to that of rat7-1 cells at 23°C. However, this growth rate was still below that of wild-type cells (FY86). Thus, high-copy RSS1 partially suppressed the growth defect of rat7-1 cells at 37°C, and its overexpression had no deleterious effect on the growth of rat7-1 or wild-type cells at 23°C.

rat7-1 cells accumulate poly(A)⁺ RNA in their nuclei rapidly after a shift to the restrictive temperature. After 15 min at 37°C, 100% of the cells show dramatically enhanced nuclear accumulation of poly(A)⁺ RNA (Gorsch et al., 1995). We examined the effect of high-copy RSS1 on mRNA export in rat7-1 cells by using fluorescence in situ hybridization. Figure 3 shows the subcellular location of poly(A)⁺ RNA in wild-type cells, rat7-1 cells, and rat7-1 cells in the presence of high-copy RSS1, both at the permissive and restrictive temperatures. In all three strains grown at 23°C, poly(A)⁺ RNA was distributed throughout the cell, although some nuclear accumulation of poly(A)⁺ RNA was seen in rat7-1 cells (Figure 3E), even in the presence of high-copy RSS1 (Figure 3I). After a 2-h shift to 37°C, nuclear accumulation of poly(A)⁺ RNA was seen in 100% of rat7-1 cells with almost no cytoplasmic signal (Figure 3G). Poly(A)⁺ RNA was found often in a pattern of multiple punc-

Figure 2. Comparison of the growth characteristics of strains FY86 (WT), LGY101 (rat7-1), and VDPY (rat7-1 and containing RSS1 on a 2μ plasmid) at 23 and 37°C. Cells were allowed to reach stationary phase and diluted; then growth was monitored at both 23 and 37°C. Duplicate samples were taken after 2, 3, or 6 h of growth, and cells were counted with a hemacytometer. Each point is an average of cell counts from duplicate samples. Strains were also grown on SC-Leu plates for 4 d at 23 or 37°C.
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Figure 3. High-copy RSS1 partially suppresses the mRNA export defect of rat7-1 cells. (A–D) WT (FY86); (E–H) rat7-1 cells; (I–L) RSS1 (rat7-1 cells transformed with pVDP7). Cells were grown to mid-log phase at 23°C and then either left at 23°C (A, B, F, I, and J) or shifted to 37°C for 2 h (C, D, G, H, K, and L). Cells were then fixed with formaldehyde and subjected to in situ hybridization by using a digoxigenin-tagged oligo (dT50) probe to detect poly(A)⁺ RNA. Hybridizing probe was visualized by FITC-conjugated anti-digoxigenin antibody (A, C, E, G, I, and K). The same fields of cells were also stained with DAPI (B, D, F, H, J, and L). Images were taken by using a cooled CCD camera. (A) WT, 23°C, FITC; (B) WT, 23°C, DAPI; (C) WT, 37°C, FITC; (D) WT, 37°C, DAPI; (E) rat7-1, 23°C, FITC; (F) rat7-1, 23°C, DAPI; (G) rat7-1, 37°C, FITC; (H) rat7-1, 37°C, DAPI; (I) high-copy RSS1, 23°C, FITC; (J) high-copy RSS1, 37°C, DAPI; (K) high-copy RSS1, 37°C, FITC; (L) high-copy RSS1, 37°C, DAPI.

tate spots, which colocalize with nucleolar antigens (Del Priore and Cole, unpublished results). When rat7-1 cells containing high-copy RSS1 were shifted to 37°C for 2 h, most of the poly(A)⁺ RNA signal was present in the nucleus, but there was also a clear cytoplasmic signal that was not present in rat7-1 cells lacking high-copy RSS1 (Figure 3, compare K with G). However, this cytoplasmic signal was much weaker than the cytoplasmic signal seen in wild-type cells shifted to 37°C (Figure 3C). No changes in signal intensities were seen when these cultures were shifted to 37°C for 4 h (Del Priore and Cole, unpublished results). We conclude that high-copy RSS1 partially suppressed the block to mRNA export caused by the rat7-1 mutation.

Fragmentation of the nucleolus after a shift to 37°C has been observed at the nonpermissive temperature in several yeast strains defective for mRNA export (Kadowaki et al., 1994, 1995; Heath et al., 1995; Goldstein et al., 1996), including strains carrying the rat7-1 allele (Heath et al., 1995). To examine nucleolar fragmentation in rat7-1 cells grown at 23°C or shifted to 37°C for 2 h, we performed indirect immunofluorescence analysis with a monoclonal antibody against the nucleolar protein, Nsrlp (monoclonal antibody 2.3B). The results are shown in Figure 4. When grown at the permissive temperature, rat7-1 cells show a distribution of the nucleolar antigen that is the same as wild type (Figure 4, compare A and E). The signal is present in a cres-
Figure 4. Indirect immunofluorescence with an anti-fibrillarin antibody to test for nucleolar fragmentation. (A–D) WT (FY86); (E–H) rat7-1; (I–L) high-copy RSS1 (rat7-1 cells transformed with pVDP7). Cells were grown to mid-log phase at 23°C and then either maintained at that temperature (A, B, E, F, I, and J) or shifted to 37°C for 2 h (C, D, G, H, K, and L). Fixation and immunofluorescence techniques were performed by following the protocol described in MATERIALS AND METHODS. The DAPI images are of the same fields of cells as the FITC images to their left. (A) WT, 23°C, FITC; (B) WT, 23°C, DAPI; (C) WT, 37°C, FITC; (D) WT, 37°C, DAPI; (E) rat7-1, 23°C, FITC; (F) rat7-1, 23°C, DAPI; (G) rat7-1, 37°C, FITC; (H) rat7-1, 37°C, DAPI; (I) high-copy RSS1, 23°C, FITC; (J) high-copy RSS1, 23°C, DAPI; (K) high-copy RSS1, 37°C, FITC; (L) high-copy RSS1, 37°C, DAPI.

cent-shaped pattern, and the DAPI signal, which represents DNA staining, seems to be excluded from the location where the intensity of the nucleolar signal reaches its maximum. When shifted to 37°C, wild-type cells displayed the same distribution of the nucleolar antigen that was seen at 23°C (Figure 4C). In contrast, after a 2-h shift to 37°C, there was a dramatic fragmentation of the nucleoli in rat7-1 cells, in agreement with our previous findings (Heath et al., 1995). In almost all cells, two to three nucleolar fragments can be seen (Figure 4G). Fragmentation was detected as soon as 20 min after shifting to the nonpermissive temperature (Gorsch and Cole, unpublished results).

When grown at the permissive temperature, the rat7-1 cells containing high-copy RSS1 displayed the same pattern for nucleolar antigen distribution as wild-type and rat7-1 cells (Figure 4, compare I with A and E). After a 2-h shift to the nonpermissive temperature of 37°C, less dramatic nucleolar fragmentation was seen in the presence of high-copy RSS1 than in similarly treated rat7-1 cells (Figure 4, compare K with G). Only a few cells showed fragmentation of the nucleolus, although few cells show the crescent-shaped morphology seen in wild-type cells. Thus, when RSS1 was present in high-copy in rat7-1 cells, the nucleolar fragmentation phenotype observed in the absence of RSS1 was partially suppressed.

In temperature-sensitive yeast strains in which nucleolar disruption is seen after a shift to the nonpermissive temperature, defects in rRNA synthesis
and processing have also been reported (Traglia et al., 1989; Aebi et al., 1990). The fact that the rat7-1 strain showed a severe nuclear disruption phenotype when shifted to the nonpermissive temperature prompted us to determine whether it also showed defects in rRNA processing. We performed a [3H]-uridine incorporation assay in wild-type and rat7-1 cells at both the permissive and nonpermissive temperatures. When rat7-1 cells were shifted to 37°C, the incorporation of [3H]-uridine into precipitable counts decreased to 20% of the level of incorporation seen at 23°C within 15 min of the shift to 37°C and to 10% after a 30-min shift. The labeled rRNA produced was analyzed by electrophoresis and fluorography (Figure 5). The autoradiograph shows that there was no significant difference in rRNA processing between wild-type and rat7-1 cells when both were grown at the permissive temperature (compare Figure 5, lanes 1 and 3). In contrast, after 1 h at 37°C, rat7-1 cells accumulated the 35S precursor, which is the primary transcript of yeast rRNA genes, and an aberrant 23S RNA (Figure 5, lane 4). Accumulation of 35S and 23S rRNAs was also seen in the control strain carrying the rna1-1 allele when it was shifted to 37°C for 40 min (Figure 5, lane 10), as has been reported previously (Traglia et al., 1989). These defects could be seen as soon as 15 min after the temperature shift of rat7-1 cells (Del Priore and Cole, unpublished results) and were more severe 2 h after the shift (Figure 5, lane 5).

To determine whether the presence of high-copy RSSI in rat7-1 cells had any effect on the level of rRNA synthesis and the fidelity of rRNA processing when this strain was shifted to 37°C, we measured the incorporation of [3H]-uridine into precipitable counts. After a 1-h shift to 37°C, incorporation in the presence of high-copy RSSI was 20% of the level of incorporation seen at 23°C and subsequently increased to 30% of the 23°C incorporation after 2 h at 37°C. We then analyzed the labeled rRNA by electrophoresis. The data show that, in the presence of high-copy RSSI, there was a partial suppression of the rRNA processing defects seen in rat7-1 cells at the restrictive temperature (Figure 5, compare lanes 7 and 8 with lanes 4 and 5). Accumulation of 35S rRNA was still detected but to a lesser degree than in rat7-1 cells, and there was no significant accumulation of 23S rRNA. Thus, in rat7-1 cells shifted to 37°C, there were defects in the levels of rRNA synthesis and in the kinetics and fidelity of processing of the 35S rRNA precursor. In the presence of high-copy RSSI, we observed partial suppression of the defects in the levels and kinetics of rRNA processing and no longer saw defects in the fidelity of processing.

**Figure 5.** Analysis of rRNA processing. Cells were labeled with [5-3H]-uridine, and total RNA was isolated from WT (FY86), rat7-1, and high-copy RSSI (rat7-1 transformed with pVDP7) cells grown either at 23°C or shifted to 37°C for 1 or 2 h. Labeled RNA was then analyzed by electrophoresis of approximately equal numbers of counts of labeled RNA in a 1.2% agarose-formaldehyde gel. As a control for rRNA processing defects, a strain carrying the rna1-1 allele was included. (1) WT grown at 23°C; (2) WT grown at 37°C for 2 h; (3) rat7-1 grown at 23°C; (4) rat7-1 grown at 37°C for 1 h; (5) rat7-1 grown at 37°C for 2 h; (6) high-copy RSSI grown at 37°C; (7) high-copy RSSI grown at 37°C for 1 h; (8) high-copy RSSI grown at 37°C for 2 h; (9) rna1-1 grown at 23°C; (10) rna1-1 grown at 37°C for 40 min. Asterisk indicates a 23S intermediate product often seen in strains with rRNA processing defects.

Rat7p Is Lost from NPCs at 37°C both in the Presence and Absence of High-Copy RSSI, but NPC Clustering Is Partially Maintained by Rss1p Overexpression

When rat7-1 cells are shifted to the nonpermissive temperature, the mutant Rat7p is rapidly lost from NPCs, as determined by immunofluorescence with an anti-Rat7p antibody (Gorsch et al., 1995). To see whether overexpression of Rss1p was sufficient to maintain Rat7p at the nuclear rim, we examined the location of Rat7p/Nup159p by indirect immunofluorescence in wild-type cells and in rat7-1 cells either lacking or containing high-copy RSSI. The results are shown in Figure 6. At 23°C, wild-type cells showed a punctuate staining pattern surrounding the nucleus (Figure 6A), which was localized by staining with DAPI (Figure 6B). As reported previously (Gorsch et al., 1995), NPCs were clustered in rat7-1 cells grown at 23°C (Figure 6F). An identical pattern of clustered NPCs was seen at 23°C in rat7-1 cells containing high-copy RSSI (Figure 6K).

When cells were shifted to 37°C for 2 h, the anti-Rat7p/Nup159p antibody continued to stain wild-type cells with the same punctate rim staining pattern seen in cells grown at 23°C (Figure 6C). In contrast, no signal for Rat7p/Nup159p was detectable in rat7-1 cells, either in the absence (Figure 6H) or presence (Figure 6M) of high-copy RSSI. Thus, the presence of high-copy RSSI was unable to prevent the loss from NPCs of mutant Rat7p/Nup159p when mutant cells were shifted to 37°C.
Clustering of NPCs is a common phenotype seen in yeast cells carrying mutant alleles of several different nucleoporins (Wente et al., 1992; Wente and Blobel, 1993, 1994; Bogerd et al., 1994; Doye et al., 1994; Aitchison et al., 1995; Gorsch et al., 1995; Heath et al., 1995; Li et al., 1995; Pemberton et al., 1995; Goldstein et al., 1996). The rat7-1 strain has a novel NPC clustering phenotype in that pores become considerably less clustered after a shift to 37°C (Gorsch et al., 1995). This altered distribution of NPCs in rat7-1 cells occurs rapidly, within 1 h of a shift to 37°C. To determine how high-copy RSS1 affected this phenotype, we performed indirect immunofluorescence with the RL-1 monoclonal antibody, which recognizes NPC antigens in both metazoan and yeast cells (Snow et al., 1987; Copeland and Snyder, 1993). At 23°C in wild-type cells and in rat7-1 cells in the presence or absence of high-copy RSS1, the pattern of staining with the RL-1 antibody was the same as that seen with the anti-Rat7p antiserum (compare Figure 6, A, F, and K, with Figure 7, A, F, and K, respectively). When shifted to 37°C for 2 h, a high percentage of rat7-1 cells showed a redistribution of NPC antigens to a pattern that was very similar to the wild-type distribution (Figure 7, compare H with C), in agreement with observations reported previously (Gorsch et al., 1995). In contrast, in the presence of high-copy RSS1, NPCs in rat7-1 cells showed a tendency to retain their clustered distribution (Figure 7M).

We also examined rat7-1 cells in the presence or absence of high-copy RSS1 by thin-section electron microscopy. In different cells, NPCs were seen to be evenly distributed, grouped, or clustered. We considered NPCs to be clustered when all or almost all of the NPCs in a thin section were clustered together, forming a single “grape-like” structure. Pores were considered to be grouped when multiple NPCs were adjacent in the plane of the nuclear envelope. Often, multiple groups were seen in a single thin section, and additional isolated NPCs were also present. Cluster-
Cells transformed K, G, S. cerevisiae. The DAPI and DIC including immunofluorescence, were examined (N) high-copy RSS1, 37°C, DAPI; (O) high-copy RSS1, 37°C, DAPI; (K) high-copy RSS1, 23°C, DAPI; (L) high-copy RSS1, 23°C, FITC; (N) high-copy RSS1, 37°C, DAPI; (O) high-copy RSS1, 37°C, DIC.

Figure 7. Indirect immunofluorescence to examine the distribution of NPCs. (A–E) WT (FY86); (F–J) rat7-1; (K–O) high-copy RSS1 (rat7-1 cells transformed with pVDP7). Cells were grown to log phase at the permissive temperature and then either maintained at 23°C (A, B, F, G, K, and L) or shifted to the nonpermissive temperature of 37°C (C–E, H–J, and M–O). After fixation and processing for indirect immunofluorescence, cells were incubated with the RL-1 monoclonal antibody, which recognizes NPC proteins from many organisms, including S. cerevisiae. The DAPI and DIC images are of the same fields of cells as the FITC images to their left. (A) WT, 23°C, FITC; (B) WT, 23°C, DAPI; (C) WT, 37°C, FITC; (D) WT, 37°C, DAPI; (E) WT, 23°C, DAPI; (F) rat7-1, 23°C, FITC; (G) rat7-1, 23°C, DAPI; (H) rat7-1, 37°C, FITC; (I) rat7-1, 37°C, DAPI; (J) rat7-1, 37°C, DIC; (K) high-copy RSS1, 23°C, FITC; (L) high-copy RSS1, 23°C, DAPI; (M) high-copy RSS1, 37°C, FITC; (N) high-copy RSS1, 37°C, DAPI; (O) high-copy RSS1, 37°C, DIC.

ing is a more extreme distribution pattern than grouping. We examined thin sections from 50 separate cells of each strain, and the data are summarized in Figure 8. Nuclei showing these different phenotypes are shown in Figure 9. Almost 80% of the thin sections of wild-type cells examined showed pores that were evenly distributed, whereas small groups of NPCs were seen in ~20% of the sections, and clusters were not observed. Figure 9, A and B, shows wild-type nuclei with NPCs that are either evenly distributed around the nucleus (A) or grouped (B). In rat7-1 cells grown at 23°C, NPCs were either clustered or grouped in >80% of thin sections examined; grouping was more common than clustering. Figure 9, C and D, shows the nuclei of representative rat7-1 cells grown at 23°C, with NPCs either clustered (C) or grouped (D). NPCs were substantially less clustered or grouped in rat7-1 cells shifted to 37°C, with NPCs distributed evenly in 66% of the sections and with NPCs either grouped or clustered in 34%. Figure 9, E and F, shows rat7-1 cells grown at 37°C, with NPCs either distributed around the nucleus (E) or grouped (F). When RSS1 was overexpressed in rat7-1 cells grown at 23°C, there was little change in the NPC distribution patterns from those observed for rat7-1 cells, with clustering or grouping observed in >70% of the cells. Figure 9, G and H, shows sections of rat7-1 cells overexpressing Rss1p and grown at 23°C, with NPCs either clustered (G) or grouped (H). Although indirect immunofluorescence suggested little change in NPC distribution when these cells were shifted to 37°C, thin-section electron microscopy revealed a modest increase in the fraction of thin sections showing evenly distributed NPCs and a slight decrease in the fraction of sections showing clustered or grouped NPCs. Figure 9, I and J, shows nuclei from rat7-1 cells overexpressing RSS1 and shifted to 37°C, in which pores are either grouped (I) or more evenly distributed (J). The data indicate that NPCs remained more clustered or grouped in rat7-1 cells shifted to 37°C when RSS1 was overexpressed than in the absence of RSS1 overexpression. This suggests that retention of
clustering and grouping may be important for partial suppression of the mRNA export defect in rat7-1 cells by RSS1.

**Immunolocalization of Rss1p**

To determine the subcellular location of Rss1p, we tagged the protein with a triple “myc” epitope. The epitope was introduced at the C-terminal end of the protein (aa 529), where a BamHI site was created by site-directed mutagenesis. The myc-tagged RSS1 gene was able to complement fully an RSS1 null strain when present on either low-copy (CEN) or high-copy (2μ) plasmids; however, it was unable to suppress the rat7-1 mutation. Indirect immunofluorescence was performed with the 9E10 anti-myc antibody. When observed with conventional fluorescence microscopy, the cells showed a strong cytoplasmic signal and a weak rim staining around the nucleus (our unpublished results). Because the intense cytoplasmic signal seemed to be partially masking the nuclear rim pattern, samples were examined by confocal fluorescence microscopy, which showed more clearly that Rss1p is located both within the cytoplasm and at the nuclear rim (Figure 10). That portion of Rss1pmyc located at the nuclear rim colocalized with nucleoporins recognized by the RL-1 monoclonal antibody (Figure 10A) and with Rat7p/Nup159p (Del Priore and Cole, unpublished results). The tagged protein was also readily detected in the cytoplasm of yeast cells, and it was primarily this cytoplasmic signal that increased when the protein was overexpressed by placing the gene encoding Rss1pmyc in a high-copy (2μ) vector (Figure 10, compare A and B). As a control, indirect immunofluorescence was also performed to localize plasmid-borne myc-tagged nucleoporins, and no cytoplasmic signal was observed for either Rat7p/Nup159pmyc or Rat2p/Nup120pmyc (our unpublished results). We conclude that Rss1p is located both at nuclear pores and in the cytoplasm.

Figure 10C shows a Western blot in which identical amounts of protein were loaded in each lane and the 9E10 anti-myc monoclonal antibody was used to detect Rss1pmyc, which migrated with an apparent molecular weight of ~66 kDa, very close to the expected position for this tagged protein. Comparison of lanes 2 and 3 indicate that Rss1pmyc was expressed at a level approximately eightfold higher when expressed from a high-copy plasmid (Figure 10C, lane 3) than when expressed from a CEN plasmid (Figure 10C, lane 2), consistent with the stronger fluorescent signal seen in Figure 10B in comparison with 10A. The 9E10 antibody also detected a band in wild-type cells (Figure 10C, lane 1) that migrated very closely to the position of Rss1pmyc. However, no immunofluorescent signal was detected in wild-type cells with this antibody (Del Priore and Cole, unpublished results).

**Depletion of Rss1p Causes an mRNA Export Defect**

Because RSS1 is an essential gene, we wished to establish the effect on yeast cells caused by loss of Rss1p function. Therefore, the Rss1p and Rss1pmyc ORFs were placed under control of the GAL1 promoter on a CEN plasmid and transformed into an RSS1 null strain, and then GAL depletion experiments were performed. Figure 11A shows that in cells lacking the genomic copy of RSS1 and in which Rss1p or Rss1pmyc expression was driven by the GAL1 promoter, growth occurred when cells were grown on galactose but not when grown on glucose. Figure 11B shows the growth curves for cells containing Rss1p or Rss1pmyc under control of the GAL1 promoter and grown in galactose or transferred to glucose-containing media at 37°C. The rate of growth decreased between 10 and 12 h after transfer.

Figure 8. Nuclear pore clustering is partially maintained in rat7-1 cells overexpressing RSS1 at 37°C. Electron microscopy was performed on wild-type cells grown at 37°C and on both rat7-1 and rat7-1 cells containing a high-copy RSS1 plasmid, grown at 23°C or shifted to 37°C. Thin sections from 50 different cells were examined for each strain, and the percentage of cells showing ungrouped (filled squares) or grouped/clusters NPCs (open squares) was determined.
of cells expressing wild-type Rss1p to glucose and between 6 and 8 h in cells expressing Rss1p$_{myc}$. The graph suggests that the depletion of Rss1p$_{myc}$ was achieved earlier than the depletion of wild-type Rss1p. This result indicates that myc-tagged Rss1p was less stable than wild-type Rss1p. To monitor the decrease in the levels of Rss1p$_{myc}$ after cells were transferred to glucose, we performed a Western blot on extracts of cells grown on the repressing (glucose) medium for different periods of time (Figure 11C). The figure shows that, within 2.5 h of growth on glucose, the level of the protein was reduced to ~10% of its level in cells grown on galactose. After 5 h of growth on glucose, Rss1p$_{myc}$ was barely detectable, indicating that the protein was readily depleted. Sec13p was used as a control for equal loading.

To determine whether depletion of Rss1p resulted in an mRNA export defect, we performed an in situ hybridization assay in cells expressing Rss1p from the GAL1 promoter. Cells were grown in galactose or transferred to glucose for 3, 6, 9, or 12 h (Figure 12A). When cells were grown on galactose, poly(A)$^+$ RNA was evenly distributed throughout the cell (Figure 12A, 0h), as is the case in wild-type cells. After the cells were grown for 3 h in glucose, no change in the distribution pattern was seen, but after 6 h in glucose, nuclear accumulation of poly(A)$^+$ RNA was detectable in ~20-30% of the cells. After 9 h, the percentage of cells accumulating mRNA in their nuclei increased to ~80%. After 12 h in glucose, essentially all cells showed nuclear accumulation of poly(A)$^+$ RNA and a substantially reduced cytoplasmic signal. This result provides strong evidence that Rss1p is involved in mRNA export.

Because the strain bearing the GAL1-driven myc-tagged RSS1 plasmid ceased growth more rapidly after transfer to glucose-containing media than cells bearing the GAL1-driven wild-type RSS1 plasmid, we tested whether this was reflected in a more rapid onset of the mRNA export defect by performing an in situ hybridization assay with the GAL1-driven myc-tagged RSS1-containing strain. Cells grown on galactose were either maintained on galactose or shifted to glucose for 5 h (Figure 12B). Even when grown on galactose, these cells showed some nuclear accumulation of poly(A)$^+$ RNA. After 5 h in

Figure 9 (cont). Representative electron micrographs of nuclei showing the different types of NPC distributions seen in wild-type cells incubated at 37°C (A and B), rat7-1 cells incubated at 23°C (C and D) or shifted to 37°C (E and F), and rat7-1 cells overexpressing RSS1 and incubated at 23°C (G and H) or 37°C (I and J). The figure shows examples of evenly distributed (ungrouped), grouped, or clustered NPCs seen in these strains. The large black arrows show examples of grouped or clustered NPCs, and the small black arrows show individual NPCs; no, nucleolus. Bar, 0.5 μm.
DISCUSSION

In this paper, we describe the identification of the RSS1 gene by using a screen for high-copy extragenic suppressors of the rat7-1 allele. RSS1 encodes a novel 538-amino-acid protein that is located both at NPCs, where it colocalizes with nucleoporins, and within the cytoplasm. By expressing Rss1p from the GAL1 promoter, Rss1p could be depleted from cells by transferring them from galactose media to glucose media. A defect in export of poly(A)^+ RNA was observed in almost all of the cells within 9–12 h of transfer to glucose. The kinetics with which growth ceased after transfer to glucose were similar to the kinetics with which cells displayed nuclear accumulation of poly(A)^+ RNA. No defects in nuclear protein import, nuclear pore distribution, or nucleolar integrity were seen when Rss1p was depleted. Together, these results suggest that Rss1p normally plays a role in nucleocytoplasmic export of poly(A)^+ RNA.

Figure 10. Rss1p localizes to nuclear pore complexes and to the cytoplasm. Immunolocalization of myc-tagged Rss1p was performed. Cells carrying a disruption of RSS1 were transformed with a CEN plasmid encoding myc-tagged Rss1p. Transformed cells were grown to log phase, fixed, and processed for indirect immunofluorescence. Images were taken with a confocal microscope. (A) FITC shows the signal obtained when anti-myc antibody 9E10 was used, and Texas Red shows the signal distribution when the same cells were costained with the RL-1 anti-nucleoporin antibody. (B) Indirect immunofluorescence performed in cells carrying a disruption of RSS1 and carrying a 2μ plasmid encoding myc-tagged Rss1p. (C) Western analysis of myc-tagged Rss1p. By SDS polyacrylamide gel electrophoresis, myc-tagged Rss1p migrates at 66 kDa and is overexpressed when produced from a 2μ vector in the RSS1 null strain. The anti-myc 9E10 antibody was used to detect myc-tagged Rss1p. (Lane 1) WT (FY86); (lane 2) rss1Δ cells transformed with a CEN plasmid encoding myc-tagged Rss1p (VDPY112); (lane 3) rss1Δ cells transformed with a 2μ plasmid encoding myc-tagged Rss1p (VDPY113). Sec13p was used as a control for equal loading, with an anti-Sec13p antibody obtained from C. Barlowe (Dartmouth Medical School, Hanover, NH).
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Figure 11. Growth at 37°C of a wild-type yeast culture and of yeast strains disrupted for the chromosomal RSS1 locus and harboring plasmid-borne GAL1::RSS1 or GAL1::RSS1myc alleles, on galactose or shifted to glucose. (B) Growth curves for the yeast strains shown in A. Cells were grown overnight, diluted to early log phase, and incubated at 37°C either in galactose-containing media or shifted to glucose-containing media at the indicated time. Cell numbers were determined with a hemocytometer. (C) Western analysis of cell extracts prepared from the strain containing the GAL1::RSS1myc allele of RSS1 at various times after transfer to glucose-containing medium. Sec13p was used as a control for equal loading.
High-copy RSS1 suppressed the growth defect of rat7-1 cells and permitted mutant cells to grow as well at 37°C as they did at 23°C in the absence of the suppressor. However, suppression was only partial, because rat7-1 cells grew considerably more slowly at 23°C than did wild-type cells (Figure 2), and there was still considerable accumulation of poly(A)^+ RNA in nuclei. Overexpression of RSS1 had no deleterious effects on the growth of either wild-type or rat7-1 cells at 23°C. Suppression was not observed when RSS1 was expressed from a CEN plasmid. On the basis of Western blot analysis (Figure 8), expression from the high-copy plasmid led to Rsslp levels that were approximately eightfold greater than with the CEN plasmid. However, CEN plasmids are maintained in yeast cells at ~1-4 copies/cell, and this should result in modest overexpression, as compared with the levels of Rss1p produced from the genomic copy. We estimate that Rss1p was overexpressed by >10-fold when expressed from the high-copy plasmid. The epitope-tagged version of Rss1p seems to have lower stability than the wild-type form, and this most likely explains...
the inability of the epitope-tagged form to suppress the rat7-1 mutation.

In addition to cessation of growth, rat7-1 cells shifted to 37°C show rapid inhibition of mRNA export, rapid fragmentation of the nucleolus, and a dramatic decrease in rRNA production. We believe that the defects in nucleolar morphology and rRNA biosynthesis are not caused directly by mutation of Rat7p/Nup159p but are most likely indirect consequences of a defect in RNA in ribosome export. Overexpression of RSS1 partially suppressed all these phenotypes. Although rat7-1 cells shifted to 37°C for 2 h display virtually no cytoplasmic signal for poly(A)+ RNA (Figure 3; Gorsch et al., 1995), the presence of RSS1 in high copy led to maintenance of a cytoplasmic signal for poly(A)+ RNA. This signal could reflect either dramatically reduced turnover of cytoplasmic poly(A)+ RNA or continued mRNA export. Only the latter explanation is consistent with the ability of rat7-1 cells to form colonies at 37°C when RSS1 is present in high copy. Therefore, we conclude that mRNA export was maintained in rat7-1 cells shifted to 37°C in the presence of high-copy RSS1 at a level adequate to support cell growth and division. Although the amount of cytoplasmic mRNA present in rat7-1 cells suppressed by high-copy RSS1 was quite low, it is noteworthy that this reduced level was sufficient to support a moderate rate of growth. We have also examined nuclear protein import in rat7-1 cells through multiple assays, and in all cases, karyophilic proteins were imported efficiently into nuclei (Gorsch et al., 1995; Del Priore and Cole, unpublished results). The fact that rat7-1 cells show defects in mRNA export but not in nuclear protein import suggests that Rat7p/Nup159p is directly involved in RNA export.

In rat7-1 cells, Rat7p is lost from the nuclear rim when mutant cells are shifted to 37°C (Gorsch et al., 1995). Because Rat7p/Nup159p is an essential gene, high-copy RSS1 could be acting to maintain Rat7p at NPCs. However, in the presence of high-copy RSS1, the mutant protein was lost from NPCs after a shift to 37°C as rapidly as it was in the absence of high-copy RSS1 (Figure 6). One of the most novel phenotypes of rat7-1 cells is that the loss of Rat7p from NPCs in rat7-1 cells shifted to 37°C is associated with a substantial reversal of the clustering of NPCs seen in these cells grown at 23°C (Gorsch et al., 1995). Interestingly, in the presence of high-copy RSS1, most of the clustering and grouping of NPCs seen at 23°C was retained in cells shifted to 37°C, suggesting that maintenance of this clustering may be important for maintaining RNA export.

The data presented in this paper suggest that RSS1, in high copy, permits rat7-1 cells to grow at the nonpermissive temperature of 37°C by acting directly to partially suppress the RNA export defect of rat7-1 cells. Three lines of evidence support the idea that Rss1p is directly involved in RNA export. First, depletion of Rss1p causes a defect in nucleocytoplasmic export of poly(A)+ RNA but no defects in nuclear protein import or NPC distribution. Second, Rss1pmyc was present both in the cytoplasm and at NPCs, a distribution similar to that seen for some factors involved in nucleocytoplasmic transport. Third, when RSS1 was overexpressed in a rat7-1 strain, it was able to suppress partially the mRNA export defect of this strain.

How Might Rss1p Act to Suppress the RNA Export Defect?

An important observation made during these studies is that high-copy RSS1 is not a bypass suppressor of a null allele of RAT7/NUP159. However, all detectable rat7-1p was lost from the nuclear rim after a shift of rat7-1 cells to 37°C, either in the presence or absence of high-copy RSS1 (Figure 6). Because rat7-1 cells continue to grow at 37°C in the presence of high-copy RSS1, the mutant rat7-1p must perform some essential function under these growth conditions. Possibly a very low level of rat7-1p remains associated with NPCs after the temperate shift, although none could be detected by indirect immunofluorescence. High-copy RSS1 could act directly to facilitate RNA export through NPCs lacking detectable rat7-1p, or it could act to facilitate limited retention of mutant rat7-1p in NPCs. We do not know whether there is a direct interaction between Rss1p and Rat7p/Nup159p. Rss1p/Nup159p was readily extracted from NPCs with 100 mM KCl and 0.2% Triton X-100. We were unable to coprecipitate Rat7p/Nup159p and Rss1p using these extraction conditions and anti-Rat7p/Nup159p polyclonal antibodies (Del Priore and Cole, unpublished results). Perhaps Rss1p does interact with Rat7p/Nup159p, but the interaction may be too weak to survive these mild extraction conditions. Alternatively, Rss1p may interact directly with components of the NPC other than Rat7p/Nup159p, or it may interact with non-NPC factors involved in mRNA export.

Two different models can be hypothesized to explain the mechanism of suppression. By one model, Rss1p could play a dynamic or enzymatic role in nucleocytoplasmic transport. By conventional fluorescence microscopy, we observed that the nuclear rim staining for Rss1pmyc was substantially less intense than the cytoplasmic signal. This distribution is similar to that of some of the soluble factors involved in nuclear protein import. For example, importin β/karyopherin β/Kap95p, one of the subunits of the heterodimeric receptor that recognizes nuclear localization signals, is found in the cytoplasm, binds with substrate to NPCs, but does not accumulate in the nucleus (Görlich et al., 1995; Moroianu et al., 1995).
small GTPase Gsp1p plays a central role in nuclear protein import, and its GTPase activating protein
Rna1p is cytoplasmic with a higher concentration near
the nucleus (Hopper et al., 1990), a pattern somewhat
similar to that of Rss1p. Gsp1p is the yeast homologue
of the metazoan Ran protein. One of the yeast Gsp1p-
binding proteins Yrb1p also has a distribution similar
to that of Rss1p (Schlenstedt et al., 1995). Perhaps
NPCs in rat7-1 cells are not absolutely defective for
RNA export, but the rate of export may be too low to
support growth of mutant cells. Overexpressed Rss1p
might act directly to increase the rate of export
through mutant NPCs, thereby restoring growth.

By a second model, Rss1 overexpression could af-
fect the structure and interactions of NPCs in mutant
cells, either by stabilizing a nuclear pore subcomplex
that normally contains Rat7p/Nup159p or by main-
taining interactions between mutant NPCs and ele-
ments of the cytoskeleton or nucleoskeleton that are
critical for RNA export. In the absence of Rat7p/
Nup159p, a subcomplex of the NPC that contains
Rat7p/Nup159p either may not assemble or may not
be further assembled into NPCs. Perhaps Rss1p acts
to ensure that the other components of a putative Rat7p
subcomplex are retained in the pore when mutant
rat7-1p is lost after a shift to 37°C. NPCs are known
to be attached to a nuclear envelope lattice in metazoan
cells (Maul, 1977; Goldberg and Allen, 1992; Ris and
Malecki, 1993), and there is evidence that a similar
attachment occurs in yeast cells (Allen and Douglas,
1989). An association of NPCs with cytoskeletal ele-
ments has been observed in metazoan cells (Franke,
1971; Jones et al., 1982), but there is no evidence yet
for similar associations in yeast cells. Because Rat7p/
Nup159p is located on the cytoplasmic face of NPCs,
at a distance consistent with it being part of the cyto-
plasmic fibrils of the NPC, Rat7p/Nup159p seems
more likely to be involved in interactions between
NPCs and cytoskeletal elements than with nucleoskel-
etal elements. Perhaps such interactions are essential
for proper transport of substrates through NPCs. If
critical connections between NPCs and cytoplasmic
structures were disrupted by mutation of the Rat7p/
Nup159p nucleoporin, then export substrates might
be unable to exit from NPCs and associate with the
translation machinery. A significant fraction of polyri-
inosomes, mRNA, and factors required for protein syn-
thesis are associated with the actin cytoskeleton (Lenk
et al., 1977; Cervera et al., 1981; Yang et al., 1990;
Zambetti et al., 1990a,b; Hesketh and Pryme, 1991;
Hansen and Ingber, 1992). Therefore, there may be
connections between the cytoskeleton and NPCs, and
mRNP exiting the pore may be actively transferred to
the actin cytoskeleton or to cytoskeleton-bound ribo-
somes. Perhaps high-copy Rss1p acts to maintain
these NPC–cytoskeletal interactions, thereby permit-
ting rat7-1 NPCs to retain the ability to export mRNP
substrate.

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