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Perturbations in the spi1p GTPase Cycle of *Schizosaccharomyces pombe* through Its GTPase-Activating Protein and Guanine Nucleotide Exchange Factor Components Result in Similar Phenotypic Consequences

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spi1p of *Schizosaccharomyces pombe* is a structural homolog of the mammalian GTPase Ran. The distribution between the GTP- and GDP-bound forms of the protein is regulated by evolutionarily conserved gene products, rna1p and pim1p, functioning as GTPase-activating protein (GAP) and guanine nucleotide exchange factor (GEF), respectively. Antibodies to spi1p, pim1p, and rna1p were generated and used to demonstrate that pim1p is exclusively nuclear, while rna1p is cytoplasmic. A loss of pim1p GEF activity or an increase in the rna1p GAP activity correlates with a change in the localization of the GTPase from predominantly nuclear to uniformly distributed, suggesting that the two forms are topologically segregated and that the nucleotide-bound state of spi1p may dictate its intracellular localization. We demonstrate that the phenotype of cells overproducing the GAP resembles the previously reported phenotype of mutants with alterations in the GEF: the cells are arrested in the cell cycle as septated, binucleated cells with highly condensed chromatin, fragmented nuclear envelopes, and abnormally wide septa. Consistent with the expectation that either an increased dosage of the GAP or a mutation in the GEF would lead to an increase of the spi1p-GDP/spi1p-GTP ratio relative to that of wild-type cells, overexpression of the GAP together with a mutation in the GEF is synthetically lethal. The similar phenotypic consequences of altering the functioning of the nuclear GEF or the cytoplasmic GAP suggest that there is a single pool of the spi1p GTPase that shuttles between the nucleus and the cytoplasm. Phenotypically, *rna1* null mutants, in which spi1p-GTP would be expected to accumulate, resemble *pim1*^{ts} and rna1p-overproducing cells, in which spi1p-GDP would be expected to accumulate. Taken together, these results support the hypothesis that the balance between the GDP- and GTP-bound forms of spi1p mediates the host of nuclear processes that are adversely affected when the functioning of different components of this system is perturbed in various organisms.

Ran is a small GTPase in the Ras superfamily, homologs of which have been identified in numerous eukaryotic organisms (10). The Ran family of GTPases, which is the subject of this paper, have been implicated in a variety of nuclear processes including cell cycle progression, nucleocytoplasmic transport of protein and RNA, initiation of DNA replication, RNA metabolism, nucleolar and chromatin structure, and transcriptional regulation (reviewed in references 10 and 37). However, the primary role(s) of this system has yet to be determined, and many other questions relating to the functioning and regulation of the Ran system remain unanswered (37).

GTPases are proteins that bind and hydrolyze GTP. The different structural conformations adopted by the GTPase, depending on whether it is bound to GDP or GTP, affect its ability to interact with specific target molecules and thus affect its function (7). The in vivo consequences of altering the normal balance between the two nucleotide-bound states differ among GTPase systems. For example, some cellular functions are sensitive to the GTP-bound active form of the protein, such as oncogenic transformation in the case of p21^{ras} (8). Others, including the Rab GTPase system, require a complete cycle of GTP exchange and hydrolysis: accumulation of the GTPase in either the GTP-bound or GDP-bound state blocks vesicular transport (16) so that neither can be defined as the active state.

Ran and its structural and functional homologs interact with proteins that modulate its nucleotide-bound state: guanine nucleotide exchange factors (GEFs) that stimulate the exchange of GDP for GTP and GTPase-activating proteins (GAPs) that catalyze the intrinsic GTPase activity, hydrolyzing the bound GTP to GDP (reviewed in reference 10). An evolutionarily conserved family of Ran-binding proteins (reviewed in references 10 and 37) bind the GTPase specifically in its GTP-bound state, and the mammalian and budding yeast proteins have been shown to enhance the GTPase-stimulating activity of the GAP in vitro (5, 39).

Some clues to the mechanism by which the Ran GTPase system is regulated come from studies of the localization of the GTPase itself and its known regulators, the GEF RCC1 and the GAP RNA1. The GEF is a nuclear protein that is associated with chromatin in vertebrates, the budding yeast *Saccharomyces cerevisiae*, and fruit flies (1, 6, 19, 24, 32, 33). In contrast, immunofluorescence localization of the GAPs in both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Rna1p and rna1p, respectively) indicates that these proteins are cytoplasmic (23, 28). The Ran family is unique among GTPases in its nuclear localization. The mammalian GTPase (Ran) (35) and its budding yeast homologs (GSP1 and GSP2)

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(2) are predominantly, but not exclusively, nuclear, and this localization depends on the functioning of the GEF (35).

The exclusively nuclear localization of the GEF and its association with chromatin suggest that in addition to its catalytic role RCC1 may directly influence chromatin structure. Consistent with this possibility is the observation that the GEF loses its chromatin association at mitosis when the chromosomes are condensed (32, 33, 35). A model in which its association with functional GEF is required to establish or maintain chromatin in its decondensed state could explain the hypercondensed chromatin associated with loss of GEF function in BHK and fission yeast cells. However, depletion of RCC1 from *Xenopus* egg extracts has no apparent deleterious effects on the assembly of histones on chromatin, although this chromatin cannot be replicated (11).

The inhibition of replication on chromatin templates assembled in RCC1-depleted extracts can be reversed by the addition of Ran-GTP. The premature chromatin condensation seen in RCC1$^-$ tsBN2 cells can also be reversed by microinjection of Ran-GTP (31). These results suggest that specific functions of the Ran system depend solely on the GEF activity of RCC1, i.e., its ability to generate Ran-GTP. However, in the fission yeast in vivo system, the loss of viability of temperature-sensitive GEF mutants cannot be rescued by expression of Ran stabilized in the GTP-bound state (38). Taken together, these studies suggest that while specific functions of the GEF depend solely on its ability to generate Ran-GTP, there are other essential functions or regulatory constraints on the system that are essential for viability. For example, the continued functioning of the Ran system in vivo may require cycling of the GTPase between its GTP and GDP states and/or the maintenance of a specific ratio between the two forms. Consistent with a requirement for cycling rather than a requirement simply for the production of the activated form of the GTPase is the observation that expression of a mutant form of Ran stabilized in its GTP-bound state cannot overcome the loss of GEF function in vivo (38) and in fact is toxic to cells (2, 35, 38).

It has been suggested by biochemical studies with mammalian cells that there is a single nuclear GEF (4) and a single cytoplasmic GAP (4). If these two activities are physically separated, then a complete round of GTP binding and hydrolysis would require shuttling of the GTPase across the nuclear envelope, although this has not been demonstrated experimentally. In contrast, the results of studies of RNA export from the nucleus have led to the suggestion that there are multiple rounds of Ran nucleotide binding and hydrolysis within the nucleus, meaning that there are separate nuclear and cytoplasmic cycles of Ran GTP binding and hydrolysis (9, 29).

In this report, we describe the in vivo approach we have taken, using the fission yeast *S. pombe*, to study the functioning and regulation of the Ran GTPase system. The components of this system are named spi1p (the GTPase), pim1p (the GEF), and rna1p (the GAP) (37). Previously, we have demonstrated that temperature-sensitive mutations in the RCC1 homolog pim1p result in an inability of fission yeast cells to complete the mitosis-to-interphase transition: cells enter and execute a normal mitosis at the restrictive temperature but then fail to decondense their chromosomes or replicate their DNA (38). We have recently discovered that although the nuclear envelope of both fission and budding yeast cells normally remains intact throughout the cell cycle, these pim1p mutant cells arrest with fragmented nuclear envelopes (13). This terminal phenotype has led to the proposal that the essential function of the spi1p GTPase system in fission yeast cells is the maintenance of the proper organization of the components of the nucleus, including the chromatin and the nuclear envelope (13), although the primary target has not yet been determined (37).

Using antibodies that we raised to pim1p, spi1p, and rna1p, the three core components of the fission yeast Ran GTPase system, we have established that the intracellular localization of components of the spi1p GTPase system is the same as that previously seen in mammals and *S. cerevisiae*: the GEF is nuclear, the GAP is cytoplasmic, and the GTPase is primarily, but not exclusively, nuclear. We have also found that the predominantly nuclear localization of spi1p is dependent on GEF function, as has been shown for mammalian cells as well. However, in neither of these in vivo systems has it been possible to determine whether the essential function of the GEF is mediated through its catalytic properties or its chromatin association because the temperature-sensitive GEF protein becomes degraded at the restrictive temperature.

This question could be addressed, however, if the effects of loss of GEF activity could be mimicked without perturbing the pim1 protein. We have devised a method of achieving this situation by overproducing the GAP rna1p. Since the GEF and the GAP have opposite effects on the nucleotide-bound state of the GTPase in vitro, the changes in the normal in vivo ratio of spi1p-GTP versus spi1p-GDP brought about by a decrease in GEF function might be mimicked by overproduction of the GAP. Consistent with this prediction is our finding that cells overexpressing rna1p arrest with the same terminal phenotype as cells harboring a temperature-sensitive mutation in the pim1p GEF: they arrest after mitosis with hypercondensed chromosomes and a medial septum. In both cases, spi1p loses its predominantly nuclear localization while the envelope is still intact. *pim1-d1^{ts}* cells are also more sensitive to rna1p overproduction than wild-type cells. This dosage synthetic lethality would be expected on the basis of known biochemical and genetic interactions between the GEF and the GAP for spi1p and is consistent with the prediction that the effects of decreased GEF activity and increased GAP activity on spi1p would be additive. On the basis of their known biochemical functions, it is likely that these effects are mediated by an accumulation of spi1p-GDP.

The one critical difference between cells with decreased pim1p function and cells with increased rna1p function is that only in the rna1p-overexpressing cells does pim1p retain its chromatin association. This suggests that the presence of chromatin-associated pim1p is not sufficient for chromatin decondensation.

Taken together, these results suggest that the essential in vivo function of pim1p is its GEF activity. They also have implications for understanding several outstanding questions relating to pim1p function: (i) the observation that a decrease in pim1p GEF function and an increase in rna1p GAP function result in similar phenotypic consequences and are synthetically lethal suggests that these effects are likely to be mediated by an increase in the proportion of spi1p-GDP relative to spi1p-GTP; (ii) the finding that this collection of phenotypes can be achieved by influencing the nucleotide-bound state of spi1p through either its nuclear GEF or its cytoplasmic GAP suggests that there is a single pool of spi1p in the cell; and (iii) if there is a single pool of spi1p in the cell but an exclusively nuclear GEF and an exclusively cytoplasmic GAP, spi1p must shuttle between the nucleus and the cytoplasm to cycle between its GTP- and GDP-bound states.

MATERIALS AND METHODS

Yeast strains and cell culture. The wild-type haploid strain (*leu1-32 ura4-D18 h$^-$*) was used for overexpression of *rna1* driven by three different strengths of the *nmt1* promoter (18, 27). Cells precultured in Edinburgh minimal medium

(EMM) containing 5 μg of thiamine per ml to repress the *nmt1* promoter were washed and grown in thiamine-free media for 20 h at 32°C to derepress the promoter unless otherwise noted. The wild-type diploid strain (*leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 h*$^{-}$*/h*$^{+}$) was used to generate the *rna1* disruption strain. Cultures were inoculated into either yeast extract (YE) medium or EMM with the appropriate supplements (30). *pim1-d1*^{ts} cells were grown at 25°C (permissive temperature) or 32°C (semipermissive temperature) and, when indicated, shifted to 36°C (restrictive temperature) for 2 or 4 h in a gyratory water bath. Phase-contrast microscopy was used to monitor the septation index. Sporulation was performed by standard methods in malt extract medium at 25°C, and germination was carried out on YE agar plates or in liquid-supplemented EMM or YE medium at 32°C (30). Sporulated diploids were dissected on YE agar plates and after growth were replica plated on uracil-selective media.

DNA manipulations. An *Nde*I site was introduced at the ATG translation start codon for both *spi1* and *pim1* cDNAs by PCR. Hemagglutinin (HA)- and His-tagged *spi1* and *pim1* cDNA constructs were made. A *spi1 Nde*I-*Bam*HI fragment was subcloned into the *Nde*I and *Bam*HI sites of the bacterial pETXHA (15) vector to make HA-tagged *spi1*. The HA-tagged *spi1* was then excised by using *Xho*I and *Bam*HI and subcloned into the fission yeast pRep3X vector so that expression is controlled from the repressible *nmt1* promoter. From the pETXHA-*spi1* construct, *spi1* was also excised with *Nde*I and *Bam*HI. This fragment was subcloned into the same sites in the bacterial pTRCHis (Invitrogen) vector to make the His-tagged construct. The same strategy was used to make the HA- and His-tagged *pim1* constructs. pGEX-*spi1*, a glutathione *S*-transferase (GST)-spi1p fusion construct, was also generated by subcloning the *spi1* cDNA in the pGEX-2TKcs (40) vector at the *Nde*I and *Bam*HI sites.

A 1.8-kb genomic clone containing the *rna1* gene in pBS II^{+} was obtained from V. Gerke (28). A 1.6-kb *Xho*I fragment containing the entire open reading frame was cloned into the *Xho*I site of the pREP3X and pREP81X vectors for overexpression from the highest and lowest strengths, respectively, of the thiamine-repressible *nmt1* promoter (18, 27).

Construction of the Δrna1-A1 strain. The 1.8-kb genomic clone containing the *rna1* gene (28) was used to make a disruption construct similar to that of Melchior et al. (28) since neither the original disruption construct nor the strain was available. A *ura4 Hin*dIII fragment was inserted into the *rna1* gene lacking the same coding region as the previous disruption construct. For ease of cloning, 113 bp was removed from the 3′ untranslated region and a *Hin*dIII linker was added at the *Stu*I site. The *ura4*-containing *rna1* construct was excised with *Eco*RI and *Xho*I. The *rna1::ura4* gel-purified 2.9-kb fragment was introduced into cells by electroporation into the diploid wild-type strain and plated on uracil-selective medium. Four Ura^{+} diploids were tested by Southern blot analysis (36) using the restriction endonuclease *Cla*I, which does not have a recognition site within the disruption construct, to verify that gene replacement had occurred at the wild-type *rna1* locus. A [^{32}P]ATP-labeled probe was generated from the 1.6-kb *Eco*RI-*Xho*I fragment of the *rna1* coding region by using Prime-It (Stratagene) according to the manufacturer's protocol. This probe was used for Southern analysis and for mapping of *rna1* with an ordered cosmid filter (22, 25) to chromosome 1. The disrupted diploid strain was transformed with pRep81X-*rna1* containing *LEU2* as a selectable marker and *rna1* under control of the lowest-strength *nmt1* promoter. The cells were sporulated under conditions in which the promoter was either repressed or derepressed, and all Ura^{+} colonies were found to also be Leu^{+}, showing that wild-type *rna1* expressed from the lowest-strength *nmt1* promoter under repressed conditions was able to restore viability to the Δrna1-A1 disrupted haploid cells. These rescued Leu^{+} colonies were inoculated into nonselective media to allow plasmid loss, but after an approximate 10^{5}-fold increase in cell number, all of the cells tested remained Leu^{+}, indicating that the plasmid encoding rna1p was required for viability.

Polyclonal antibody production. rna1 protein purified from *S. pombe* was kindly provided by Bischoff and Ponstingl (4) and used as an antigen for raising polyclonal anti-rna1p antiserum (20). The antiserum is normally used in Western blotting (immunoblotting) at a 1:10,000 dilution.

To produce spi1 protein for antibody production, pGEX-*spi1* was expressed in the *Escherichia coli* BL21(DE3)(pLysS) strain (Stratagene). The GST-spi1p was purified by using glutathione-Sepharose beads (Pharmacia). Two different antigens (fusion and cleaved) were used to raise polyclonal antibodies. Purified native GST-spi1p was used as the fusion antigen. In a separate preparation, the fusion protein was proteolyzed with thrombin (Sigma), and the spi1p portion was recovered as a single band from a denaturing polyacrylamide gel and used as the cleaved antigen. Both antisera are used at a 1:10,000 dilution for Western blot analysis.

To raise antibodies to the pim1 protein, the His-tagged pim1p construct was transformed into the BL21 (DE3) (pLysS) bacterial strain and the His-tagged protein was purified by standard methods (Qiagen). His-tagged pim1 protein isolated directly from the nickel agarose beads or a protein preparation further purified and recovered as a single band on a denaturing polyacrylamide gel was used to raise polyclonal antibodies. Both antisera recognize the pim1 protein in Western blot analysis at a 1:10,000 dilution.

Affinity purification and antibody specificity. Antisera to the spi1 fusion protein and pim1 gel-purified protein were both affinity purified by using the respective purified His-tagged antigens. The His-tagged spi1p and pim1p were prepared as described for His-tagged pim1p above. For the spi1 protein, the nickel column eluate was used, whereas for the pim1 protein, gel-purified protein was used. The purified proteins were dialyzed against phosphate-buffered saline (PBS), concentrated, and conjugated to Affigel-10 resin (Bio-Rad). Antibodies were purified according to the manufacturer's protocol, concentrated, dialyzed against PBS, and stored at 4°C. Both the affinity-purified anti-spi1p and anti-pim1p antibodies were used at a dilution of 1:5,000. Anti-HA antibodies (BAbCO) were used at a dilution of 1:2,500.

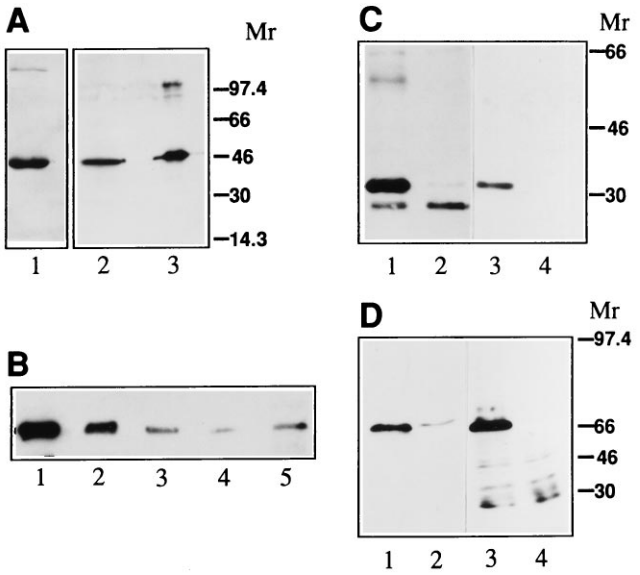


FIG. 1. Western blot analysis showing the specificity of anti-rna1p, affinity-purified anti-spi1p, and affinity-purified anti-pim1p antibodies. (A) Coomassie brilliant blue staining of purified rna1p (lane 1), Western blot analysis of total fission yeast cell extract (lane 2) and purified rna1p (lane 3) probed with anti-rna1 antibodies. (B) Determination of the amount of rna1p in overexpressing cells compared with that of wild-type cells. Cell extract from wild-type cells (lane 5) cells overexpressing rna1p from the pREP3X-*rna1* plasmid (lanes 1 to 4) was probed with anti-rna1p antibodies. The following amounts of total proteins were loaded: lane 1, 0.2 μg; lane 2, 0.1 μg; lane 3, 40 ng; lane 4, 20 ng; and lane 5, 2 μg. The film shown was exposed so that the signal in lane 4 is barely detected. (C) Cell extracts from wild-type cells containing plasmid encoded HA-spi1p transcribed from the *nmt1* promoter grown under derepressed conditions (lanes 1 and 3) or repressed conditions (lanes 2 and 4) using affinity-purified anti-spi1p antibodies (lanes 1 and 2) or anti-HA antibodies (lanes 3 and 4). (D) Cell extracts from wild-type cells containing plasmid-encoded HA-pim1p transcribed from the *nmt1* promoter grown under derepressed conditions (lanes 1 and 3) or repressed conditions (lanes 2 and 4) using affinity-purified anti-pim1p antibodies (lanes 1 and 2) or anti-HA antibodies (lanes 3 and 4). Molecular weights are given in thousands.

Antibody specificity was determined by Western blotting (20) and chemiluminescent detection (Amersham ECL kit) according to the manufacturer's directions. Wild-type fission yeast cells were transformed with *rna1*, HA-tagged *spi1*, or HA-tagged *pim1* constructs controlled by the *nmt1* promoter, and total cell extracts from these samples were probed with anti-rna1p, affinity-purified anti-pim1p, affinity-purified anti-spi1p, or anti-HA antibodies as described for Fig. 1.

Fluorescence microscopy. Cells were stained with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) to visualize the nuclear envelope (13), Hoechst 33342 to visualize the DNA (13), calcofluor to visualize the septum (30) in living cells, and 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA in fixed cells (30). Cells were observed and photographed with a Zeiss Axioskop fluorescence microscope.

For indirect immunofluorescence microscopy, fixation and staining were carried out as previously described (13) with the following changes: cell walls were digested by using 1 mg of Novozyme 234 (Interspex) per ml and 0.3 mg of Zymolyase 20T (Seikagaku America) per ml for 10 to 40 min. Monoclonal antibodies to nuclear pore proteins (MAb414; BAbCo) were used at a 1/10 dilution. Anti-rna1p, affinity-purified anti-spi1p, and affinity-purified anti-pim1p antibodies were used at dilutions of 1/100, 1/100, and 1/50, respectively. The secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Pierce) or Texas red-conjugated goat anti-rabbit antibodies (Cedarlane), were applied at a 1/50 dilution for 60 min at 20°C.

RESULTS

Construction of an *rna1*-overexpressing strain. To address the physiological relevance of the GAP activity attributed to

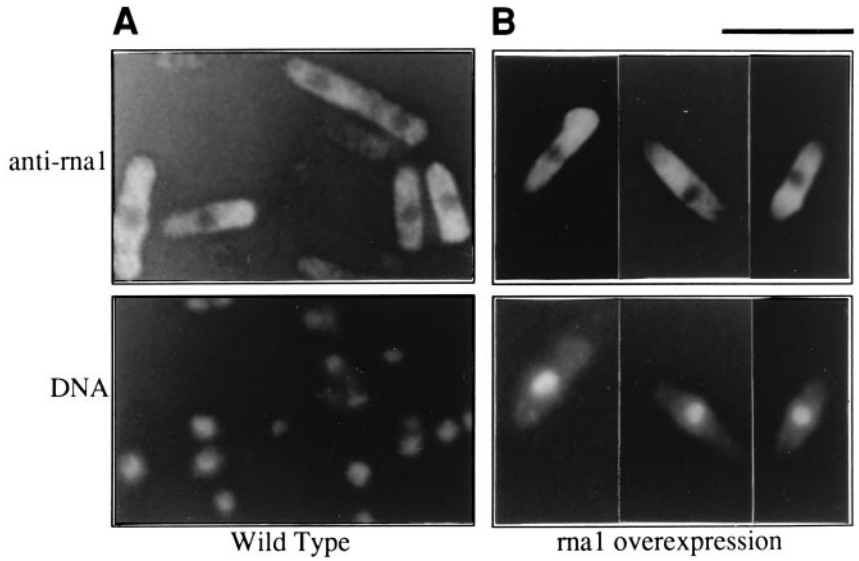


FIG. 2. Immunolocalization of rna1p. (A) rna1p and DNA localization in wild-type cells. The exposure time was 15 s. (B) rna1p and DNA localization in wild-type cells overexpressing plasmid-encoded *rna1* from the *nmt1* promoter. The exposure time was 1 s. Times used for printing and developing the negatives and prints were identical for panels A and B. Bar = 10 μm.

rna1p in vitro, the *rna1* gene was overexpressed in wild-type fission yeast cells from the thiamine-repressible *nmt1* promoter and the phenotypic consequences were monitored. Polyclonal antibodies that specifically recognized rna1 protein in Western blot analysis were raised. Anti-rna1p antibodies recognize a single band of 43 kDa, the predicted molecular mass of the protein, in total cell extracts (Fig. 1A, lane 2) that comigrates with purified rna1p detected by either Coomassie brilliant blue staining (Fig. 1A, lane 1) or Western blotting (Fig. 1A, lane 3). Furthermore, the intensity of the rna1 protein band increased over the endogenous level when the *rna1* gene was transcribed from the highest-strength *nmt1* promoter by using the pREP3X-*rna1* plasmid (Fig. 1B, compare lanes 1 and 5). Western blotting with this antibody was also used to determine that rna1p was overexpressed approximately 50-fold in these cells (Fig. 1B, compare lanes 3 and 5). It should be noted, however, that our previous experience with the *nmt1* promoter showed various degrees of expression in individual cells, so fluctuations from this average value can be expected on the single-cell level. Immunolocalization of rna1p, using anti-rna1p antibodies that specifically recognize rna1p (Fig. 1A and B), demonstrated that rna1p is cytoplasmic (Fig. 2A) and that this localization pattern did not change upon overproduction (Fig. 2B).

The morphological phenotype of the cells overexpressing rna1p resembles that of *pim1* mutants. The activities of the pim1p GEF and the rna1p GAP counteract each other. Since cells overexpressing rna1p and *pim1-d1^{ts}* mutant cells would be expected to have similar alterations in the nucleotide-bound state of spi1p compared with wild-type cells, we tested whether they had similar morphological phenotypes. Binucleated, septated cells with highly condensed chromatin accumulate among both *pim1-d1^{ts}* mutant cells at the restrictive temperature (38) and cells in which rna1p is overexpressed (Fig. 3A). The fact that we did not see mononucleate cells with condensed chromosomes indicates that, as has been shown for the *pim1-d1^{ts}* mutant (38), *rna1*-overexpressing cells must undergo mitosis before the chromatin condensation phenotype is observed. Previously, it has been shown that *pim1-d1^{ts}* cells undergo dramatic changes in nuclear envelope morphology and integrity following mitosis as determined by using the vital membrane-binding dye $DiOC_6$, the nucleoporin-specific monoclonal antibody MAb414, and electron microscopy (13). Cells overexpressing rna1p arrested as binucleated septated cells with condensed chromosomes (Fig. 3B, bottom panel) and also lost the characteristic outline of the nucleus (Fig. 3C, bottom panel) seen in normally growing cells (13) and in cells transformed with pREP3X-*rna1* that have not yet achieved their terminal phenotype (Fig. 3C, top panel). Indirect immunofluorescence localization of a nuclear pore complex antigen showed pore clustering similar to that seen in the *pim1-d1^{ts}* mutant at the restrictive temperature (13), confirming that the nuclear envelope is defective in *rna1*-overexpressing cells (see Fig. 5E, panel 3, and F).

A dramatic widening of the septum was observed in *rna1*-overexpressing cells 24 h after derepression of the promoter (Fig. 3D) compared with that of wild-type cells (data not shown) and transformed cells that were presumably expressing low levels of rna1p (Fig. 3D). This abnormal septum formation is also a feature of the *pim1-d1^{ts}* cells which has not been previously described but was apparent upon prolonged incubation at the restrictive temperature (Fig. 3E).

On the basis of the appearance of binucleated cells with condensed chromatin, fragmented nuclear envelopes, and a wide medial septum, we conclude that overexpression of *rna1* in *S. pombe* leads to the same morphological changes that result from mutation in the *pim1* gene.

***pim1* mutation and *rna1* overexpression are synthetically lethal.** To test the hypothesis that the terminal phenotypes of cells overexpressing *rna1* and *pim1-d1^{ts}* arrested cells were mediated by a common mechanism, we tested whether there was a detrimental synergistic effect on cell growth of *rna1* overexpression in the *pim1-d1^{ts}* strain. Serial dilutions of nontransformed *pim1-d1^{ts}* cells, *pim1-d1^{ts}* cells transformed with pREP3X-*rna1*, and wild-type cells transformed with pREP3X-*rna1* grown in liquid media lacking thiamine for 18 h to induce *rna1* transcription were spotted on plates without (promoter ON) or with (promoter OFF) thiamine at 32°C, the semipermissive temperature for the *pim1-d1^{ts}* mutant (Fig. 4). After derepression of the *nmt1* promoter (Fig. 4, promoter ON), *pim1-d1^{ts}* cells overexpressing rna1p had a much more pro-

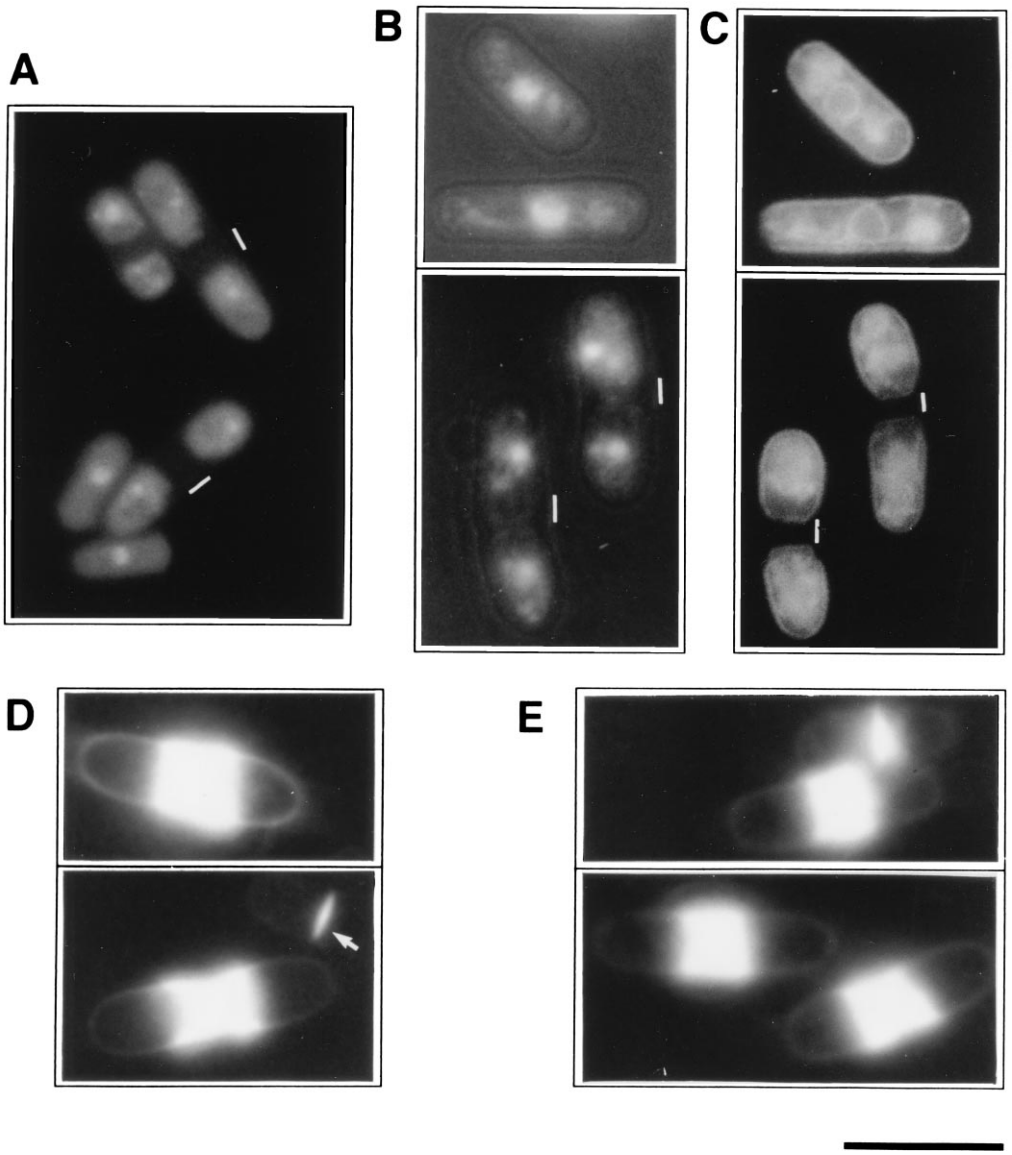


FIG. 3. Phenotypes of cells overexpressing rna1p. (A) DAPI staining of the DNA. (B and C) Hoechst DNA and $DiOC_6$ membrane double staining, respectively, showing examples of cells with normal interphase chromatin in which the outline of the nuclear envelope can be observed (top panels) and examples of arrested, binucleated cells with condensed chromosomes lacking an intact nuclear envelope (bottom panels). (D) Calcofluor staining reveals the formation of medial septa in *rna1*-overexpressing cells that are wider than in cells that have not reached the terminal phenotype (arrow). (E) Calcofluor staining reveals the formation of wide medial septa in *pim1-d1*^{ts} cells at the restrictive temperature. White lines indicate the positions of the medial septum in terminally arrested cells. Bar = 10 μm.

nounced growth inhibition than wild-type cells overexpressing rna1p or nontransformed *pim1-d1*^{ts} cells. This synthetic lethality was also observed when transcription of *rna1* was controlled by two lower-strength versions of the *nmt1* promoter (data not shown). When transcription from the *nmt1* promoter was repressed (Fig. 4, promoter OFF), the growth of the three strains was comparable. In wild-type cells and cells transformed with pREP3X-*rna1* under promoter-repressing conditions, 10 to 15% of the population was septated. When the promoter was derepressed, *rna1*-overexpressing cells were significantly inhibited for growth and the septation index increased to 30 to 45%. The *pim1-d1*^{ts} pREP3X-*rna1* cells also showed this characteristic of the wild-type *rna1*-overexpressing cells, but at a frequency of up to 70%. Because the defect in the pim1p GEF was not detrimental to cell growth at the semipermissive temperature of 32°C, these data suggest that the mutation lowers the tolerance threshold for rna1p GAP expression at the permissive temperature, resulting in a higher percentage of *rna1*-overexpressing cells that display the characteristic *pim1-d1*^{ts} phenotype.

spi1p nuclear localization is dependent on both the GEF and the GAP. The nucleotide-bound state of spi1p is regulated by the balance between the pim1p GEF activity and the rna1p GAP activity. To determine if the nucleotide-bound state of spi1p influences its localization, the intracellular distribution of spi1p was monitored in wild-type, *rna1*-overexpressing, and *pim1-d1*^{ts} cells by using affinity-purified anti-spi1p antibodies. To demonstrate the specificity of the anti-spi1p antibody, equal amounts of total protein from cells expressing HA-spi1p from the *nmt1* promoter under repressed or derepressed conditions were analyzed by Western blot analysis (Fig. 1C). The anti-spi1p antibody recognized the endogenous, untagged spi1 pro-

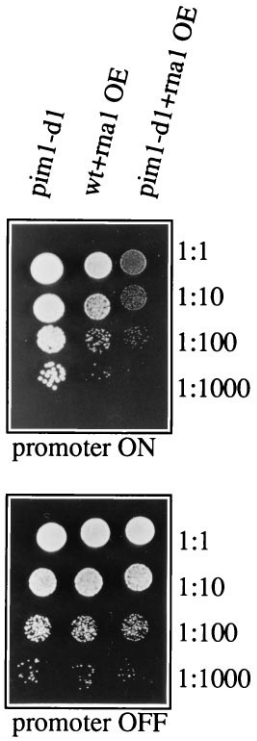


FIG. 4. Synthetic lethality of *rna1* overexpression with the *pim1-d1*^{ts} mutation. A total of 5×10^4 *pim1-d1*^{ts} cells, wild-type cells transformed with pREP3X-*rna1* (wt + rna1 OE), and *pim1-d1*^{ts} cells transformed with pREP3X-*rna1* (pim1-d1 + rna1 OE) were grown in liquid media lacking thiamine for 18 h to induce transcription from the *nmt1* promoter and then spotted on the top row of each plate and in 10-fold serial dilutions on the rows beneath them. One plate lacking thiamine, on which the *nmt1* promoter is derepressed (promoter ON), and one plate containing thiamine, on which the *nmt1* promoter is repressed (promoter OFF), were incubated at 32°C. OE, overexpressing; wt, wild type.

tein with the predicted molecular mass of 25 kDa under both the promoter-on (Fig. 1C, lane 1) and promoter-off (Fig. 1C, lane 2) conditions and also recognized a higher-molecular-mass species only when transcription of HA-spi1p was activated (Fig. 1C, compare lanes 1 and 2). To confirm the identity of this *nmt1* transcription-dependent protein as HA-spi1p, the same protein samples were probed with the anti-HA antibody (Fig. 1C, lanes 3 and 4).

The localization of the pim1 protein was monitored by using affinity-purified anti-pim1p antibodies to determine if pim1p was localized exclusively to the chromatin-containing region of the nucleus and if the localization of pim1p was altered in the *pim1-d1*^{ts} strain. To demonstrate the specificity of the anti-pim1p antibody, equal amounts of total protein from cells expressing HA-pim1p from the *nmt1* promoter under repressed or derepressed conditions were analyzed by Western blot analysis (Fig. 1D). The anti-pim1p antibody recognized the endogenous, untagged pim1 protein with the predicted molecular mass of 58 kDa when the promoter is off (Fig. 1D, lane 2). The endogenous protein and the HA-pim1 protein comigrated, but the increase in the intensity of this band was dependent upon activation of the promoter (Fig. 1D, compare lanes 1 and 2). To confirm the identity of this *nmt1* transcription-dependent protein as HA-pim1p, the same protein samples were probed with the anti-HA antibody (Fig. 1D, lanes 3 and 4).

In *pim1-d1*^{ts} cells at the permissive temperature (Fig. 5A) and wild-type cells (data not shown), affinity-purified anti-spi1p antibodies detected spi1p predominantly in the nucleus, coincident with the DNA and surrounded by the nuclear pores. pim1p, immunolocalized by using affinity-purified anti-pim1p antibodies, was exclusively nuclear but was not found exclusively in the DNA-containing regions of the nucleus (Fig. 5B). In mitosis, pim1p was clearly detected in the region between the separating chromosomes (Fig. 5B, panel 1). In interphase, pim1p was detected throughout the nucleus (Fig. 5B, panel 1).

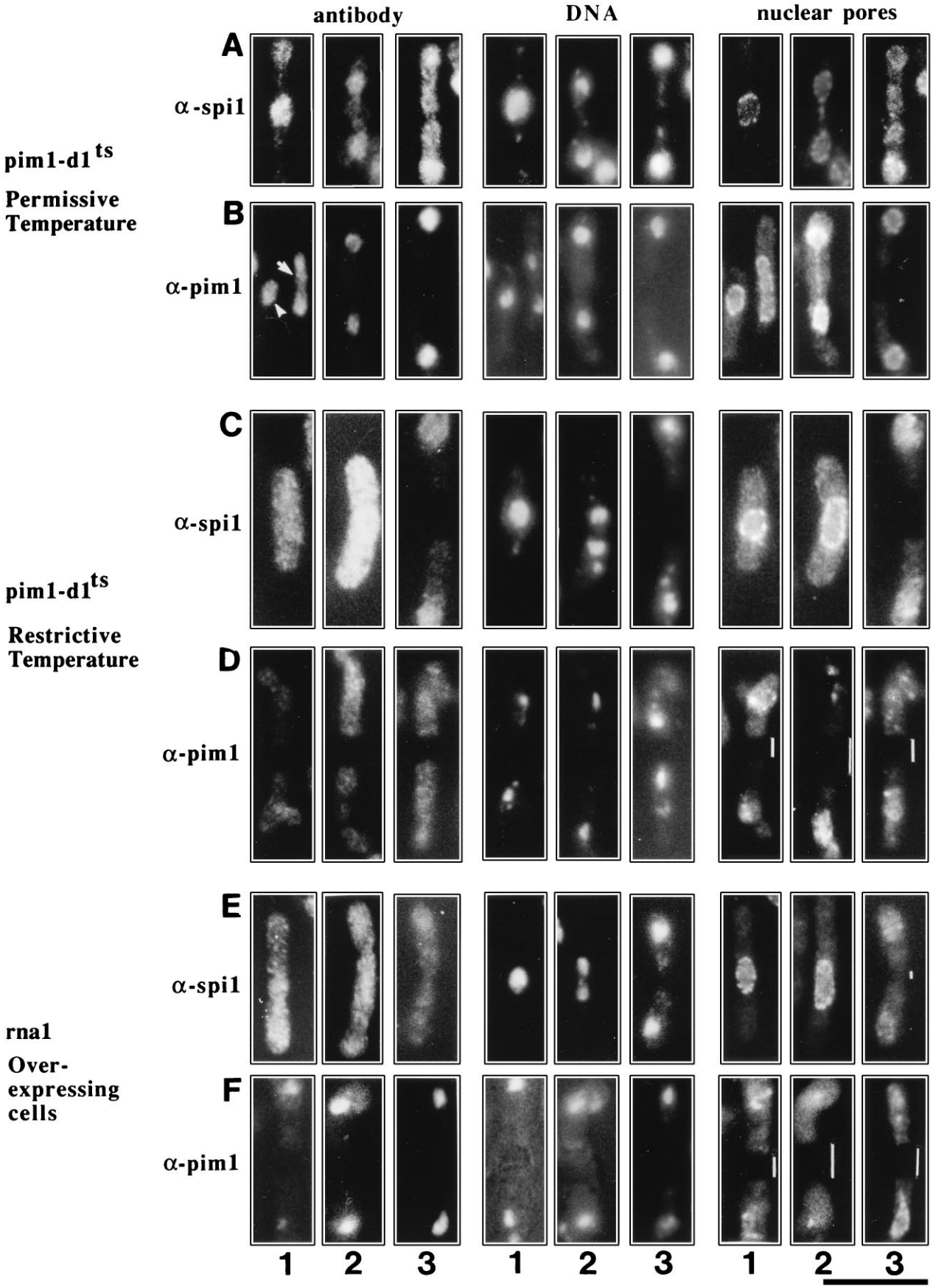
In the *pim1-d1*^{ts} strain at the restrictive temperature, pim1p was degraded (data not shown), a characteristic previously reported for the *pim1-46*^{ts} mutant (26). Under these conditions, cells arrest after mitosis with a wide medial septum, condensed chromosomes, and a fragmented nuclear envelope (Fig. 5C, panel 3, and D). Indirect immunofluorescence microscopy demonstrated that in these terminally arrested cells, both spi1p (Fig. 5C, panel 3) and pim1p (Fig. 5D) lost their nuclear localization. However, after incubation at the restrictive temperature for just 2 h, even before nuclear envelope fragmentation was detected microscopically, spi1p was found equally in the cytoplasm and nucleus (Fig. 5C, panels 1 and 2). In *rna1*-overexpressing cells that arrest with a terminal phenotype identical to that of the *pim1-d1*^{ts} mutant, immunofluorescence microscopy indicated that spi1p was also uniformly distributed throughout cells both before (Fig. 5E, panels 1 and 2) and after (Fig. 5E, panel 3) nuclear envelope fragmentation. In contrast to our observations with *pim1-d1*^{ts} cells, in which the pim1 protein is degraded, in *rna1*-overexpressing cells pim1p remains colocalized with the DNA even after nuclear envelope fragmentation (Fig. 5F).

Disruption of the *rna1* gene is lethal. Overexpression of *rna1* in wild-type cells resulted in growth arrest with a *pim1-d1*^{ts} phenotype. We also examined the phenotypic consequences of loss of *rna1*, which should have an opposite effect on the nucleotide-bound state of spi1p. Although disruption of the *rna1* gene in fission yeast cells has previously been described (28), no physiological or morphological characterization was reported and the strain was not available for the present study. To study the phenotypic consequences of disruption of the *rna1* gene and to establish that the gene is essential, we reconstructed and unambiguously identified *rna1*-disrupted strains.

The linearized disruption construct (see Materials Methods) was integrated into a wild-type diploid strain. Southern blot analysis showed that the parental strain had a single *Cla*I fragment of approximately 5.1 kb (Fig. 6A, lane 2) that hybridized to the *rna1* coding region probe and was not present as a discrete band in uncut DNA (Fig. 6A, lane 1). The Δrna1-A1 disrupted diploid strain contained a wild-type 5.1-kb fragment and a 6.2-kb fragment, the size of the *ura4*-containing gene replacement (Fig. 6A, lane 3). The same *rna1* coding region probe was hybridized to an ordered cosmid library filter (22, 25), and the chromosomal positions of the positively hybridizing cosmids were used to map the *rna1* locus to chromosome 1 between *rad1* and *tps19*.

Tetrad analysis of Δrna1-A1 diploids yielded 13 of 14 dissected tetrads with two viable colonies and two single germinated spores. Replica plating to selective media indicated that the growing colonies were all Ura$^-$ and therefore wild type for *rna1* (Fig. 6B) and that the Δrna1-A1 spores were unable to form colonies. Further confirmation that the *rna1* gene was disrupted in the Δrna1-A1 diploid was obtained by plasmid rescue (see Materials and Methods).

We took advantage of our observation that the *rna1* gene product was required for mitotic growth but not for spore germination to examine the phenotype of *rna1*::*ura4* germinated spores. The Δrna1-A1 diploid strain was sporulated, and random spore analysis was performed. As expected from the


antibody
DNA
nuclear pores
A
α-spi1
pim1-d1ts
Permissive
Temperature
B
α-pim1
C
α-spi1
pim1-d1ts
Restrictive
Temperature
D
α-pim1
E
α-spi1
rna1
Over-
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cells
F
α-pim1
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tetrad analysis, only wild-type (Ura^-) colonies grew, but microscopic examination also revealed a number of single cells and microcolonies. Of 290 such cells, 79% were arrested as germinated spores (Table 1). Of these single germinated cells, 75% arrested with a medial septum, typical of *pim1-d1^{ts}* and *rna1*-overproducing cells. The random spores from the Δrna1-A1 diploid strain were also examined after germination in liquid minimal media lacking uracil to inhibit the germination of wild-type Ura^- spores. The Δrna1-A1 spores germinated within 16 h at 25°C, comparable to the time required for wild-type spores to germinate. Twenty-four hours after germination was initiated, 23% of the Δrna1-A1 cells were septated (Fig. 6C, upper panel) compared with 11% of wild-type cells (Fig. 6C, lower panel). A total of 32% of the Δrna1-A1 cells had abnormally condensed chromatin that is clearly distinguishable from the chromatin of wild-type cells (Fig. 6C, compare upper and lower panels).

DISCUSSION

The nucleotide-bound state of GTPases of the Ran family, including fission yeast spi1p, are regulated by the antagonistic activities of the GEF, which stimulates the exchange of GDP for GTP, and the GAP, which catalyzes the hydrolysis of GTP to GDP. A RanGAP1-related protein, rna1p, has been found in *S. pombe* and shown to have GAP activity in vitro (4, 28). Here, we report genetic evidence that links rna1p as a physiologically relevant component of the *S. pombe* spi1p GTPase system in vivo. We have monitored the effects of perturbing the functioning of the spi1p GTPase system in vivo, using a variety of methods, and conclude that cells require a precise balance between the GDP- and GTP-bound forms of spi1p, that the nucleotide-bound state of the GTPase correlates with its intracellular localization, that there is a single pool of spi1p GTPase in fission yeast cells that shuttles between the nucleus and cytoplasm, and that the essential function of pim1p is as the spi1p GEF and not as a structural component of chromatin.

Decreased nuclear GEF activity and increased cytoplasmic GAP activity, both of which would be expected to shift the balance to the GDP form of spi1p, have similar phenotypic consequences. Presumably because both lead to an increase of spi1p-GDP relative to spi1p-GTP, we found that overexpression of rna1p led to the same phenotypic changes as a loss of the pim1p GEF with regard to nuclear morphology, subcellular localization of spi1p, and septum formation. Furthermore, overexpression of rna1p was synthetically lethal with a mutation in *pim1*, suggesting that their effects are additive and are likely to be mediated by an imbalance between the two nucleotide-bound states of spi1p. Further support for this model comes from additional experiments in which spi1p-GDP was expected to accumulate. Wild-type cells overexpressing either a mutant form of spi1p stabilized in the GDP-bound state (14) or the recently identified sbp1 (spi1p-binding protein 1) (21), a fission yeast protein approximately 55% identical and 70% similar at the amino acid level to the human (RanBP1) and budding yeast (YRB1) GAP-activating proteins (4, 34, 39), displayed phenotypes similar to those of cells overexpressing the rna1p GAP. These effects on nuclear morphology associated with perturbing the nucleotide-bound state of spi1p that we observe in fission yeast cells are similar to those seen in *Xenopus* nuclear assembly systems (12). In the presence of the dominant Ran mutant stabilized in its GDP-bound state, nuclei form but cannot grow and cannot assume the normal interphase state. This suggests that the defects in nuclear architecture associated with alterations in the spi1p-GDP concentration are not specific features of *S. pombe* but are also found in other eukaryotic organisms.

Loss of rna1p, which is expected to shift the balance to the GTP form of spi1p, mimics overexpression of rna1p, which is expected to shift the balance to the GDP form. We were unable to monitor the effects of depletion of rna1p from cells in which the endogenous *rna1* gene had been disrupted by either repression of the ectopic promoter or plasmid loss. However, because *rna1* is essential for mitotic growth but not for spore germination in *S. pombe*, we could examine the terminal phenotype of the Δ*rna1* spores. We found that after germination, spores lacking the *rna1* gene product, presumably having a decreased spi1p-GDP/spi1p-GTP ratio, had a phenotype similar to those of *pim1-d1^{ts}* mutants and cells overexpressing rna1p. These results suggest that cells require the proper balance between spi1p-GTP and spi1p-GDP to complete the mitosis-to-interphase transition and that disruption of this balance in favor of either form results in similar phenotypic changes. This observation is in accordance with previous findings that temperature-sensitive mutations in *RNA1* (GAP) and *PRP20* (GEF) have similar effects on RNA processing in budding yeast cells (17).

If, as our results suggest, increasing the relative abundance of spi1p-GDP or spi1p-GTP results in similar phenotypic consequences, this would shed light on the general mode of action of spi1p and other Ran-related GTPases. Some cellular processes dependent on G-proteins, such as Rab-dependent vesicle fusion (16) and EF-G-dependent ribosome translocation during translation (3), are sensitive to an overabundance of either the GTP- or the GDP-bound form of the GTPase. In this respect, they differ from other processes, such as oncogenic transformation, which is mediated by expression of the GTP-bound form but not the GDP-bound form of p21^{ras} (8).

Our observation of similar terminal phenotypes in *pim1-d1^{ts}* cells, cells overexpressing *rna1*, and cells in which the *rna1* gene has been deleted suggests a model in which either the balance between spi1p-GTP and spi1p-GDP or the continual cycling between these two states is required for cell viability. In all three of these situations, the normal balance of the two forms of the GTPase is perturbed. Further examination of the second possibility is warranted because although cycling would be impaired in the absence of functional pim1p or rna1p, it would be expected to occur, although perhaps not in a normal manner, when rna1p is overexpressed in cells containing wild-type pim1p.

spi1p GEF and GAP activities are localized in separate intracellular compartments. We raised antibodies to pim1p,

FIG. 5. Immunolocalization of spi1p and pim1p relative to the DNA and the nuclear pores. (A) spi1p, DNA, and nuclear pore localization in *pim1-d1^{ts}* cells at the permissive temperature. (B) pim1p, DNA, and nuclear pore localization in *pim1-d1^{ts}* cells at the permissive temperature reveals pim1p localized throughout the nucleus of interphase cells (arrowhead) and in both the DNA- and the non-DNA-containing regions of the nucleus in mitotic cells (arrow). (C) spi1p, DNA, and nuclear pore localization in *pim1-d1^{ts}* cells at the restrictive temperature for 2 h (panels 1 and 2), which have not yet undergone nuclear envelope fragmentation, or 4 h (panels 3), which have undergone nuclear envelope fragmentation. (D) pim1p, DNA, and nuclear pore localization in *pim1-d1^{ts}* cells at the restrictive temperature for 4 h that have undergone nuclear envelope fragmentation. (E) spi1p, DNA, and nuclear pore localization in *rna1*-overexpressing cells that have intact (panels 1 and 2) or fragmented (panels 3) nuclear envelopes. (F) pim1p, DNA, and nuclear pore localization in *rna1*-overexpressing cells that have undergone nuclear envelope fragmentation. White lines indicate the positions of the medial septum in terminally arrested cells. Bar = 10 μm.

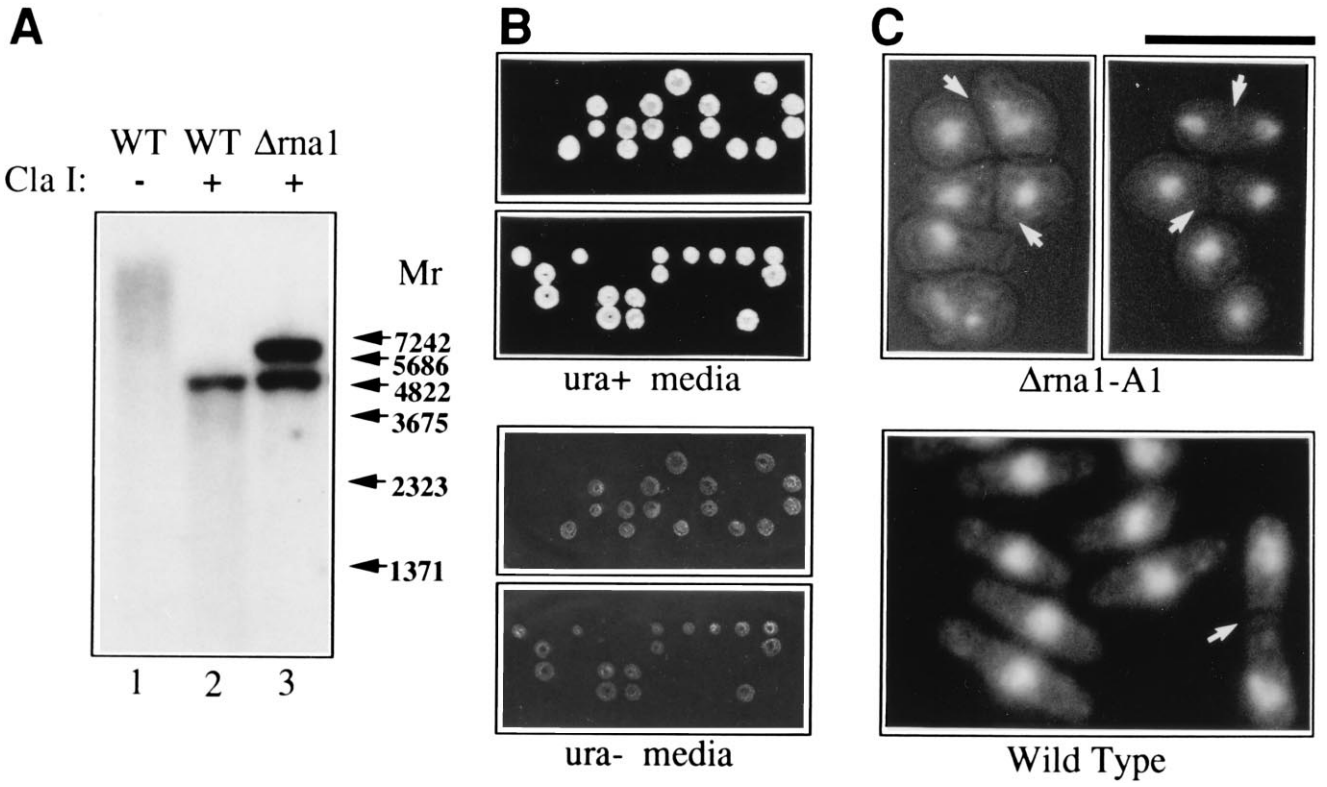

FIG. 6. Disruption of the *rna1* gene. (A) Southern blot analysis of diploid parental undigested DNA (lane 1), parental *Cla*I digested DNA (lane 2), and strain Δrna1-A1 DNA (lane 3) probed with a 1.6-kb fragment of the *rna1* coding region. The digested Δrna1-A1 DNA has one fragment of 5.1 kb corresponding to the wild-type copy of the *rna1* gene and one fragment of 6.2 kb corresponding to the disrupted copy, whereas the parental DNA contains just the wild-type copy and the undigested DNA has neither. (B) Δrna1-A1 tetrads replica plated to minimal media with (top) or without (bottom) uracil. (C) DAPI staining of the DNA of Δrna1-A1 (top) and wild-type spores (bottom) germinated in liquid medium for 24 h. Compared with wild-type cells, Δrna1-A1 cells accumulate as septated cells (arrows) that have abnormally condensed chromatin. WT, wild type. Bar = 10 μm.

spi1p, and rna1p and found that the GEF was exclusively nuclear but not exclusively chromatin associated in both interphase and mitotic cells. This is in contrast to previous studies of mitotic mammalian cells in which the GEF did not colocalize with DNA (32, 33, 35). We also found that the GAP was exclusively cytoplasmic and the GTPase was predominantly but not exclusively nuclear. These localization patterns are consistent with observations for other eukaryotic systems (reviewed in references 10 and 37).

There is a single intracellular pool of the spi1p GTPase. Perturbations in either the nuclear GEF or the cytoplasmic GAP have similar phenotypic consequences, which we believe are mediated by an imbalance between the GTP- and GDP-bound forms of spi1p. These observations are consistent with a model in which there is a single pool of the GTPase that shuttles between the nucleus and the cytoplasm in order to undergo a complete round of nucleotide binding and hydrolysis.

TABLE 1. Growth characteristics of germinated Δrna1 spores

Colony size (no. of cells)	% of cells[a]
0 (ungerminated spores)	10
1	79
2	7
3	1
4	1
>4	2

[a] A total of 290 cells were counted.

The localization of the spi1p GTPase correlates with its nucleotide-bound state. spi1p lost its predominant nuclear localization in both *rna1*-overexpressing cells and *pim1-d1*^{ts} cells prior to loss of nuclear envelope integrity. Under these two conditions, the relative abundance of spi1p-GDP is expected to increase, demonstrating a correlation between the nucleotide-bound form of the GTPase and its localization. We predict that nuclear spi1p is predominantly GTP bound, whereas cytoplasmic spi1p is predominantly GDP bound. Our observations, together with the topological separation of the GAP and the GEF in the cytoplasm and the nucleus, respectively, suggest that the distinctive properties of the spi1p-GTP and spi1p-GDP species are executed in different cellular compartments.

The essential function of chromatin-associated pim1p is its GEF activity. Our results have direct bearing on the question of whether the GEFs for the Ran family of GTPases have functions other than activation of guanine nucleotide exchange. RCC1, the GEF for Ran, is normally localized to the chromatin but is rapidly degraded in the temperature-sensitive tsBN2 mutant which undergoes premature chromatin condensation. On the basis of this observation and others, it has been proposed that RCC1 may have a structural role in chromatin and nuclear morphology (11, 24). However, because the DNA replication defect of chromatin assembled in a *Xenopus* extract depleted of RCC1 and the premature chromatin condensation defect in tsBN2 cells can be rescued by the addition of Ran-

GTP (12, 31), it has been suggested that the essential function of RCC1 is its exchange activity. In *pim1-d1^{ts}* cells, the pim1 protein level decreased dramatically after a shift to the restrictive temperature, probably because of degradation, and the exclusively nuclear immunolocalization of pim1p was lost. We have shown that wild-type cells overexpressing rna1p had the same morphological phenotype as *pim1-d1^{ts}* arrested cells but that pim1p retained its bright nuclear staining. pim1p was colocalized with the DNA in *rna1*-overexpressing cells even after loss of nuclear envelope integrity, suggesting that at least some pim1p remained chromatin bound. These data are consistent with the hypothesis that the aberrant cellular morphology seen in *pim1-d1^{ts}* arrested cells and *rna1*-overexpressing cells is caused by an abnormal increase of spi1p-GDP relative to spi1p-GTP and is not due exclusively to the loss of chromatin-associated pim1 protein. Thus, the previously characterized phenotype of the *pim1* mutant is likely to be due not to a structural loss of the protein at the restrictive temperature but to the loss of its guanine nucleotide exchange activity.

Summary. We have characterized the phenotypic consequences of perturbing the spi1p system of *S. pombe* and have found that cells responded identically to an overabundance of rna1p or a lesion in pim1p, both of which are expected to increase the spi1p-GDP/spi1p-GTP ratio. We have also shown that loss of rna1p, which presumably has an opposite effect on the spi1p-GDP/spi1p-GTP ratio, also resulted in a phenotype similar to that of GEF mutation and GAP overexpression, suggesting that cells are sensitive to the balance between the two forms of the GTPase.

The crucial remaining questions for elucidation of the molecular function of spi1p are what biological signals regulate the levels of spi1p-GTP and spi1p-GDP in normal cells and what processes they regulate. There is a considerable amount of evidence that Ran and its regulators affect diverse processes in eukaryotic cells, but as yet there is no conclusive evidence regarding which of these processes are directly regulated by this GTPase system (37).

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A.M. and K.D. contributed equally to this work.

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