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Overexpression of *RPI1*, a Novel Inhibitor of the Yeast Ras-Cyclic AMP Pathway, Down-Regulates Normal but Not Mutationally Activated Ras Function

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A high-copy-number plasmid genomic library was screened for genes that when overexpressed down-regulate Ras protein activity in *Saccharomyces cerevisiae*. We report on the structure and characterization of one such gene, *RPI1*, which potentially encodes a novel 46-kDa negative regulator of the Ras-cyclic AMP pathway. Three lines of evidence suggest that the *RPI1* gene product operates upstream to negatively regulate the activity of normal but not mutationally activated Ras proteins: (i) overexpressed *RPI1* lowers cyclic AMP levels in wild-type yeast cells but not in yeast cells carrying the *RAS2*$^{Val-19}$ mutation, (ii) overexpressed *RPI1* suppresses the heat shock sensitivity phenotype induced by overexpression of normal *RAS2* but does not suppress the same phenotype induced by *RAS2*$^{Val-19}$, and (iii) disruption of *RPI1* results in a heat shock sensitivity phenotype which can be suppressed by mutations that lower normal Ras activity. Thus, *RPI1* appears to encode an inhibitor of Ras activity that shares a common feature with Ras GTPase-activating proteins in that it fails to down-regulate activated *RAS2*$^{Val-19}$ function. We present evidence that the down-regulatory effect of *RPI1* requires the presence of one of the two Ras GTPase activators, *IRA1* and *IRA2*.

Ras proteins and Ras-like proteins, such as Sec4 and Cdc42, are members of a group of eukaryotic GTPases that utilize "on" and "off" states, triggered by binding and hydrolysis of GTP, to act as molecular switches that participate in several cellular functions, such as growth control, cell morphogenesis, and secretion (4, 5, 18). Some of the best-studied Ras or Ras-like proteins are the Ras1 and Ras2 proteins of budding yeast cells which, in their GTP-bound on state, stimulate adenylate cyclase to synthesize cyclic AMP (cAMP) and which are the yeast proteins most closely related to the mammalian proto-oncogenic p21^{ras} proteins (3, 7, 13, 15). Studies of various Ras-cAMP pathway mutants have indicated that an important physiological function of the Ras-cAMP pathway involves sensing the availability of metabolites and nutrients, such as glucose, and conveying this information to processes essential for cell growth (9, 25, 26, 41, 42). Exactly how nutrients or metabolites regulate the activity of the yeast Ras-cAMP pathway is not known, but it is likely to involve control of the ratio of GTP to GDP nucleotides bound to Ras proteins (16, 39).

The mechanisms by which Ras and Ras-like proteins are regulated appear to differ in interesting ways from the regulation of the more thoroughly studied heterotrimeric G proteins. For one, the intrinsic GTPase activity of Ras and Ras-like proteins is much slower than that of G proteins, such that the cellular GTPase activity of Ras and Ras-like proteins, unlike that of G proteins, depends upon specific GTPase-activating proteins (GAPs) (4, 5, 27). Physiological signals that regulate Ras and Ras-like proteins may work through the regulation of GAP activity, as suggested by the recent demonstration of T-cell antigen receptor activation of p21^{ras} proteins, which correlated with a decrease in the activity of the p21^{ras} GAP (10). In contrast, the primary physiological signals that regulate the activity of heterotrimeric G proteins are transduced by transmembrane receptors which catalyze the conversion of inactive GDP-bound G proteins to the active GTP-bound form (4, 5, 14). Although indirect evidence suggests that Cdc25 acts analogously as a guanine nucleotide exchange factor for Ras proteins in yeast cells (8, 9, 12, 32, 33) and although similar factors may exist for mammalian p21^{ras} and the Ras1 protein of *S. pombe* (11, 20, 46), there is still no strong indication that Ras and Ras-like proteins are physiologically regulated by changes in the activity of guanine nucleotide exchange factors. Thus, unlike the situation with G proteins, it is possible that negative regulators of Ras and Ras-like proteins may be more physiologically prominent than positive regulators. Recently, Sasahi et al. (34) and Ueda et al. (45) discovered a new type of negative regulator for certain Ras-like proteins, which they term the guanine nucleotide dissociation inhibitor and which acts to stabilize the inactive GDP-bound state.

In yeast cells, one important form of negative regulation of Ras1 and Ras2 activity is governed by two GAP homologs, *IRA1* and *IRA2* (37–40). *IRA1* and *IRA2* potentially encode extremely large proteins (over 300 kDa) that are 45% identical to each other. Both Ira proteins share a stretch of about 380 amino acids that is structurally and functionally related to the catalytic domain of the mammalian p21^{ras} GAP (2, 23, 38–40), and an *IRA2*-encoded peptide that spans this catalytic domain stimulates the GTPase activity of Ras2 proteins (37). Interestingly, the negative regulation of Ras proteins mediated by these two different Ira proteins is not totally redundant, since either the loss of *IRA1* function alone or the loss of *IRA2* function alone significantly increases Ras activity (38–40). Nonredundant Ras GAPs may also exist in animal cells, in which two distinct proteins with GAP activity for p21^{ras} have been discovered: cytoplasmic GAP and the *NF1* gene product (1, 24, 43, 47, 48). Although investigation into the function of Ras GAPs is still a rapidly evolving area of research, it seems likely that one important aspect of their function is the regulation of Ras protein activity (4, 5, 27). Ras GAPs themselves may be regulated; indeed, recent reports demonstrate that increased activity of

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TABLE 1. Yeast strains used in this study

S. cerevisiae strain	Genotype	Reference or source
SP1	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2*	42
JHY10[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ADH1p:CYR1*	This study
JHY102[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 rpi1::LEU2*	This study
JHY105[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 rpi1::URA3*	This study
JHY252[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ADH1p:CDC25-Δ1*	This study
JHY254[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ADH1p:CDC25-Δ2*	This study
JHY311[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ADH1p:RAS2*	This study
JHY450[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ira1::URA3*	This study
PT1-6[a]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 gal2 ras1::URA3 RAS2*$^{\text{Val-19}}$	42
RS62-22A[b]	*MATα leu2 ura3 his3 trp1 ade8 can1 ras2::URA3*	This study
RS11-2D[b]	*MATα leu2 ura3 trp1 his3 ade8 can1 ras1::HIS3 RAS2*$^{\text{Val-19}}$	This study
SLCIII-5[b]	*MAT***a** *leu2 ura3 trp1 his3 ade2 ade8 can1 cyh4*	This study
KT27-1D	*MATα leu2 ura3 trp1 his3 ira2::HIS3*	K. Tanaka
JHY430	*MATα leu2 ura3 trp1 his3 ade8 can1 cyh4 ira2::HIS3*	This study
JHY451[c]	*MAT***a** *leu2 ura3 his3 trp1 ade8 can1 cyh4 ira1::URA3*	This study
JHY431[c]	*MATα leu2 ura3 his3 trp1 ade8 can1 cyh4 ira2::HIS3*	This study
JHY442[c]	*MATα leu2 ura3 his3 trp1 ade8 can1 cyh4 ira1::URA3 ira2::HIS3*	This study
LRA26	*MATα leu2 ura3 his4 cdc25-5*	K Tatchell
KT626	*MAT***a** *leu2 ura3 his4*	K Tatchell
JHY210[d]	*MAT***a** *leu2 ura3 his4 rpi1::URA3*	This study

[a] Derived from SP1 by DNA transformation.
[b] Congenic to SP1.
[c] Derived from the same diploid that was formed by crossing JHY450 with JHY430. JHY430 was derived from a cross between KT27-1D and SLCIII-5.
[d] Derived from KT626 by DNA transformation.

protein kinase C or increased production of certain lipids, such as arachidonic acid, negatively affect the GTPase-stimulating activity of the cytoplasmic p21^{ras} GAP (10, 44). It is possible that the activities of Ras GAPs are also regulated by positive effectors.

We sought to devise a simple genetic means of finding novel negative regulators of Ras activity in *S. cerevisiae*. To do so, we used a high-copy-number plasmid genomic library and looked for normal genes which, when overexpressed, could down-regulate the Ras-cAMP pathway. To avoid examining negative regulators acting downstream from the site of control of Ras activity, we included in the screening a step to exclude overexpressed genes which could suppress *RAS2*$^{\text{Val-19}}$, which encodes a mutant Ras protein that is relatively insensitive to control by either the *IRA* or the *CDC25* gene product (8, 33, 39).

MATERIALS AND METHODS

Yeast strains. The yeast strains used in this study are described in Table 1. Transformation of yeast cells with DNA was performed by the lithium acetate method, with the modification that 25 mM dithiothreitol or 25 mM β-mercaptoethanol was included in the transformation buffers. Integration of DNA at the expected site was checked by Southern analysis.

Construction of plasmids that overexpress *CDC25*, *CYR1*, and *RAS2*. Integration plasmid pJHK600 was constructed by inserting the 6.0-kbp *Sal*I-*Cal*I fragment of the episomal *ADH1p:CYR1* plasmid pEF-*CYR1* (13) into the *URA3*$^{+}$ vector YIp5. Digestion of pJHK600 with *Xho*I directs the integration of the plasmid into the *CYR1* locus, resulting in the endogenous *CYR1* promoter driving the expression of an adenylate cyclase gene missing C-terminal coding sequences and the *ADH1* promoter driving the expression of an intact adenylate cyclase coding sequence. We refer to this mutant allele as *ADH1p:CYR1*. Plasmid pJHK410 was constructed by inserting the 2.5-kbp *Bam*HI fragment of YE*ADH1p:RAS2* (kindly provided by T. Kataoka) into centromere plasmid pRS316; the mutant *RAS2* allele is referred to as *ADH1p:RAS2*. Plasmid pJHK302 contains the 2.6-kbp *Sph*I fragment of plasmid pM*CDC25*-A inserted into YCp50 such that the *ADH1* promoter drives the expression of a *CDC25* gene missing nonessential sequences 5′ to the *Bgl*II site at codon 877 (8); this mutant allele is referred to as *ADH1p:CDC25-Δ1*. pM*CDC25*-B is an episomal plasmid that utilizes the *ADH1* promoter to direct the expression of the C-terminal 563 amino acids of *CDC25*; this mutant allele is referred to as *ADH1p:CDC25-Δ2*. Details of the construction of plasmids pM*CDC25*-A and pM*CDC25*-B will be published elsewhere.

Localization of gene activity. *RPI1* high-copy-number plasmids p1 through p5 were isolated from a genomic library constructed in vector YEp13M4 by Junichi Nikawa (29). The ability of library isolate p1 to suppress *ADH1p:CYR1* indicated that sequences outside of the vector-insert junctions (specifically, the right-hand vector-insert junction of p1 shown in Fig. 1) were not required for activity. Thus, sequences to the right of the *Sau*3AI site shown in Fig. 1 are not essential sequences of the *RPI1* gene. Similarly, sequences to the left of the *Sal*I site shown in Fig. 1 are not essential sequences of the *RPI1* gene, on the basis of the retention of biological activity of plasmid pJHK105, which was derived from library isolate p2 by deletion of sequences from the *Sal*I site within the insert to the nearby *Sal*I site within the vector. These results indicated that the entire *RPI1* gene or at least an active fragment of the *RPI1* gene resided within the 2.2-kbp stretch of DNA (flanked by a *Sal*I site and a *Sau*3AI site) shown in Fig. 1. Other deletion mutants that were derived from the original library isolates and that were missing sequences within this 2.2-kbp region were negative for suppressing activity.

Construction of the complete *RPI1* coding sequence disruption plasmid. The plasmid used for the complete disruption of the coding sequence of *RPI1* by the *URA3* marker was constructed by use of oligonucleotide-directed deletion mutagenesis. We synthesized an oligonucleotide (5′-TTTATT

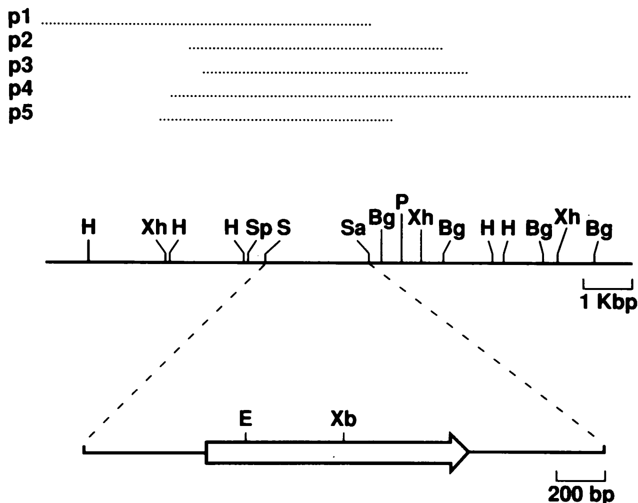

FIG. 1. Composite restriction site map of the *RPI1* locus. Shown is a composite restriction site map of the 12.3 kbp of DNA defined by the overlapping genomic inserts of plasmids p1 through p5, all of which suppressed the heat shock sensitivity phenotype induced by overexpression of *CYR1* (see the text). All sites for *Bgl*II (Bg), *Xho*I (Xh), *Hin*dIII (H), *Pst*I (P), and *Sal*I (S) are displayed, as are two other sites that are not (*Sau*3AI [Sa]) or may not (*Sph*I [Sp]) be unique within the insert but are important for describing the analysis of *RPI1* or the construction of certain plasmids. Shown on a magnified scale is the 2.2-kbp biologically active fragment of DNA containing the *RPI1* coding sequence. The *Eco*RI (E) and *Xba*I (Xb) sites within this 2.2-kbp stretch of DNA are unique.

ACTGATATTTATAAAGCTTAAAGTATGTATCGGGAGTC-3′) that begins with the 21-nucleotide sequence immediately 5′ to the initiation codon of *RPI1*, followed by a *Hin*dIII site, followed by the 21-nucleotide sequence immediately 3′ to the final amino acid codon. Hybridization of the oligonucleotide to a single-stranded template prepared from phagemid pJHK200 (see below) and subsequent steps of the mutagenesis procedure were carried out by standard procedures with the *dut ung* strain CJ236. Candidates were screened for reduced size and then sequenced. One correct candidate, pJHK223, was digested with *Hin*dIII and ligated to the *Hin*dIII fragment of *URA3* to generate pJHK224. pJHK224 was digested with *Sal*I and *Xba*I (the *Xba*I site is in the polylinker) to generate the DNA fragment used for the disruption of *RPI1*.

Other plasmids. Four plasmids containing DNA which spanned the entire *RPI1* gene (Fig. 1) were constructed for use in sequencing, construction of gene disruptions, or construction of alternate high-copy-number plasmids. The 2.5-kbp *Sal*I-*Bgl*II fragment was cloned into pBLUESCRIPT SK(+) to generate pJHK200; the 2.9-kbp *Hin*dIII-*Bgl*II fragment was cloned into KS(+) to generate pJHK203; the 4.2-kbp *Hin*dIII-*Bgl*II fragment was cloned into pUC118 to generate pJHK220 (the *Bgl*II site used extends beyond the proximal *Bgl*II site); and the 2.9-kbp *Sph*I-*Bgl*II fragment was cloned into YEp24 to generate the high-copy-number plasmid pJHK120. The *rpi1::LEU2* disruption plasmid was constructed stepwise by digestion of pJHK220 with *Xba*I and *Sal*I, blunt-ending with the Klenow fragment, and religation to generate pJHK221, which retains the *Sal*I site within the *RPI1* locus but suffers a deletion of 539 nucleotides upstream of the initiation codon and the first 556 nucleotides of the coding sequence. The *Sal*I-*Xho*I fragment of *LEU2* was inserted into the *Sal*I site of pJHK221 to generate pJHK222. The DNA fragment that was used to disrupt *RPI1* was isolated by digestion of this plasmid with *Hin*dIII and *Pst*I.

We also used the following high-copy-number plasmids containing the *LEU2* marker: YEp13, YEp-*PDE2* (35), YEp-*PDE1* (29), and pAAH5-GAP (39). Other plasmids used included YEp24, pd13 (YEp24-*IRA1*) (38), and pKT16 and pKP11, which utilize the glyceraldehyde-3-phosphate dehydrogenase promoter to overexpress active fragments of *IRA2* and *NF1*, respectively (37, 47).

Determination of heat shock sensitivity. One-day-old patches of cells grown on either YPD or synthetic medium plates were replica plated to prewarmed plates, placed for 10 to 45 min in an incubator kept at 55 to 56°C, and allowed to recover for 1 to 3 days at normal temperatures. The length of the heat shock depended upon the strain background and the particular mutation being examined. In general, $RAS2^{Val-19}$, *ira2*, and *ira1 ira2* mutants were heat shocked for 10 to 20 min, whereas *ira1*, *ADH1p:CYR1*, *ADH1p:CDC25*, *ADH1p:RAS2*, and *rpi1* mutants were heat shocked for 20 to 45 min.

DNA sequencing and analysis. Nested deletions of phagemid pJHK200 were used to generate single-stranded templates suitable for sequencing the entire 2.2-kbp biologically active region of DNA containing *RPI1*. The other strand was sequenced with oligonucleotides designed from prior sequencing by use of phagemid pJHK203 for the production of a template. Sequenases 1.0 and 2.0 were used in accordance with the instructions provided by the manufacturer. Analysis of the *RPI1* coding sequence for homologies to previously sequenced genes and proteins was performed with the FASTA and TFASTA programs of the GCG software package of the University of Wisconsin. No scores of any significance were found when *RPI1* was compared with the current data bases. The highest scoring matches were well below those of several weak but significant homologies, such as those between bovine GAP and *IRA1*, *RAM1* and *ORF2*, or *CDC25* and *LTE1* (1, 6, 30).

cAMP assays. Cells taken from log-phase cultures were inoculated at low densities into standard synthetic medium and incubated with vigorous shaking at 30°C, and growth was monitored by measuring the optical density. Cultures were allowed to reach the early stationary phase and to further incubate for 12 to 14 h to ensure the depletion of glucose. Glucose depletion was monitored by the use of indicator sticks. During this relatively brief starvation period, the viability of the strains, including the $RAS2^{Val-19}$ mutant, did not decrease. Glucose was added to a final concentration of 2%. One-milliliter aliquots were removed before and after glucose addition, collected onto polycarbonate filters by vacuum filtration, extracted with 1.5 ml of 1 M formic acid saturated with butanol, centrifuged to remove insoluble material, dried with a Speed-Vac, and resuspended in 50 mM sodium acetate buffer (pH 4.75) for the measurement of cAMP content by a radioimmunoassay as described previously (19). Duplicate 1-ml aliquots were collected for the measurement of protein content as described previously (22).

RESULTS

Isolation of *RPI1* from a high-copy-number genomic library. High-copy-number yeast genomic expression libraries screened for members that can suppress a mutant phenotype have been useful in the identification of additional gene products that act in a particular pathway. For example,

the *CLN1* and *CLN2* G1-cyclin genes were identified as high-copy-number suppressors of temperature-sensitive *cdc28* mutants, and the *PDE1* and *PDE2* cAMP phosphodiesterase genes were identified as high-copy-number suppressors of the hyperactive cAMP pathway phenotype induced by *RAS2*$^{Val-19}$ (17, 29, 35). In the latter case, the hyperactivity induced by *RAS2*$^{Val-19}$ prevents cells from becoming heat shock resistant following nutrient depletion and arrest in the stationary phase (29, 35). Acquisition of increased resistance to heat shock is a normal response of wild-type cells to growth arrest in G_0, and this phenotypic change is manifested in individual colonies after 5 or 6 days of colony growth, when sufficient numbers of the cells have entered the stationary phase (21). Overexpression of either *PDE2* or *PDE1* on high-copy-number plasmids allows *RAS2*$^{Val-19}$ mutant colonies to display normal heat shock resistance, and such colonies can be readily distinguished in a screening of a high-copy-number plasmid library (29, 35).

We wished to use a high-copy-number genomic library to find genes which, when overexpressed, acted to down-regulate normal Ras function, as opposed to mutationally activated *RAS2*$^{Val-19}$ function; to do so, we examined various genetic manipulations of the Ras-cAMP pathway for a suitable heat shock sensitivity phenotype. We wished to find a mutation that could induce a heat shock sensitivity phenotype that would be dependent upon normal Ras activity. We examined the phenotype induced by overexpression of the full-length normal adenylate cyclase gene *CYR1*. Strains were constructed to contain a single integrated copy of a plasmid that expresses full-length adenylate cyclase by use of the *ADH1* promoter (see Materials and Methods). These strains, containing a single copy of the *ADH1p:CYR1* allele, were found to have a hyperactive cAMP pathway phenotype, as evidenced by increased heat shock sensitivity and a reduction in the sporulation efficiency of diploids from 40 to 10%. The heat shock sensitivity phenotype induced by *ADH1p:CYR1* was fully suppressed by the partial loss of Ras function (*ras2*), indicating that overexpressed wild-type adenylate cyclase still requires intact Ras function for full activity (data not shown). In addition, we found that overexpression of full-length adenylate cyclase only very weakly suppressed *ras1 ras2* or *cdc25* growth defects (data not shown). Therefore, overexpressed full-length adenylate cyclase activates the pathway, but this hyperactivity is totally dependent upon normal levels of Ras activity. We reasoned that overexpressed genes which could down-regulate normal Ras activity should be able to suppress *ADH1p:CYR1*.

To search for such genes, we transformed a strain carrying the *ADH1p:CYR1* allele (JHY10) with DNA from a yeast genomic library constructed in the high-copy-number plasmid YEp13M4 (29). After allowing the transformants to grow for 5 days, we subjected the colonies to heat shock (see Materials and Methods). Of approximately 10,000 library transformants analyzed, 189 colonies exhibited increased resistance to heat shock. We patched these candidates and allowed them to grow for 2-days for reevaluation of their heat shock resistance compared with that of the original *ADH1p:CYR1* mutant strain. Only 62 of the 189 candidates showed clear heat shock resistance upon reexamination. Of these 62, 13 were Ura$^-$, suggesting that the integrated *ADH1p:CYR1* plasmid (which contains the *URA3*$^+$ gene as a selectable marker) had been lost by a mitotic crossing-over event.

Of the remaining 49 candidates, we expected that some might contain high-copy-number plasmids which could act downstream of Ras to suppress the phenotype induced by *ADH1p:CRY1*. To test this idea, we mated each of these 49 candidates to a *RAS2*$^{Val-19}$ haploid of the opposite mating type (RS11-2D) and selected for diploids while maintaining selective pressure for the presence of the high-copy-number plasmids. We patched these diploids and, after 2 days of growth, tested them for heat shock resistance. Nine of the 49 candidates yielded diploids which were heat shock resistant, indicating that they contained high-copy-number plasmids which could suppress *RAS2*$^{Val-19}$; these candidates were not studied further. DNA was prepared from the remaining 40 candidates and, following transformation into *Escherichia coli*, we succeeded in rescuing plasmids with genomic inserts from 26 of these candidates. Each of these 26 plasmids was transformed into the original *ADH1p:CYR1* mutant and assayed for the ability to suppress the heat shock sensitivity of this mutant. Only 9 of these 26 plasmids exhibited clear activity in this assay; of these 9 plasmids, only 5 were unique. Restriction enzyme analysis indicated that all of the 5 unique plasmids contained overlapping inserts that defined a region of DNA spanning approximately 12 kbp. A composite restriction map of the locus covered by the inserts of the five unique plasmids is shown in Fig. 1. Subcloning experiments (see Materials and Methods) indicated that the suppressing activity of this stretch of DNA resided in the 2.2-kbp fragment shown in Fig. 1. We named the gene contained within this region *RPI1*, for Ras-cAMP pathway inhibitor. On the basis of the restriction map of the *RPI1* locus and the restriction maps of the *IRA1* and *IRA2* loci, we concluded that *RPI1* is distinct from these two known negative regulators of normal Ras activity in yeast cells (38, 40).

Characterization of the ability of overexpressed *RPI1* to suppress hyperactive cAMP pathway mutants. Our goal was to find a gene which, when overexpressed, could down-regulate normal Ras activity, but not the *RAS2*$^{Val-19}$ gene product, since several lines of evidence suggest that the protein encoded by *RAS2*$^{Val-19}$ possesses activity that is not sensitive to regulation. For one, this protein is deficient in intrinsic GTPase activity compared with normal Ras2 protein and, unlike normal Ras2, its GTPase activity is not activated by a purified fusion fragment of the *IRA2* gene product (37). In addition, the expression of *RAS2*$^{Val-19}$ bypasses the essential requirement for *CDC25* function, and the heat shock sensitivity phenotype induced by *RAS2*$^{Val-19}$ is not significantly attenuated by the loss of *CDC25* function (8). In contrast, high-level overexpression of normal *RAS2* driven by the *ADH1* promoter induces a heat shock sensitivity phenotype, but the hyperactivity of Ras in this case is still dependent upon upstream activation, since overexpression of wild-type *RAS2* does not bypass the essential requirement for *CDC25* function (8). Therefore, we tested whether transformation of high-copy-number *RPI1* could suppress the heat shock sensitivity phenotype of cells in which overexpression of wild-type *RAS2* is driven by the *ADH1* promoter. As controls, we also tested the effect of overexpression of the cAMP phosphodiesterase genes *PDE1* and *PDE2*. We found that high-copy-number *RPI1* significantly suppressed the heat shock sensitivity phenotype induced by high-level overexpression of normal *RAS2* (Fig. 2). The level of suppression exerted by high-copy-number *RPI1* was as strong as that exerted by high-copy-number *PDE2* or *PDE1* (Fig. 2). In contrast, we found that high-copy-number *RPI1* did not suppress the *RAS2*$^{Val-19}$ mutant PT1-6, compared with the strong suppression by high-copy-number *PDE2* and the weaker suppression by high-copy-number *PDE1* (Fig. 2). Thus, overexpression of *RPI1* clearly

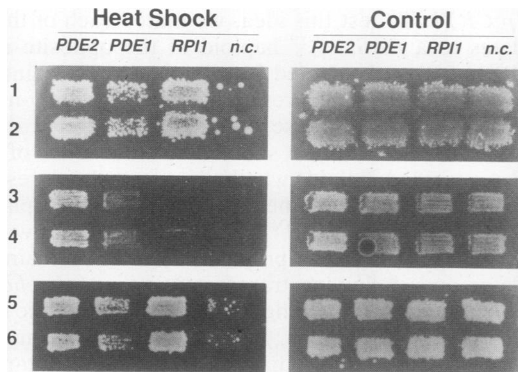

FIG. 2. Suppression of the heat shock sensitivity phenotype of cAMP pathway mutants by overexpressed *RPI1*. Different cAMP pathway mutants were transformed with various high-copy-number plasmids, and the ability of these plasmids to suppress their heat shock sensitivity phenotype was assayed. Colonies were patched on synthetic medium plates, grown for 1 day, replica plated, immediately heat shocked at 55 to 56°C for various times, allowed to recover, and grown for 1 to 3 days at 30°C. The right panel shows the control, non-heat-shocked replica, and the left panel shows the heat-shocked replica. Rows 1 and 2, for each plasmid, two independent transformants of host strain JHY311 (*ADH1p:RAS2*), 40-min heat shock; rows 3 and 4, for each plasmid, two independent transformants of PT1-6 (*RAS2*$^{Val-19}$), 20-min heat shock; rows 5 and 6, for each plasmid, independent transformants of JHY252 (*ADH1p: CDC25-Δ1*), 40-min heat shock. Column 1, high-copy-number *PDE2* transformants; column 2, high-copy-number *PDE1* transformants; column 3, high-copy-number *RPI1* transformants; column 4, negative control transformants (*n.c.*) (YEp13).

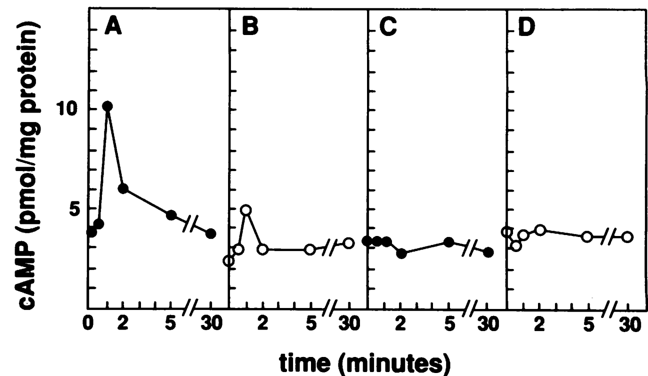

FIG. 3. Effect of overexpressed *RPI1* on cAMP levels. Cells were grown to the stationary phase in synthetic medium to maintain the presence of plasmids, incubated for an additional 12 h, and stimulated by the addition of glucose to a final concentration of 2%. Aliquots were removed before and after glucose addition at the indicated times for the determination of cAMP content, and the values were normalized to protein content. Duplicate samples deviated less than 15%. (A) Wild-type strain SP1 carrying control plasmid YEp13. (B) SP1 carrying the high-copy-number *RPI1* plasmid. (C) *RAS2*$^{Val-19}$ mutant PT1-6 carrying control vector YEp13. (D) PT1-6 carrying the high-copy-number *RPI1* plasmid.

suppresses the hyperactivity induced by overexpression of the normal Ras2 protein but does not suppress *RAS2*$^{Val-19}$, consistent with a role for *RPI1* in the regulation of normal Ras activity that is somehow lost in *RAS2*$^{Val-19}$ mutants.

We have found that *ADH1* promoter-driven overexpression of certain active gene fragments of the Ras protein activator *CDC25* induces a heat shock sensitivity phenotype (see Materials and Methods; data not shown). Since *CDC25* operates upstream of Ras proteins, we reasoned that if overexpressed *RPI1* can act to down-regulate normal Ras activity, it may suppress the heat shock sensitivity phenotype of cells that overexpress active fragments of *CDC25*. We tested this idea and found that high-copy-number *RPI1* could suppress *ADH1p:CDC25-Δ1* (Fig. 2). As with the suppression of *ADH1p:RAS2*, the suppressing activity of high-copy-number *RPI1* was stronger than that of high-copy-number *PDE1* but was comparable to that of high-copy-number *PDE2*. Thus, in both cases, high-copy-number *RPI1* is capable of suppressing a hyperactive cAMP pathway phenotype when it is induced by an increase in the activity of normal Ras proteins, either by overexpression of normal *RAS2* or by increased activity of the positive regulator *CDC25*.

We also examined the ability of high-copy-number *RPI1* to suppress two heat shock-sensitive mutants that contain lesions downstream of Ras proteins in the cAMP pathway. *bcy1* mutant cells, which possess constitutively high protein kinase A activity (25), were not suppressed by high-copy-number *RPI1*, and *pde1 pde2* mutant cells (28, 29), which lack the ability to degrade cAMP, were not suppressed by high-copy-number *RPI1* (data not shown). Thus, the ability of overexpressed *RPI1* to down-regulate the Ras-cAMP pathway is absent in mutants that have lesions downstream of Ras proteins.

Effect of overexpression of *RPI1* on cAMP levels. The ability of glucose to induce a cAMP signal in glucose-starved cells is Ras dependent; thus, measuring the glucose induction of cAMP levels is likely to be good barometer of Ras activity in yeast cells (26). On the basis of the genetic experiments described above, we suspected that high-copy-number *RPI1* was acting to down-regulate normal Ras activity. To evaluate this idea biochemically, we examined the effect of high-copy-number *RPI1* on cAMP levels. In three independent experiments, wild-type strains showed both lower basal cAMP levels and an attenuated cAMP response to added glucose when *RPI1* was overexpressed (Fig. 3). This result is consistent with a model in which overexpressed *RPI1* down-regulates normal Ras activity. In the same three experiments, we observed no significant effect of overexpressed *RPI1* on cAMP levels in the *ras1 RAS2*$^{Val-19}$ mutant PT1-6 (Fig. 3). Thus, overexpressed *RPI1* is not likely to be acting directly on adenylate cyclase or phosphodiesterases, and its ability to lower cAMP levels is dependent upon normal Ras function.

We did not observe significantly higher cAMP levels in the *RAS2*$^{Val-19}$ mutant than in the wild type in these experiments, in contrast to published reports (42). Our results may differ from others because of the use of a synthetic growth medium rather than a rich medium. However, during the course of this work, we observed that *RAS2*$^{Val-19}$ mutants exhibited higher cAMP levels only when the cells were allowed to remain in the stationary phase for several additional hours. Even under such conditions, overexpressed *RPI1* did not alter cAMP levels in the *RAS2*$^{Val-19}$ mutant (data not shown). Similar to the results of others, we did not detect any significant response of the *ras1 RAS2*$^{Val-19}$ mutant to added glucose (26).

Sequence analysis of *RPI1*. To determine the primary structure of the *RPI1* gene product and to guide our construction of gene disruptions of *RPI1*, we determined the nucleotide sequence of the 2.2-kbp biologically active region

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-540  GTCGACGGGAATGGAATTTTCTCTGGTATTTGCCGCAGCTTGCAGATAGTAGAGCCGATCTTGTGAGGTTGACACCGCATATGGCATTCT  -451
-450  GTTGCAAAGTTAAAATGCGTCGCCCACCCTAGCACCTAGGGTCGAGTACTGTTTTAAACTTTTCAAGACCGGACGTACCATTGTGCTTG  -361
-360  TTCCTTACTTCGTTATTTCTTGCATATTATATTGGTTGAGATTATTTCTATGGACTATATATAAATAAGGGAGGTCGTATTGTTCTTTT  -271
-270  ATGTCTGCATCCTAAAGAGTATTTTTTTTTATAGTCTTGTTTTCTTGTTCAAATTAAAGAAAGATGTAATAGTTTTCGTAATACTGAAA  -181
-180  CTACTCTTTTTTTTTTCCTTACAATCTGACTTTTTTTGTATTTCCTTTTTTTCCGTTTGGTTATCCAGACTACAGTTCATGGTAGTATA  -91
 -90  TCATATTATCGTATTCGTTTAACTATTTCTCAGTCCTTTTTTTAAGTCTTTTTTTTTTTTTCTCATCATTTTATTACTGATATTTATAAA  -1

   1  MetTyrLeuGluTyrLeuGlnProLysLeuAsnLeuMetAspGluSerSerThrIleSerLysAsnPheProAspTyrSerProAsnLeu  30
   1  ATGTACTTGGAATATCTTCAACCGAAGTTGAACCTAATGGATGAGTCTAGCACTATAAGCAAAAATTTCCCTGATTATTCACCAAATTTA  90

  31  AsnThrProIleThrSerLysPheAsnGluGluThrGlySerAspCysSerLeuValThrProArgIleIleSerSerSerAsnSerAsn  60
  91  AACACACCCATTACTTCAAAGTTTAATGAAGAAACGGGTTCGGACTGCTCTTTAGTTACTCCAAGAATAATTTCCAGTTCAAATTCGAAT  180

  61  SerAsnSerAsnSerAsnSerAsnSerAsnProGlySerIleAspGluAsnGluLeuAsnAsnSerAsnSerSerSerSerSerValArg  90
 181  TCGAACTCCAATTCTAATTCGAACTCCAACCCAGGCTCTATTGACGAAAATGAGCTAAACAACTCTAATTCATCCTCCTCGTCTGTAAGA  270

  91  GlnIleArgLysLysTrpLysGluProGluAspIleAlaPheIleThrThrIleMetAsnAsnSerGlnLeuLeuThrPheValGluTyr  120
 271  CAGATAAGGAAAAAATGGAAAGAGCCTGAGGATATAGCATTCATTACTACAATAATGAATAATTCTCAATTACTAACATTCGTAGAATAT  360

 121  PheLysProMetLysAsnPheTrpLysLysIleSerLysIleLeuPheGlnGlnTyrGlyTyrGluArgAsnSerArgGlnCysHisAsp  150
 361  TTCAAGCCTATGAAGAATTTTTGGAAAAAAATTTCAAAAATCCTATTCCAACAATATGGGTACGAGAGAAACTCTCGTCAATGTCATGAT  450

 151  ArgPheLysValLeuTyrThrLysSerLeuLysValHisProSerLysLysLysLysSerLysGlnGluAlaGlySerAsnLeuAsnPhe  180
 451  AGATTTAAAGTCTTATACACAAAGTCTTTGAAAGTGCATCCATCAAAGAAAAAAAAATCAAAACAAGAGGCAGGCTCAAACTTAAATTTT  540

 181  AspProSerLysLeuSerArgMetGlnTyrLeuLeuValGlnLeuGlnAsnThrPheSerPheValAsnGlyAsnIleIleLeuLysSer  210
 541  GATCCGTCAAAATTATCTAGAATGCAGTATTTGCTAGTGCAGTTACAAAACACTTTTAGTTTTGTAAACGGAAACATTATACTCAAGTCG  630

 211  GlnLysThrLeuLysProAsnLysAsnGlyThrAsnAspAsnIleAsnAsnHisTyrTyrAsnAsnSerAsnSerAsnAsnAsnAsnIle  240
 631  CAAAAAACATTGAAGCCGAACAAGAATGGTACTAATGATAATATTAATAACCATTATTATAATAATAGTAACAGTAACAATAATAATATT  720

 241  AsnAsnSerAsnAsnAsnAsnAsnSerAsnAsnSerAsnAsnAsnAsnSerAsnAsnIleAsnArgAsnSerAsnHisSerThrAsnVal  270
 721  AACAATAGTAACAATAATAATAATAGTAACAATAGCAACAATAATAATAGCAATAATATTAATAGGAATAGTAATCATAGTACTAATGTT  810

 271  PheSerThrProGluHisIleGlnSerSerIleAsnLeuAspLysLeuGluSerLeuProAlaLeuAspThrLysGlyGluProSerPhe  300
 811  TTTAGTACACCAGAGCATATTCAGTCAAGTATCAACCTCGATAAATTAGAATCTTTGCCAGCTTTGGATACCAAAGGAGAACCATCCTTC  900

 301  IleSerProAlaGlnPheSerLeuLeuSerSerAlaProAlaAspAsnLeuIleLeuGlnThrProProSerProPhePheGlnGlnThr  330
 901  ATTAGCCCGGCTCAATTTTCCCTTTTGTCATCAGCACCGGCAGACAATCTAATCCTGCAAACTCCACCATCGCCATTTTTCCAGCAAACA  990

 331  MetProIleGlnLeuProArgAspAlaGlnGlnGluGlnIleSerProValPheSerThrAspValIleTyrMetTrpGlnThrMetPhe  360
 991  ATGCCTATACAGCTACCGCGCGATGCACAACAAGAACAAATTTCCCCAGTTTTCTCTACAGATGTCATATACATGTGGCAAACAATGTTT  1080

 361  AsnThrIleGluAsnLeuLysGluGlnValAsnCysLeuLysAsnGluValLysGlnLeuAsnHisLysPheTyrGlnGlnAsnLysPro  390
1081  AACACTATTGAAAATTTAAAGGAACAAGTAAATTGCTTAAAAAATGAAGTTAAGCAATTAAACCATAAATTTTACCAACAAAATAAACCG  1170

 391  LeuHisAsnMetSerThrSerAspSerGluAsnPheMetGlnGlnHisEnd                                         420
1171  TTGCATAATATGTCAACTTCAGACTCAGAAAATTTTATGCAACAACATTAAAGTATGTATCGGGAAGTCTCCACCATAAATTTTCTACCC  1260
1261  CTTTAATTCTAGCACATTCCTTGTTTCCCTTCACCGTCATCAATAATTTTCAATTCGTCGTCGTTTCACCTTTTAATTAATTTTAAATGC  1350
1351  CCATTTCATTTCTGTTTTAAATTTTATGTCTGTTTTGTATGCTATTTCATTTTTCATTTACTTCTCCAGGGAAACAAGACAATATTACTT  1440
1441  ATATATATATTTATATTTATGCCCATTCAACATCCGATTTTTTTCTATAAAATAAGTGAAAAAAATAATGAAACTTAACGATTCTGTCAC  1530
1531  CAAGAAACGTCTTATTGTTCATTTTGGATTTTCTCAAGTTTTATCTTTGGTTGAAGCAGTTTATG  1595
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FIG. 4. Nucleotide sequence of *RPI1*. Shown is the nucleotide sequence of 2,135 bp of the 2.2-kbp *Sal*I-*Sau*3AI fragment containing *RPI1*. The sequence begins at the *Sal*I site 540 bp upstream of the initiator codon, proceeds through the major open reading frame, which is translated to display the proposed 406-amino-acid *RPI1* peptide sequence, and terminates with a 374-nucleotide sequence downstream of the *RPI1* termination codon. Sequences (approximately 85 bp) near the terminal *Sau*3AI site that were not accurately determined are not shown.

of DNA described in Fig. 1. One open reading frame of 1,218 nucleotides that could potentially encode a protein of 406 amino acids was found (Fig. 4). To 374 bp upstream of the initiation codon lay a stretch of nucleotides that is highly rich in adenine and thymidine residues, a feature common to many yeast promoters (36). No protein or coding sequence that was significantly homologous to *RPI1* was found in any data base (see Materials and Methods); therefore, *RPI1* does not appear to belong to a previously described family of proteins that are related in structure.

Phenotypic analysis of cells that contain a disruption of *RPI1*. To examine the effects of the loss of *RPI1* function, we constructed gene disruption plasmids and used these plasmids to transform yeast cells. One of these was a complete null allele, *rpi1::URA3*, which replaces the entire coding sequence of *RPI1* with the *URA3* gene, while leaving all flanking sequences intact (see Materials and Methods). The other disruption allele, *rpi1::LEU2*, removes sequences upstream of the initiation codon plus over half of the coding region of *RPI1* (see Materials and Methods). Both of these gene disruption plasmids could be used to disrupt the *RPI1* gene in haploid yeast cells, as confirmed by Southern blotting analysis. Several *rpi1::URA3* and *rpi1::LEU2* derivatives of wild-type strain SP1 were examined for heat shock sensitivity, and all of them displayed a heat shock sensitivity phenotype (data not shown). Longer heat shock periods were required to effectively kill *rpi1* mutants than to kill *RAS2*$^{Val-19}$ mutants (see Materials and Methods). *rpi1* mutants showed lower levels of glycogen than did the wild-type strain, as assayed by staining with iodine solutions (data not shown; 43); thus, by two criteria, the loss of *RPI1* function activates the Ras-cAMP pathway. These effects are similar to the somewhat more severe effects of the loss of *IRA1* or *IRA2* function (38, 40).

We also examined the effect of the loss of *RPI1* function on the glucose induction of cAMP levels in starved cells. In three separate experiments, we observed a small (<40%) increase in the amplitude of the cAMP peak at 1 min but did not observe any defect in the ability to return to near base-line levels after 2 min or more (data not shown). Thus, *RPI1* does not have an essential role in the transduction or feedback control of the glucose signal to the Ras-cAMP pathway. In addition, homozygous *rpi1* diploids could sporulate efficiently under standard sporulation conditions (data not shown). In these respects, the loss of *RPI1* function differs from the loss of *IRA1* or *IRA2* function, since the loss of *IRA* function significantly affects sporulation and is also reported to accentuate the glucose induction of cAMP levels (38, 40).

Epistasis relationships of *RPI1* and *RAS2*. One possibility we wished to examine was that disruption of *RPI1* results in a heat shock sensitivity phenotype by activating some component of the Ras-cAMP pathway downstream from Ras. To address this issue, we tested the effect of attenuated Ras

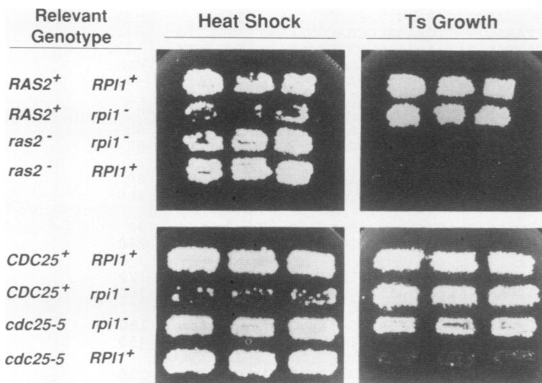

FIG. 5. Epistasis relationships among *RPI1*, *RAS2*, and *CDC25*. JHY102 (*rpi1::LEU2 RAS2*$^+$) was crossed with RS62-22A (*RPI1*$^+$ *ras2::URA3*), and the resulting diploid was sporulated to generate parental and recombinant haploid progeny. Three haploids of each genotype were patched and tested for heat shock sensitivity (45-min heat shock; upper left panel) and the ability to grow at 35°C on plates containing 2% glycerol as the carbon source (Ts growth) (upper right panel). Similarly, JHY210 (*rpi1::URA3 CDC25*$^+$) was crossed with LRA26 (*RPI1*$^+$ *cdc25-5*), and the resulting diploid was sporulated to generate parental and recombinant haploid progeny. Three haploids of each genotype were grown at 25°C and tested for heat shock sensitivity (30-min heat shock; lower left panel) and the ability to grow at 36°C on standard YPD plates (Ts growth) (lower right panel).

function on the ability of the *rpi1* mutation to induce a heat shock sensitivity phenotype. Previously, Tanaka and coworkers showed that the loss of Ras activity resulting from the *ras2* mutation suppresses the heat shock sensitivity phenotype induced by either *ira1* or *ira2* mutations (38, 40). If *RPI1*, like *IRA1* or *IRA2*, encodes a negative regulator of Ras activity, then one would expect the loss of Ras activity to suppress *rpi1*. An *rpi1::LEU2* haploid was crossed with an isogenic *ras2::URA3* haploid, and the resulting diploid was sporulated to generate recombinant double mutants (*rpi1::LEU2 ras2::URA3*) along with appropriate controls. Haploid progeny from 10 complete tetrads were analyzed for heat shock sensitivity; in all cases, double mutants (*rpi1::LEU2 ras2::URA3*) were as heat shock resistant as either wild-type or *ras2::URA3* haploids, indicating that the loss of Ras activity resulting from the *ras2::URA3* mutation suppresses the heat shock sensitivity phenotype induced by the loss of *RPI1* function. Representative results are shown in Fig. 5. We also assayed the ability of *rpi1* to suppress the growth defect phenotype of *ras2* mutants, which are unable to grow at 35°C on nonfermentable carbon sources (41). The results shown in Fig. 5 indicate that *rpi1* cannot suppress *ras2*. Thus, by two criteria, *RAS2* is epistatic to *RPI1*, consistent with the *RPI1* gene product acting upstream of Ras proteins.

Epistasis relationships of *RPI1* and *CDC25*. If the loss of *RPI1* function activates the Ras-cAMP pathway by activating Ras proteins, then the loss of *CDC25* function, which is absolutely required for normal Ras function, should suppress the *rpi1* phenotype. We could not test this possibility with the null allele of *CDC25*, since *rpi1* does not bypass the essential requirement for *CDC25* function (data not shown). In this respect, the loss of *RPI1* function differs from the loss of *IRA1* or *IRA2* function, each of which can suppress *cdc25*, although *ira2* is reported to only weakly bypass essential *CDC25* function (38, 40). To test whether a less drastic attenuation of *CDC25* function might suppress *rpi1*, we crossed an *rpi1::URA3* strain with a *cdc25-5* mutant (see Materials and Methods). The *cdc25-5* mutation attenuates *CDC25* function even at the permissive growth temperature of 25°C (31). Haploid progeny from 10 complete tetrads were examined for heat shock sensitivity after growth at 25°C; in all cases, the *cdc25-5* mutation fully suppressed *rpi1* (Fig. 5). Thus, the loss of normal Ras activity, whether it results from the *ras2* mutation or from attenuated *CDC25* function, suppresses *rpi1*. We were able to monitor the segregation of the *cdc25-5* allele in these haploids by an absolute growth defect at 37.5°C, regardless of the presence or absence of *RPI1*. However, at 35°C, we noted that the double mutants (*rpi1 cdc25-5*) could grow but that *RPI1*$^+$ *cdc25-5* haploids could not (Fig. 5). Therefore, the loss of *RPI1* function is able to compensate, to a considerable extent, for the growth defect caused by the *cdc25-5* mutation. This result is in marked contrast to the complete inability of *rpi1* to suppress the partial loss of Ras function (*ras2*). In addition, *rpi1* did not suppress temperature-sensitive *ras1 ras2* mutants, even at the lowest nonpermissive temperature of 35°C (data not shown). We suggest that these results argue that the *RPI1* gene product operates upstream of Ras proteins but that it does not act upstream as a negative regulator of the *CDC25* gene product.

Epistasis relationships among *RPI1*, *IRA1*, and *IRA2*. We noted that the effects of mutations in *RPI1* were similar to the effects of mutations in *IRA1* or *IRA2*. First, overexpression of *RPI1*, which suppresses other Ras-cAMP pathway mutants, fails to suppress *RAS2*$^{Val-19}$, similar to the effects of overexpression of *IRA1* and *IRA2* (38, 40). Second the loss of *RPI1* function results in a heat shock sensitivity phenotype which is fully suppressed by the loss of *RAS2* function or the loss of *CDC25* function, and the same properties are observed with the *ira1* and *ira2* mutations (38, 40). Finally, *rpi1*, like the loss of *IRA1* or *IRA2* function, cannot suppress growth defects resulting from the loss of Ras function but can suppress growth defects resulting from the loss of *CDC25* function (38, 40). These parallels prompted us to explore the possibility that *RPI1* may work through a mechanism involving the *IRA1* and *IRA2* gene products. We tested the ability of high-copy-number *RPI1* to suppress the heat shock sensitivity phenotype induced by the *ira1* or *ira2* mutations. We found that high-copy-number *RPI1* could suppress both of the single disruption mutants, either *ira1* or *ira2*, with activity that was comparable to the exerted by high-copy-number *PDE1* (Fig. 6). Since the heat shock sensitivity phenotype of both of the single disruption mutants (either *ira1* or *ira2*) is dependent upon normal Ras function (38, 40), these results are consistent with *RPI1* acting to negatively regulate normal Ras activity.

Surprisingly, high-copy-number *RPI1* failed to suppress the heat shock sensitivity phenotype of the double *ira1 ira2* mutants, even though the weak suppression by high-copy-number *PDE1* was still apparent in *ira1 ira2* mutants (Fig. 6). We repeated this experiment with two different sets of *ira* mutants and obtained the same results (data not shown); thus, the marked inability of overexpressed *RPI1* to suppress the *ira* double disruption mutant is not due to strain variation. This result demonstrates that the suppressor effects of overexpression of *RPI1* require the presence of at least one of the two *IRA* genes and suggest that *RPI1* may act upstream of *IRA* gene products as a positive regulator of their ability to down-regulate Ras activity. Clearly, if this is the case, *RPI1* can act through either *IRA1* or *IRA2*, since high-copy-number *RPI1* can effectively suppress the heat

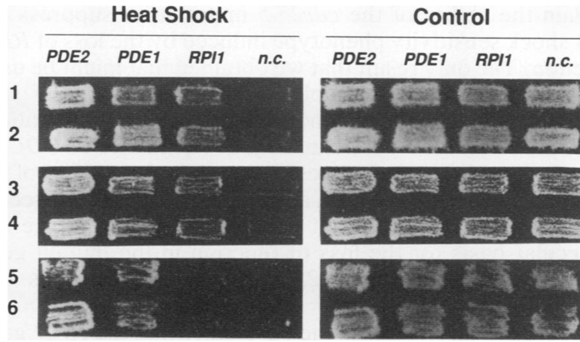

FIG. 6. Suppression by overexpressed *RPI1* of the heat shock sensitivity phenotype of single but not *ira* double disruption mutants. Three different mutants, JHY451 (*ira1 IRA2*$^+$), JHY431 (*IRA1*$^+$ *ira2*), and JHY442 (*ira1 ira2*), were transformed with the high-copy-number *RPI1* plasmid and control plasmids, and the ability of these plasmids to suppress the heat shock sensitivity phenotype of the host strain was assayed as described in the text. Column 1, high-copy-number *PDE2* transformants; column 2, high-copy-number *PDE1* transformants; column 3, high-copy-number *RPI1* transformants; column 4, negative control (*n.c.*) transformants (YEp13). Rows 1 and 2, *ira1 IRA2*$^+$ mutants (40-min heat shock); rows 3 and 4, *IRA1*$^+$ *ira2* mutants (15-min heat shock); rows 5 and 6, *ira1 ira2* mutants (10-min heat shock). The heat-shocked replicas were allowed to recover for 1 day at 30°C.

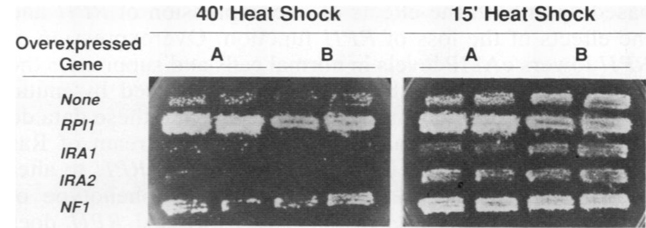

FIG. 7. Effect of overexpressed *IRA* and *NF1* genes on the heat shock sensitivity phenotype of *rpi1* and *ADH1p:CDC25-Δ2* mutants. Two different heat-shock-sensitive mutants, JHY102 (*rpi1::LEU2*; column A) and JHY254 (*ADH1p:CDC25-Δ2*; column B), were transformed with five different high-copy-number plasmids, and the ability of these plasmids to suppress the heat shock sensitivity phenotype of the host strain was assayed as described in the text. The right panel shows recovery for 40-min-heat-shocked replicas, and the left panel shows recovery for 10-min-heat-shocked replicas. Row 1, control plasmid YEp24 transformants; row 2, high-copy-number *RPI1* transformants; row 3, high-copy-number *IRA1* transformants; row 4, *IRA2* overexpression plasmid pKT16 (37, 40) transformants; row 5, *NF1* overexpression plasmid pKP11 (47) transformants. The first two columns of patched cells show two independent transformants for each plasmid of the *rpi1* mutant; the second two columns show two independent transformants for each plasmid of the *ADH1p:CDC25-Δ2* mutant.

shock sensitivity phenotype of either single disruption mutant.

If indeed *RPI1* acts upstream of *IRA1* and *IRA2*, then one might expect overexpression of *IRA1* or *IRA2* to suppress the heat shock sensitivity phenotype induced by the *rpi1* mutation. We tested this idea and found that overexpression of *IRA1* or *IRA2* failed to significantly suppress *rpi1* (Fig. 7). In contrast, overexpression of the human Ras GTPase activator *NF1* suppressed *rpi1* (Fig. 7). We also observed the suppression of *rpi1* by overexpression of bovine GAP cDNA (data not shown). Assuming that overexpression of *NF1* or GAP can down-regulate yeast Ras activity, these latter two results are consistent with the results described above which showed that the heat shock sensitivity phenotype induced by *rpi1* is dependent upon normal levels of Ras activity. However, the inability of overexpressed *IRA1* or *IRA2* to suppress *rpi1* seems at odds with these results. Especially puzzling is the result that the overexpressed *IRA2* fragment, utilizing the high-level-expression glyceraldehyde-3-phosphate dehydrogenase promoter, had no effect on *rpi1*, even though it was fully active in suppressing *ira1* and *ira2* (Fig. 7) (37, 39, 40). We consider two possible explanations: (i) increasing gene dosage or message for Ira proteins fails to produce an amount of Ira proteins significantly larger than the wild-type amount or (ii) the amount of Ira proteins above a certain threshold is not rate limiting for the negative regulation of Ras activity, perhaps because a positive effector of Ira becomes rate limiting. If the latter were true, then the epistasis relationship between *RPI1* and *IRA* genes would be similar to the relationship between *CDC25* and *RAS* genes: even though *CDC25* clearly acts upstream of *RAS*, overexpression of either *RAS1* or *RAS2* fails to suppress the loss of *CDC25* function (8, 33).

Overexpression of *IRA1* or *IRA2* is known to down-regulate Ras activity in *ira1* or *ira2* mutants, in which it is reasonable to presume that the amount of Ira proteins is rate limiting (38, 40). If, as we proposed above, overexpression of *IRA* genes in *IRA1*$^+$ *IRA2*$^+$ cells does not significantly decrease Ras activity, then overexpressed *IRA1* or *IRA2* genes should fail to suppress the heat shock sensitivity phenotype induced by any mutation that increases Ras activity (other than *ira1* or *ira2*). To test idea, we examined the effects of overexpressed *IRA1*, *IRA2*, and *NF1* on the heat shock sensitivity phenotype of the *ADH1p:CDC25-Δ2* mutant (see Materials and Methods). As a control, we confirmed that high-copy-number *RPI1* suppressed *ADH1p:CDC25-Δ2* (Fig. 7). We found that overexpressed *NF1* suppressed *ADH1p:CDC25-Δ2* but that overexpressed *IRA1* or *IRA2* had no effect (Fig. 7). Therefore, it would appear that overexpressed *IRA1* or *IRA2* cannot decrease Ras activity in *IRA1*$^+$ *IRA2*$^+$ cells, and this result may also explain why we did not isolate *IRA1* or *IRA2* in our screening for genes which, when overexpressed, could negatively regulate Ras function.

DISCUSSION

At least part of the manner in which budding yeast cells adjust growth-related processes in response to changes in nutrient levels is directed by the Ras-cAMP pathway (7). How nutrients or metabolite levels modulate the Ras-cAMP pathway is likely to involve *CDC25*, *IRA1*, *IRA2*, and other upstream regulators of Ras proteins (7, 39). Although little is known about the physiological roles of *CDC25*, *IRA1*, and *IRA2* in regulating Ras activity, our current understanding of their function and our current limited knowledge of gene products that regulate Ras activity in mammalian cells serve for the time being as the best-understood Ras protein system, in terms of both identification and characterization of interacting proteins and in terms of how Ras proteins are regulated. One avenue for further understanding of the regulation of Ras proteins is to identify and study other gene products that in some fashion can control Ras activity. In this paper, we have characterized a new gene, *RPI1*, that encodes a negative regulator of the Ras-cAMP pathway, one that appears to operate upstream of Ras proteins.

The evidence that *RPI1* acts upstream of Ras proteins is

based upon both the effects of overexpression of *RPI1* and the effects of the loss of *RPI1* function. Overexpression of *RPI1* lowers cAMP levels in normal cells and suppresses the hyperactive cAMP pathway phenotype induced by mutations that increase normal Ras activity. While these data do not directly indicate that *RPI1* operates upstream of Ras proteins, the marked failure of overexpressed *RPI1* to alter the cAMP level or heat shock sensitivity phenotype of *RAS2*$^{Val-19}$ mutants argues that overexpressed *RPI1* does not affect the mutationally activated *RAS2*$^{Val-19}$ protein or downstream components of the Ras-cAMP pathway. The effects of *RPI1* appear to be restricted to the control of normal, wild-type Ras activity. Further confirmation of this upstream regulatory role for *RPI1* comes from analysis of the phenotypic effects of the loss of *RPI1* function. The *rpi1* mutation induces a heat shock sensitivity phenotype and decreases the amount of stored glycogen, both of which indicate increased Ras-cAMP pathway activity. These phenotypic effects of *rpi1* are likely to be mediated by Ras proteins, since *rpi1* can be suppressed by mutations that lower normal Ras activity. All of these results are consistent with a model in which *RPI1* encodes a negative regulator of normal Ras function.

The *RPI1* gene product is a new member of the expanding family of gene products that regulate normal Ras activity but do not appreciably affect the hyperactivity of the mutationally activated *RAS2*$^{Val-19}$ protein. As discussed earlier, neither the activity of the *CDC25* gene product nor the activity of *IRA* gene products can alter *RAS2*$^{Val-19}$ function (8, 37, 39). Moreover, as further evidence that the *RAS2*$^{Val-19}$ protein fails to respond to physiological signals, both we and Mbonyi et al. have failed to detect glucose induction of cAMP levels in strains that contain *RAS2*$^{Val-19}$ as their only *RAS* gene (26). Because of these properties, *RAS2*$^{val-19}$ is very useful in genetic experiments designed to test or search for upstream regulators of normal Ras activity.

Inability of overexpressed *IRA1* or *IRA2* to alter the phenotype of yeast cells. Curiously, we did not detect the two known negative regulators of Ras activity, *IRA1* and *IRA2* (38, 40), in our screening for genes which, when overexpressed, can down-regulate normal Ras activity. Further examination of the effects of overexpressed *IRA1* or *IRA2* suggests that overexpression of either of these genes cannot down-regulate normal Ras activity in yeast cells and that the phenotypic effects of overexpression of *IRA1* or *IRA2* are restricted to complementing *ira1* or *ira2* mutations (38, 40). In contrast, we found that overexpressed mammalian Ras GTPase activator GAP or *NF1* can act to down-regulate Ras activity in yeast cells, even in *IRA1*$^+$ *IRA2*$^+$ cells. We suggest that overexpressed *IRA1* or *IRA2*, unlike overexpressed GAP or *NF1*, may require other cellular factors for full activity in yeast cells. Alternatively, there may be negative regulators of *IRA* gene products that counteract the effects of overexpression, or there simply may be a failure of overexpressed *IRA* genes to increase the amount of Ira proteins. However, the biochemical results to Tanaka and coworkers argue strongly against this last possibility (37).

Does *RPI1* act through any of the three known regulators of Ras activity? We wish to discuss the possibility that *RPI1* operates through one or more of the three known regulators of Ras activity in *S. cerevisiae*: *CDC25*, *IRA1*, or *IRA2*. First, we consider the possibility that *RPI1* encodes a negative regulator of the *CDC25* gene product. The inability of overexpressed *RPI1* to suppress *RAS2*$^{Val-19}$, which is relatively insensitive to *CDC25* function (8), would be consistent with this hypothesis. Also, this model would readily explain the ability of the *cdc25-5* mutation to suppress the heat shock sensitivity phenotype induced by the loss of *RPI1* function. The only result that we obtained that might be used to argue against this possibility is that *rpi1* partially suppressed the growth defects induced by the *cdc25-5* mutation. If indeed *RPI1* is an upstream negative regulator of *CDC25*, then the *cdc25-5* mutation would attenuate the activity of the *CDC25* gene product in such a way that it could be corrected by the loss of an upstream negative regulator. Since the molecular basis for the loss of function in the mutant gene product encoded by the *cdc25-5* allele is not clear, this idea remains a possibility.

We now wish to discuss the possibility that the *RPI1* gene product may exert its effects by stimulating the Ras GTPase-stimulating activity of the proteins encoded by *IRA1* and *IRA2*. The argument for this possibility arises from the striking failure of overexpressed *RPI1* to suppress the heat shock sensitivity phenotype of double *ira1 ira2* mutants, suggesting that in the absence of *IRA* function, overexpressed *RPI1* cannot affect the Ras-cAMP pathway. Interestingly, if this hypothesis is correct, the *RPI1* gene product must be able to act through either *IRA1* or *IRA2* gene products, since overexpressed *RPI1* can suppress both the single *ira1* or the single *ira2* mutation. We are in the process of biochemically testing the hypothesis that the *RPI1* gene product is a positive regulator of *IRA*-directed Ras GTPase-activating function. Unfortunately, one cannot detect Ras GTPase-activating function in wild-type yeast extracts (37), unlike the situation with animal cells, precluding a simple and direct test of this hypothesis. In yeast cells, Ras GTPase-activating function has only been observed in extracts from mutant cells that overexpress either animal cell GAP or *NF1* or in extracts prepared from mutants that, by use of a powerful promoter, overexpress active fragments of *IRA2* (1, 37, 47). It is not clear that such artificially induced activity will be subject to physiologically relevant regulation. Nevertheless, we are in the process of testing the effect of overexpressed *RPI1* and the loss of *RPI1* function on Ras GTPase-activating function in such mutants.

Alternatively, the inability of *RPI1* to suppress the double *ira1 ira2* mutants might be explained if either *IRA1* or *IRA2* function were required in some unknown fashion for Ras proteins to exert their normal effects. If this were the case, there might not be any phenotypic effect of lowering Ras activity in double *ira1 ira2* mutants. This idea leaves open the possibility that overexpressed *RPI1* down-regulates normal Ras activity by an alternate mechanism, perhaps involving direct inhibition of *RAS* function in a manner analogous to guanine nucleotide dissociation inhibition of other, Ras-like proteins (34, 45). It does seem clear that whatever biochemical mechanism the *RPI1* gene product utilizes to lower Ras activity, *IRA* gene products play a critical role in *RPI1* function. A more detailed analysis of *RPI1* and *IRA* gene products will be required to fully understand the negative regulation of Ras activity and how this regulation relates to the physiological signals that control Ras.

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