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MSI1, a negative regulator of the RAS–cAMP pathway in *Saccharomyces cerevisiae*

(yeast/guanine nucleotide-binding regulatory protein)

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ABSTRACT **We have previously demonstrated that the *IRA1*-encoded protein inhibits the function of the RAS protein in a fashion antagonistic to the function of the CDC25 protein in the RAS–cAMP pathway in *Saccharomyces cerevisiae*. In an attempt to identify genes involved in the regulation of this pathway, high-copy-number plasmid suppressors of the heat shock sensitivity of the *ira1* mutation were isolated. One such suppressor, *MSI1*, was found to encode a putative protein of 422 amino acids that shows homology to the β subunit of the mammalian guanine nucleotide-binding regulatory proteins. Overexpression of the *MSI1* gene could suppress the heat shock sensitivity and the defect in sporulation caused by the *ira1* and *RAS2*^{Val19} mutations but not those of the *bcy1* mutation. Furthermore, the high level of intracellular cAMP in *ira1* and *RAS2*^{Val19} cells was reduced by the *MSI1* gene carried on a YEp-based plasmid. These results suggest that the MSI1 protein is a negative regulator of the RAS-mediated induction of cAMP in *S. cerevisiae*.**

In the yeast *Saccharomyces cerevisiae* the signal transduction for growth in response to nutrients is mediated by the RAS–cAMP pathway (1, 2). The yeast RAS proteins play an important role in this process: they activate adenylate cyclase, which induces the formation of cAMP (3, 4). The levels of this second messenger regulate, by means of cAMP-dependent protein phosphorylation, the progression of the cell cycle through the early G_1 phase (1).

The ras proteins are highly conserved in evolution (5). The extended structural homology between the yeast and the mammalian ras proteins suggests that they may serve a fundamental function in cellular proliferation. Mutations in mammalian ras proteins have been associated with cell transformation (5); likewise, the analogous alteration in the yeast RAS2 sequence (*RAS2*^{Val19}) causes defects in the cell cycle control exerted by nutrients (3). Although numerous indications suggest that mammalian ras proteins do not activate adenylate cyclase (6, 7), their analogy to guanine nucleotide-binding regulatory proteins (G proteins) has directed considerable attention to the study of RAS-mediated signal transduction in yeast, as this simple eukaryote is amenable to genetic approaches.

Like their mammalian counterparts, yeast RAS proteins oscillate between a GDP- and a GTP-bound state, an inactive and an active form, respectively. The RAS2 protein has been shown to activate adenylate cyclase when bound to GTP, but not when bound to GDP (8). The *CDC25* gene product, which is required for the regulation of adenylate cyclase, has been found to regulate the RAS proteins and may mediate nucleotide exchange of GDP for GTP (9, 10). Recently, we identified *IRA1*, a gene whose product negatively regulates the RAS–cAMP pathway (11). Genetic evidence indicates that this protein may operate on RAS proteins in a manner opposite to the function of the CDC25 protein. *IRA1* gene disruption can suppress the lethality of the *cdc25* mutation and causes an increased level of intracellular cAMP (11). These phenotypes are typical of the *RAS2*^{Val19} mutant, which has a reduced intrinsic GTPase activity (4). These observations, together with its partial homology to mammalian GAP (GTPase activating protein) (12), suggest that *IRA1* acts to down-regulate RAS activity, and hence adenylate cyclase, by stimulating the GTPase activity of RAS proteins.

In an attempt to further elucidate the components operating in the RAS–cAMP pathway, we isolated multicopy suppressor genes of the heat shock sensitive phenotype caused by the *ira1* mutation. In this report, we describe one such suppressor gene, *MSI1*, and show that *MSI1* negatively regulates the cAMP level in response to glucose.¶

MATERIALS AND METHODS

Yeast Strains, Media, and Plasmids. The relevant genotype of each strain is described in the text and in the figure legends. YPD medium contains 2% Bacto peptone, 1% Bacto yeast extract, and 2% glucose. SD medium contains 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose; SC medium is SD medium supplemented with auxotrophic requirements. Sporulation medium contains 1% potassium acetate. Plasmid pd3-L was derived from pd3 (see Fig. 1) by substituting the *URA3* gene with the 1.9-kilobase (kb) *Bam*HI–*Acc* I *LEU2* fragment from plasmid YEp13.

Analysis of Heat Shock Sensitivity and Sporulation Efficiency. Heat shock experiments were performed as follows. Fresh cells were grown at 25°C on YPD or SC medium, patched on YPD or SC plates, and treated for 20 min at 57°C or 1 hr at 50°C, respectively. After heat shock treatment, the plates were cooled immediately on ice and, after a 2-day incubation at 25°C, heat sensitivity was scored. For the determination of sporulation efficiency, fresh diploid cells were grown on SC medium and transferred to sporulation plates. After a 96-hr incubation at 25°C, sporulated cells were counted under a microscope.

cAMP Assay. Glucose-induced cAMP formation was determined in stationary-phase cells as previously described (13).

mRNA Analysis. Total RNA was extracted as described by Sherman *et al.* (14). Poly(A)$^+$ RNA, purified by oligo(dT)-cellulose chromatography, was electrophoresed on a 1%

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; T_β, the β subunit of transducin.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27300).

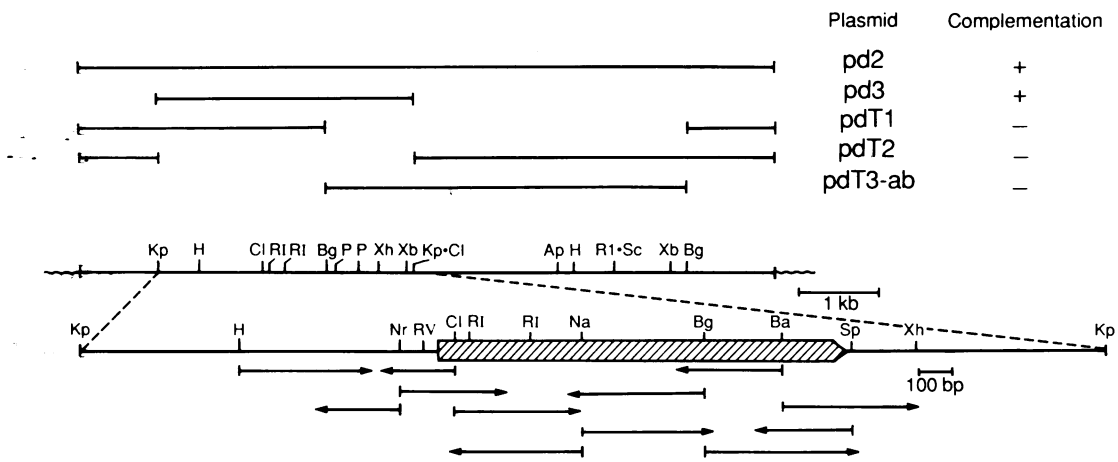

FIG. 1. Deletion analysis of an *MSI1* clone, as well as the physical map, subcloning, and sequencing strategy of the *MSI1* locus. Deletion analysis of clone pd2 is shown. Horizontal lines indicate the DNA fragments that were tested for complementation ability after deletion. The complementation test refers to suppression of heat shock sensitivity after transformation of C70-3D cells (*MATa ira1-1 ura3 trp1 his3 lys2 ade8*) with the plasmids shown. Kp, *Kpn* I; H, *Hin*dIII; Cl, *Cla* I; RI, *Eco*RI; Bg, *Bgl* II; P, *Pst* I; Xh, *Xho* I; Xb, *Xba* I; Ap, *Apa* I; Sc, *Sca* I; Nr, *Nru* I; RV, *Eco*RV; Na, *Nar* I; Ba, *Bal* I; Sp, *Sph* I. The open reading frame is indicated by the hatched arrow. DNA regions that were subcloned and sequenced are indicated by the horizontal arrows.

agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized, as previously described (15), to a 1.3-kb *Eco*RI–*Sph* I ^{32}P-labeled *MSI1* probe.

RESULTS

Isolation of the *MSI1* Gene. We have searched for genes that, on a multicopy plasmid, could suppress the heat shock-sensitive phenotype of the *ira1* mutation. Yeast strain C70-3D (*ira1-1*) was transformed with a yeast genomic library constructed in the high-copy vector YEp24 (16). When colonies were visible, plates were incubated at 50°C for 1 hr. Heat shock-resistant transformants were obtained from these colonies. Segregation analysis showed that cells that lost the plasmid were heat shock sensitive, and cells were resistant to heat shock only if they retained the *URA3* marker. The plasmids in these cells were transferred to *Escherichia coli* DH1, and their DNA inserts were compared by restriction enzyme analysis. Seven different plasmids were isolated. Three of these seven contained known genes: Two plasmids contained *IRA1* itself (11); one contained *PDE2*, which encodes the high-affinity cAMP phosphodiesterase (17). Two of the four remaining plasmids (pd2 and pd3) contained overlapping restriction fragments and represented a common locus (Fig. 1). Deletion analysis indicated that this overlapping region is responsible for the suppressor activity (Fig. 1). We named this gene *MSI1* for *M*ulticopy *S*uppressor of *ira1* and describe its characterization in this report. The other two plasmids contained two different genes, which we called *MSI2* and *MSI3*.

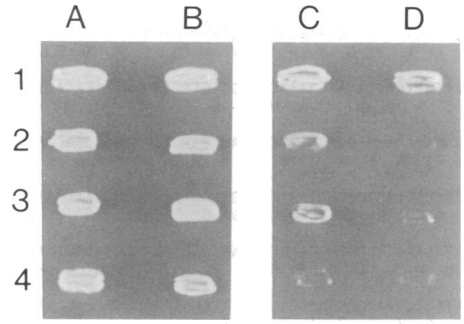

FIG. 2. Heat shock sensitivity of strains overexpressing the *MSI1* gene. Yeast strains carrying the control plasmid YEp13 (*B* and *D*) or plasmid pd3-L (*A* and *C*) were patched on YPD plates, heat-shocked for 20 min at 57°C (*C* and *D*) or untreated (*A* and *B*), and incubated at 25°C for 2 days. A photograph of the resulting patches is shown. The strains used in this experiment are 1, wild-type strain RAY3A-D-1C (*MATa ura3 leu2 trp1 his3*); 2, the *RAS2*^{Val19} strain TK161-R2V (*MATa RAS2*^{Val19} *leu2 ura3 trp1 his3 ade8 can1*); 3, the *ira1*::*URA3* strain KT12-2A (*MATa ira1*::*URA3 ura3 leu2 trp1 his3*); 4, the *bcy1*::*URA3* strain, R146-2C (*MATa bcy1*::*URA3 ura3 leu2*).

Suppression by *MSI1*. The isolation of the *MSI1* gene as a suppressor of the *ira1* mutation suggested that this gene acts negatively on the RAS–cAMP pathway. To determine at which step of the pathway the *MSI1* gene product exerts its action, we undertook a genetic analysis. The *ira1* mutation causes a phenotype that is very similar to that of *RAS2*^{Val19} and *bcy1* mutants; that is, cells carrying these mutations are very sensitive to heat shock treatment and do not sporulate efficiently (3, 11). These two phenotypes were used to study the epistasis of *MSI1* on different components of the RAS–cAMP pathway. Fig. 2 and Table 1 show that the *MSI1* gene on a multicopy plasmid could suppress both phenotypes in *ira1* and *RAS2*^{Val19} but was not able to suppress the *bcy1* mutation, which is deficient in the regulatory subunit of the cAMP-dependent protein kinase (18, 19). These results indicated that *MSI1* works upstream of cAMP-dependent protein kinase.

Overexpression of *MSI1* Decreases cAMP Levels in *ira1* and *RAS2*^{Val19} Cells. The heat shock sensitivity and the defect in sporulation of *ira1* and *RAS2*^{Val19} cells is caused by an increase in cAMP levels (4, 11). Suppression of these phenotypes by *MSI1* was, therefore, expected to operate by means of a reduction of cAMP levels in the cell. To examine this possibility, we studied the effect of *MSI1* on cAMP formation. In the experiments shown in Fig. 3, the kinetics of

Table 1. Sporulation efficiencies of *MSI1* overexpressing cells

Strain	Genotype	Sporulated cells	
		YEp13	pd3-L
RAY3A-D	Wild type	12%	21%
TK161-R2V-D	*RAS2*^{Val19}	0/1000	3%
KT12-2A-D	*ira1*::*URA3*	0/1000	4%
R146-2C-D	*bcy1*::*URA3*	0/1000	0/1000

The genotype of each strain is described in the legend to Figs. 2 and 3. Fresh cultures of diploid strains were patched on sporulation plates, and the frequency of tetrads was determined after 4 days of incubation at 25°C. About 1000 cells from eight independent transformants were counted for each strain.

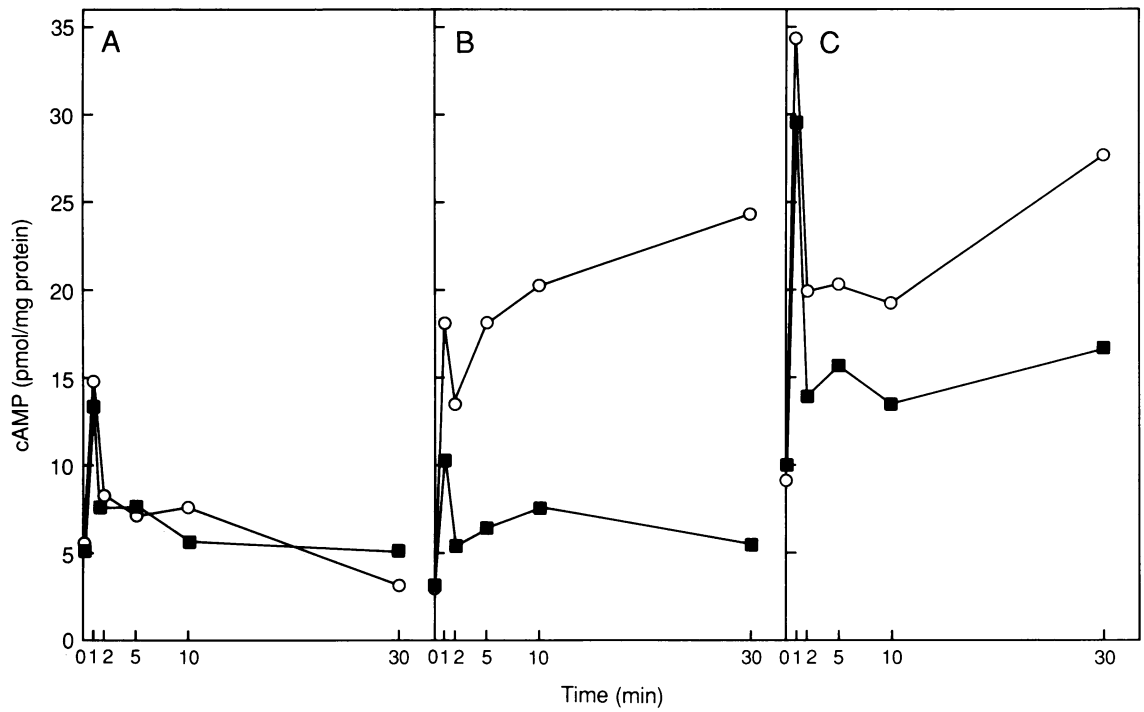

FIG. 3. Effect of *MSI1* on the cAMP levels in $RAS2^{Val19}$ and *ira1* cells. Cells were stimulated with 0.5% glucose and incubated at 30°C. After various periods of time, aliquots were harvested and the cAMP content was determined. ○, Strains transformed with the control plasmid YEp24; ■, strains carrying the plasmid pd3. (*A*) Wild-type RAY3A-D strain. (*B*) *ira1*::*LEU2* strain KT6-1A-D (*MATa*/*MATα ira1*::*LEU2*/*ira1*::*LEU2 ura3*/*ura3 leu2*/*leu2 trp1*/*trp1 his3*/*his3*). (*C*) $RAS2^{Val19}$ strain TK161-R2V-D. The diploid strains (designated by a D) used in this experiment were obtained from the corresponding haploid strains by transformation with the *HO* gene.

cAMP formation in response to glucose were measured. The *ira1* and $RAS2^{Val19}$ cells showed a large increase in cAMP levels in response to addition of glucose compared with wild-type cells. On the other hand, introduction of pd3 into the *ira1* and $RAS2^{Val19}$ strains decreased the level of glucose-induced synthesis of cAMP. These results suggest that overexpression of *MSI1* suppresses the phenotypes of the *ira1* and $RAS2^{Val19}$ mutants by reducing their high levels of cAMP.

The *MSI1* Gene Product Has Homology to the β Subunit of Transducin (T_β). Nucleotide sequence analysis of pd3 revealed that the *MSI1* gene encodes a protein of 422 amino acid residues (Fig. 4). When the putative protein sequence was compared with the National Biomedical Research Foundation sequence data bank by using the TFASTA program, some homology was found to T_β, the mammalian signal-transducing G protein that functionally couples rhodopsin to cGMP phosphodiesterase in phototransduction (20). A

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-123        CGAAAAGTAAAAATTTTAAAAATTAGAGCACCTTGAACTTGCGAAAAAGGTTCTCATCAACTGTTTAAAAGGAGGATATCAGGTCCTATTTCTGACAAACAATATACAAATTTAGTTTCAAAG

  1 ATGAATCAGTGCGCGAAGGACATAACTCATGAAGCCTCCAGTATACCCATCGATTTGCAAGAAAGATACTCGCACTGGAAGAAAAACACTAAACTACTTTATGATTACCTAAACACGAATTCAACAAAGT
  1 M N Q C A K D I T H E A S S I P I D L Q E R Y S H W K K N T K L L Y D Y L N T N S T K W

131 GGCCGTCCTTAACGTGCCAGTTCTTTCCTGATTTAGATACCACTTCGGATGAGCATCGCATCTTGTTATCCTCATTTACATCTTCCCAAAAACCTGAAGATGAGACCATATATATTAGCAAAATATCCAC
 45   P S L T C Q F F P D L D T T S D E H R I L L S S F T S S Q K P E D E T I Y I S K I S T

261 GTTGGGTCATATAAAATGGTCATCTTTAAATAATTTCGACATGGACGAAATGGAATTCAAACCGGAGAACTCGACAAGGTTTCCCTCCAAACACTTAGTAAATGACATCAGTATTTTCTTCCCAAACGGG
 88   L G H I K W S S L N N F D M D E M E F K P E N S T R F P S K H L V N D I S I F F P N G

391 GAATGCAATAGGGCAAGATATTTGCCTCAAAATCCAGATATTATAGCCGGCGCCTCTTCAGATGGTGCAATCTACATATTCGATAGAACAAAACACGGCTCTACTAGAATAAGACAGTCCAAAATTTCAC
131 E C N R A R Y L P Q N P D I I A G A S S D G A I Y I F D R T K H G S T R I R Q S K I S H

521 ATCCCTTTGAGACAAAGCTGTTTGGTTCACATGGTGTTATTCAAGACGTGGAGGCAATGGATACTTCTTCGGCAGATATAAATGAGGCGACTTCTTTAGCCTGGAACTTGCAGCAGGAGGCCCTTTTACT
175   P F E T K L F G S H G V I Q D V E A M D T S S A D I N E A T S L A W N L Q Q E A L L L

651 TTCTTCTCACTCCAACGGCCAAGTTCAAGTTTGGGACATTAAACAATATTCGCATGAGAACCCTATAATAGATTTACCCTTAGTGTCAATAAACAGCGACGGAACAGCGGTGAATGATGTAACTTGGATG
218   S S H S N G Q V Q V W D I K Q Y S H E N P I I D L P L V S I N S D G T A V N D V T W M

781 CCAACACACGATTCCCTCTTTGCTGCTTGTACTGAAGGAAATGCGGTCTCCCTATTAGATCTGAGGACTAAGAAAGAGAAGCTCCAGAGTAACCGTGAAAAACACGATGGTGGAGTAAACTCCTGTAGAT
261   P T H D S L F A A C T E G N A V S L L D L R T K K E K L Q S N R E K H D G G V N S C R F

911 TTAACTATAAGAACTCTTTAATTCTAGCATCTGCAGATTCAAATGGGAGGCTAAATTTATGGGATATTAGAAACATGAACAAAAGCCCAATCGCTACCATGGAGCACGGTACTTCCGTTTCAACTTTAGA
305   N Y K N S L I L A S A D S N G R L N L W D I R N M N K S P I A T M E H G T S V S T L E

1041 ATGGAGTCCAAATTTCGATACTGTATTGGCAACGGCTGGCCAAGAAGATGGGTTAGTCAAGCTATGGGATACCTCCTGCGAAGAAACTATATTTACCCATGGTGGTCATATGCTCGGTGTGAACGACATT
348   W S P N F D T V L A T A G Q E D G L V K L W D T S C E E T I F T H G G H M L G V N D I

1171 TCGTGGGACGCTCATGACCCTTGGTTAATGTGCAGTGTGGCAAATGATAATTCAGTTCACATATGGAAACCTGCAGGAAACCTTGTTGGACATTCGTGACCTAAACGTTCCTTGAAGCCAGGCATGCTAA
391   S W D A H D P W L M C S V A N D N S V H I W K P A G N L V G H S

1301 TGGCTCCTGTCCCTATTACATATTCAACCATTTGATAGACGATATACAAACATAAATAGAGTTTATAAAATATGCTCAAGACTTGCTACAATAAACACTACGCTCAGACTTCATGCTGATTTCAATATTG

1431 TATTTCGAAGAAAGTGGTTGC
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FIG. 4. DNA sequence of the *MSI1* gene and its derived amino acid sequence. The putative starting ATG triplet and the TGA stop codon are boxed.

search for internal repeats in the amino acid sequence revealed a degree of internal homology in the *MSI1* protein that resembles the periodic motif in T_β (21). An optimal alignment between the sequences of two cloned G protein β subunits and the putative *MSI1* peptide sequence is shown in Fig. 5.

Gene Disruption and Expression of *MSI1*. To examine the effect on cells of perturbing the *MSI1* gene, we constructed a plasmid containing the *MSI1* gene in which the *Eco*RI–*Bgl* II internal fragment was replaced by a 1.2-kb *Hind*III fragment of *URA3*. This construction eliminated the ability of *MSI1* to suppress the heat shock sensitivity and sporulation deficiency of *ira1* (data not shown). The 3.2-kb *Xho* I–*Bam*HI fragment containing the Δ*msi1*::*URA3* allele was used to disrupt one *MSI1* locus of the wild-type diploid strain RAY3A-D. The heterozygous diploid strain (*MSI1*$^+$/Δ*msi1*::*URA3*) was sporulated, and the asci were dissected to obtain haploid progenies. In every tetrad analyzed, all four spores developed into normal colonies. DNA was prepared from haploid progenies and analyzed by Southern hybridization. The results indicated that Ura$^+$ progenies possessed the disrupted *MSI1* gene (data not shown). Thus, the *MSI1* gene is not essential for the growth of yeast cells. Cells carrying the Δ*msi1*::*URA3* mutation were indistinguishable from wild-type cells with regard to growth rate, sporulation efficiency, and heat shock sensitivity. Although Southern blot analysis at low stringency failed to clearly indicate the presence of genes homologous to *MSI1* (data not shown), we cannot exclude the possibility that other genes could supply the *MSI1* function in the absence of the *MSI1* gene.

To identify the RNA encoded by *MSI1*, poly(A)$^+$ RNA was prepared from growing cultures of two haploid cell types (**a** and α) and a diploid cell type (**a**/α). Northern blot analysis revealed a single band of about 1.9 kb in all three cell types (data not shown). No such band was observed in the Δ*msi1*::*URA3* strain. The multicopy plasmid carrying the *MSI1* gene (pd3-L) increased the level of *MSI1* transcript, which was quantified by densitometer scanning and found to be about 10-fold higher than the level in wild-type cells carrying the control plasmid YEp13.

Mapping of *MSI1*. The map position of *MSI1* was determined by chromosome blotting and tetrad analysis. Hybridization of the *MSI1* sequence to a Southern blot of *S. cerevisiae* chromosomes, separated by orthogonal-field alternating gel electrophoresis, indicated that the *MSI1* gene maps to chromosome II (data not shown). This assignment was confirmed by tetrad analysis. A three-factor cross involving *tyr1*, *lys2*, and Δ*msi1*::*URA3* localized *msi1* on chromosome II ≈4 centimorgans from *tyr1* and 33 centimorgans from *lys2*. The tetrad data showed a parental ditype/non-parental ditype/tetratype (PD/NPD/T) ratio of 14:0:28 for *msi1* and *lys2* and 39:0:3 for *msi1* and *tyr1*, whereas the *lys2* and *tyr1* combination showed a 13:0:29 ratio. These data clearly indicate that the order of genes on chromosome II is centromere–*lys2*–(*msi1*–*tyr1*).

DISCUSSION

IRA1 is an inhibitory regulator of the RAS–cAMP pathway in yeast (11). Strains carrying disruption of this gene have increased levels of cAMP and display phenotypes associated with the *bcy1* and *RAS2*^{Val19} mutations, such as sporulation deficiency and sensitivity to heat shock. Because of the ability of the *ira1* disruption mutation to suppress the lethality caused by disruption of the *CDC25* gene and its partial

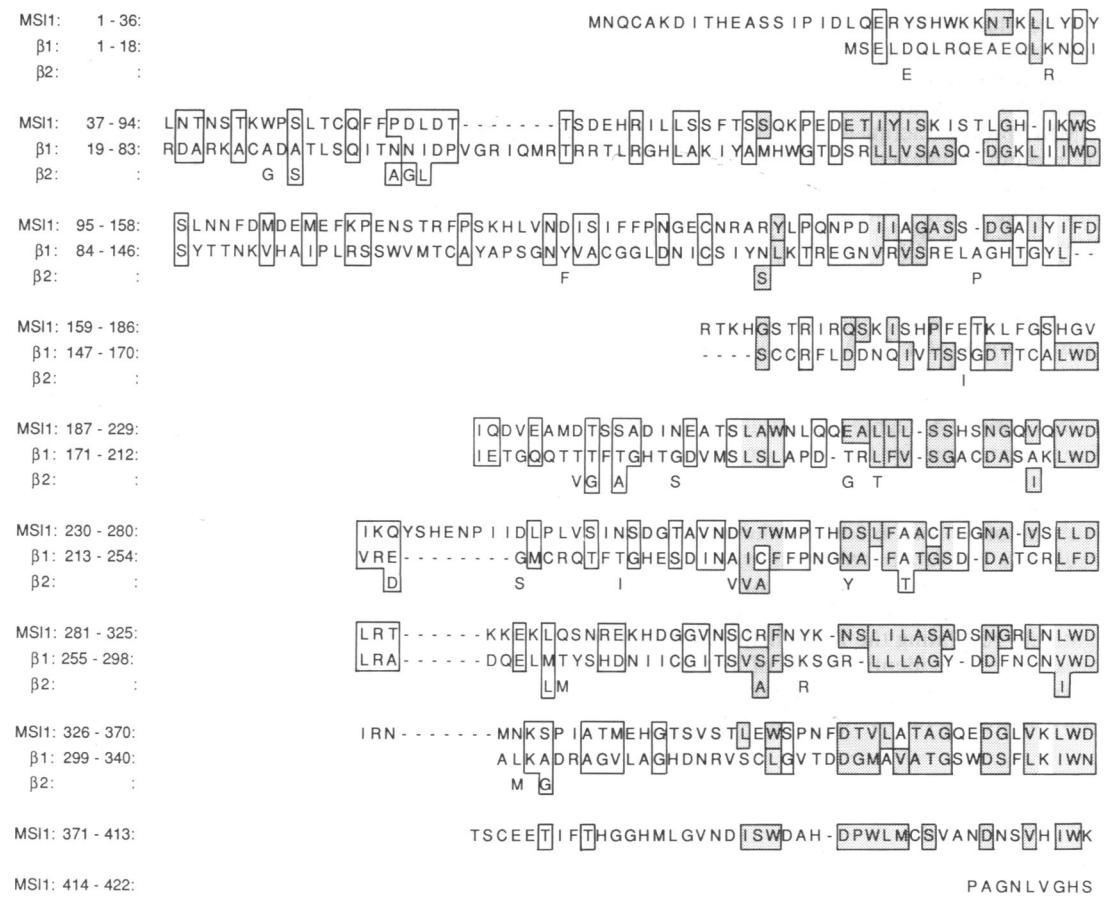

FIG. 5. The deduced amino acid sequence of MSI1 compared to that of two mammalian β subunits, $T_{\beta 1}$ (β1) and $T_{\beta 2}$ (β2). The alignment shown maximizes the frequency of sequence identity with the β1 (20) and β2 (22) proteins. Identical and conserved residues in the three proteins are boxed. Stippled boxes indicate the internal repeat homology.

homology to the carboxyl-terminal catalytic domain of mammalian GAP (GTPase-activating protein) (11), the *IRA1* gene product has been proposed to stimulate the intrinsic GTPase activity of yeast RAS proteins. In the absence of this function, the RAS proteins are thought to accumulate in the GTP-bound state and constitutively activate adenylate cyclase. This situation is analogous to that caused by the $RAS2^{Val19}$ mutation, which has a reduced intrinsic GTPase activity (23, 24). The similar phenotypes of these two mutants would be, therefore, caused by the continuous active state of the RAS proteins. Overexpression of the *MSI1* gene suppresses the *ira1* disruption and the $RAS2^{Val19}$ mutations and decreases their high levels of cAMP. One possible function of the MSI1 protein could be to directly inhibit the RAS proteins, which have regions of amino acid homology to the α subunit of G proteins and are thought to play a role analogous to that of the α subunit in that they bind and hydrolyze GTP (5). The α subunit of trimeric G proteins interacts with the $\beta\gamma$ subunits (25). In an analogous manner, the MSI1 protein may interact with the yeast RAS proteins and thereby regulate their function. Another possibility is that the MSI1 protein may interact and regulate adenylate cyclase. Alternatively, the effect of the MSI1 protein could be indirect as a consequence of overexpression of its gene.

The finding that the MSI1 protein sequence shares some homology with the β subunit of mammalian G proteins is of interest, especially since this homology is extended to the internal structural organization of repeated units, typical of the β subunits of G proteins. This homology could suggest a possible role of the MSI1 protein similar to that of β subunits of mammalian G protein—that is, to interact with the α subunit. The *GPA2* gene, which encodes a G protein α subunit that affects the cAMP pathway, has been identified in yeast by cross-hybridization to cDNAs encoding the α subunit of mammalian G_i (an inhibitory G protein) and G_o (a G protein of unknown function) (13). We tested the possibility that the MSI1 protein, acting as a β subunit, interacted with the *GPA2* protein. However, a high copy number of the *MSI1* gene did not affect the high levels of cAMP caused by overexpression of the *GPA2* gene (data not shown). Therefore, it is unlikely that the MSI1 protein interacts with the GPA2 protein. Moreover, the homology of the MSI1 protein to T_β is lower than that observed between T_β and the STE4 protein (26). This is the putative β subunit interacting with the yeast G protein α subunit, the GPA1 protein, that mediates the response to mating pheromones (15, 27). These results suggest that the MSI1 protein may not be a member of the trimeric G protein family. A degree of homology to mammalian T_β, similar to that found for the MSI1 protein, has been reported for another yeast protein, the *CDC4* gene product (21). There is, however, no evidence that the CDC4 protein works as a β subunit of G proteins and that the repeated motif within the β subunits has functional meaning.

The data reported in this paper suggest that the *MSI1* gene product is a component of the cAMP pathway in yeast and works by negatively regulating the RAS-mediated induction of cAMP formation. The identification of the molecular interactions between the MSI1 protein and other elements of the cAMP pathway awaits further investigation.

We thank Jun-ichi Nikawa and Michael Wigler for communicating, prior to publication, that the sequence of their gene *JUN1* is identical to that of *MSI1*, except for the last 21 amino acids. We also thank Naoki Nakayama and Charlie Brenner for useful suggestions and Ikuko Miyajima for constructing plasmid pd3-L. We are grateful to Allan Waitz for his critical reading of the manuscript.

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