

Posttranscriptional Regulation of *HO* Expression by the Mkt1-Pbp1 Complex†

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Cells of budding yeast give rise to mother and daughter cells, which differ in that only mother cells express the *HO* endonuclease gene and are thereby able to switch mating types. In this study, we identified the *MKT1* gene as a positive regulator of *HO* expression. The *MKT1* gene encodes a protein with two domains, XPG-N and XPG-I, which are conserved among a family of nucleases, including human XPG endonuclease. Loss of *MKT1* had little effect on *HO* mRNA levels but resulted in decreased protein levels. This decrease was dependent on the 3' untranslated region of the *HO* transcript. We screened for proteins that associate with Mkt1 and isolated Pbp1, a protein that is known to associate with Pab1, a poly(A)-binding protein. Loss of *PBP1* resembles a *mkt1Δ* deletion, causing decreased expression of *HO* at the posttranscriptional level. Mkt1 and Pbp1 cosedimented with polysomes in sucrose gradients, with Mkt1 distribution in the polysomes dependent on Pbp1, but not vice versa. These observations suggest that a complex of Mkt1 and Pbp1 regulates the translation of *HO* mRNA.

Gene expression can be regulated at multiple steps, including transcription, splicing, mRNA transport, mRNA stability, and translation. Regulation of mRNA translation depends on a temporally and spatially orchestrated sequence of protein-protein, protein-RNA, and RNA-RNA interactions. Posttranscriptional regulation plays an important role during the development of a wide variety of organisms (7). For example, the asymmetric patterns of gene expression in *Xenopus* and *Drosophila* embryos are determined by the differential localization, stabilization, and translation of maternally synthesized mRNAs. Translational regulation also plays a key role in germ line sex determination in *Caenorhabditis elegans* (6, 14, 39). In most of the cases studied in detail, posttranscriptional regulation is mediated by sites in the 3' untranslated region (UTR) of the target mRNA.

The *HO* endonuclease initiates mating-type switching in *Saccharomyces cerevisiae* by creating a double-stranded break at the *MAT* locus (9). The *HO* gene is only transcribed in mother cells, i.e., cells that have previously budded and given birth to a daughter cell. In mother cells, *HO* is transcribed transiently during the cell cycle, shortly before budding and DNA replication (24). Activation of *HO* transcription depends on at least 10 genes, named *SWI1* through *SWI10*. *SWI4* and *SWI6* encode subunits of a cell cycle-regulated transcription factor, SCB-binding factor, that activates a number of genes at the G₁/S boundary (17). *SWI1*, *SWI2*, *SWI3*, and *SWI10* encode

components of a large multisubunit complex that is needed for the expression of many yeast genes (26).

Asymmetric transcription of the *HO* gene results from the preferential accumulation of a negative regulatory protein, Ash1, in daughter nuclei (3, 32). *ASH1* mRNA is targeted to the distal tip of daughter buds in postanaphase cells (18, 35). Localization of *ASH1* mRNA depends on an intact actin cytoskeleton and five *SHE* genes (15, 18, 35). *SHE1* encodes a type V myosin motor, Myo4, that colocalizes with *ASH1* mRNA at the tip of daughter cells (2, 8, 23, 36). Thus, *ASH1* mRNA is localized to the bud tip by an actomyosin-based transport mechanism. The RNA-binding proteins Loc1 and Khd1 are also involved in *ASH1* mRNA localization and translation (12, 19). These factors interact with and are required for efficient localization of the *ASH1* mRNA. Overexpression of Khd1 decreases the level of Ash1 protein, suggesting that Khd1 could be involved in the translational control of the *ASH1* mRNA during its transport to the bud tip (12).

HO expression is also regulated posttranscriptionally by the yeast Puf (for Pumilio and FBF) homolog Mpt5/Puf5 (34). Mpt5 binds to the 3' UTR of *HO* mRNA and represses *HO* expression. Repression requires the 3' UTR of *HO*, which contains a tetranucleotide, UUGU, that is also found in the binding sites of Pumilio and FBF. Mpt5 may affect mRNA stability or turnover via binding to the *HO* 3' UTR. Null mutation of *MPT5* confers mating-type switching on daughter cells, suggesting that Mpt5 provides a second mechanism for preventing the synthesis of *HO* protein in daughter cells.

Here we describe the identification of another gene, *MKT1*, that is required for *HO* expression at a posttranscriptional step. *MKT1* encodes a protein containing two domains, XPG-N and XPG-I, that are conserved in human XPG endonuclease. We observed that disruption of *MKT1* caused a decrease in *HO* protein and that this inhibition required the 3' UTR of *HO*. We screened for proteins that interact with Mkt1 and isolated

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† This paper is dedicated to the memory of Ira Herskowitz (deceased 28 April 2003).

TABLE 1. Strains used in this study

Strain	Genotype ^a	Reference or source
W303	a <i>ho ade2 trp1 can1 leu2 his3 ura3 GAL psi⁺</i>	32
K1107	a <i>HOp-LacZ-HO 3'UTR</i>	25
K5552	α <i>ASH1-myc</i>	15
10B	α <i>HOp-ADE2-HO 3'UTR</i>	34
YKEN104	α <i>HOp-ADE2-HO 3'UTR myo4Δ::CgHIS3</i>	This study
YKEN301	α <i>HOp-ADE2-HO 3'UTR kanMX6::GAL1p-KHD1</i>	12
YKEN303	α <i>HOp-ADE2-HO 3'UTR myo4Δ kanMX6::GAL1p-KHD1</i>	12
YKEN304	α <i>HOp-ADE2-HO 3'UTR ash1Δ</i>	12
YKEN306	α <i>HOp-ADE2-HO 3'UTR myo4Δ ash1Δ</i>	12
YKEN451	α <i>ASH1-myc myo4Δ::CgHIS3</i>	This study
YKEN9152	α <i>HOp-ADE2-HO 3'UTR mkt1-19 kanMX6::GAL1p-KHD1</i>	This study
YKEN9152a	α <i>HOp-ADE2-HO 3'UTR mkt1-19 ash1Δ</i>	This study
TTC4	a <i>HOp-LacZ-HO 3'UTR myo4Δ::CgLEU2</i>	This study
TTC46	a / α <i>HO/HO</i>	34
TTC47	α <i>HOp-ADE2-ADH 3'UTR</i>	34
TTC237	α <i>ASH1-myc mkt1Δ::CgHIS3</i>	This study
TTC478	α <i>HOp-ADE2-HO 3'UTR mkt1Δ::CgHIS3</i>	This study
TTC798	α <i>HOp-ADE2-HO 3'UTR pbp1Δ::CgHIS3</i>	This study
TTC848	α <i>HOp-ADE2-HO 3'UTR MKT1-HA::kanMX6</i>	This study
TTC929	a / α <i>HO/HO mkt1Δ::CgHIS3/mkt1Δ::CgHIS3</i>	This study
TTC993	a / α <i>HO/HO pbp1Δ::CgHIS3/pbp1Δ::CgHIS3</i>	This study
TTC996	α <i>HOp-ADE2-HO 3'UTR PBP1-HA::kanMX6</i>	This study
TTC999	a <i>HOp-ADE2-HO 3'UTR PBP1-HA::kanMX6</i>	This study
TTC1049	a <i>HOp-ADE2-HO 3'UTR PBP1-HA::kanMX6 MKT1-myc::kanMX6</i>	This study
TTC1085	a <i>HOp-LacZ-HO 3'UTR mkt1Δ::CgHIS3</i>	This study
TTC1089	a <i>HOp-LacZ-HO 3'UTR pbp1Δ::CgHIS3</i>	This study
TTC1163	a <i>HOp-LacZ-HO 3'UTR mkt1Δ::CgHIS3 pbp1Δ::CgHIS3</i>	This study
TTC1108	α <i>HOp-ADE2-ADH 3'UTR mkt1Δ::CgHIS3</i>	This study
TTC1233	α <i>HOp-ADE2-HO 3'UTR PBP1-HA::kanMX6 mkt1Δ::CgHIS3</i>	This study
TTC1234	α <i>HOp-ADE2-HO 3'UTR MKT1-HA::kanMX6 pbp1Δ::CgHIS3</i>	This study
TTC1257	α <i>HOp-ADE2-HO 3'UTR mkt1Δ::CgTRP1 pbp1Δ::CgHIS3</i>	This study
TTC1302	a <i>HOp-ADE2-HO 3'UTR PBP1-HA::kanMX6 MKT1ΔC-myc::kanMX6</i>	This study
TTC1313	a <i>ho mkt1Δ::CgLEU2</i>	This study
TTC1316	a <i>ho pbp1Δ::CgLEU2</i>	This study
TTC1319	a <i>ho myo4Δ::CgLEU2</i>	This study
TTC1333	α <i>HOp-ADE2-ADH 3'UTR mkt1Δ::CgTRP1 pbp1Δ::CgHIS3</i>	This study
TTC1334	α <i>HOp-ADE2-ADH 3'UTR pbp1Δ::CgHIS3</i>	This study
PJ69-4A	a <i>trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ</i>	13

^a All strains except PJ69-4A were isogenic derivatives of W303.

the *PBP1* gene, which was previously shown to interact with the poly(A)-binding protein Pab1 (21). Loss of *PBP1* causes a phenotype similar to that caused by *mkt1 Δ* with regard to *HO* expression. Mkt1 and Pbp1 cosedimented with polysomes in sucrose gradients, and the association of Mkt1 in the polysome fraction was Pbp1 dependent, but not vice versa. These observations suggest that a complex of Mkt1 and Pbp1 regulates the translation of *HO* mRNA.

MATERIALS AND METHODS

Strains and general methods. *Escherichia coli* DH5 α was used for DNA manipulations. The yeast strains used in this study are described in Table 1. Standard procedures were followed for yeast manipulations (16). The media used in this study included yeast extract-peptone-dextrose (YPD) medium, synthetic complete medium (SC), synthetic minimal medium (SD), and sporulation medium (16). SC media lacking amino acids or other nutrients (e.g., SC-Ade lacks adenine) were used to select transformants and to score *ADE2* reporter activity. SG was identical to SC except that it contained 2% galactose and raffinose instead of 2% glucose. Recombinant DNA procedures were carried out as described previously (29).

Plasmids. The plasmids used in this study are described in Table 2. Plasmids YCpSHE2, YCpSHE3, and YCpBN1 were kindly provided by R. P. Jansen. pGBD-MKT1 expresses Mkt1 fused to the Gal4 DNA-binding domain. The *MKT1* open reading frame (ORF) was amplified by PCR with the 5' primer

5'-CTCAGATCTATGGCAATCAAGTCATTGGAATCG-3', incorporating a BglII site, and the 3' primer 5'-CTCCTCGAGTCAGCTAGATAGAGCTGTTGTAGT-3', incorporating an XhoI site. A 2.5-kb BglII-XhoI fragment generated by PCR was inserted into the BamHI-SalI gap of the pGBD-C1 vector (13) to generate pGBD-MKT1. pGBD-MKT1 Δ C1 (amino acids [aa] 1 to 760) and pGBD-MKT1 Δ C2 (aa 1 to 570) express Mkt1 Δ C (aa 1 to 760) and Mkt1 Δ C (aa 1 to 570), respectively, fused to the Gal4 DNA-binding domain. The *MKT1* ORF was amplified by PCR as described above, and the resultant fragment was digested with EcoT22I or SalI to truncate the region encoding Mkt1-C (aa 761 to 830) or Mkt1-C (aa 571 to 830). The resultant BglII-EcoT22I and BglII-SalI fragments were inserted into the BamHI-PstI and the BamHI-SalI gaps of the pGBD-C1 vector to create pGBD-MKT1 Δ C1 and pGBD-MKT1 Δ C2, respectively. pGAD-PBP1 expresses Pbp1 (aa 475 to 722) fused to the Gal4 transcriptional activation domain. pGAD-PBP1 was cloned from Y2HL yeast genomic two-hybrid libraries (13). pK508 is YCp50 carrying a 14.5-kb fragment of chromosome XIV that contains the *MKT1* gene. pK508 was cloned from a YCp50-based genomic library (27). YCplac33MKT1 is YCplac33 carrying *MKT1*. The 3.5-kb fragment encoding the *MKT1* 5' UTR (0.7 kb), the *MKT1* ORF, and the *MKT1* 3' UTR (0.3 kb) was amplified by PCR with the 5' primer 5'-CTCCCCGGCAATGAATTACGCGAGTCGCG-3', incorporating an SmaI site, and the 3' primer 5'-CTCCTCGAGTTCCTTTTCTTCTCTCTGCCCCG-3', incorporating an XhoI site. The resultant SmaI-XhoI fragment was inserted into the SmaI-SalI gap of the YCplac33 vector to generate YCplac33MKT1. YCplac33MKT1 (D32A) expresses a mutated allele of *MKT1* containing one amino acid change, from Asp-32 to Ala. The 5' UTR and mutated NH₂-terminal portion of *MKT1* were amplified by PCR with the 5' primer 5'-CTCCCCGGCAATGAATTAC

TABLE 2. Plasmids used in this study

Plasmid	Relevant markers	Reference or source
pFA6a-kanMX6-GAL1p	<i>kanMX6-GAL1p</i> sequence	20
pFA6a-3HA-kanMX6	<i>3HA-ADH1 3'UTR-kanMX6</i> sequence	20
pFA6a-13MYC-kanMX6	<i>13MYC-ADH1 3'UTR-kanMX6</i> sequence	20
pCgHIS3	<i>C. glabrata HIS3</i> in pUC19	28
pCgLEU2	<i>C. glabrata LEU2</i> in pUC19	28
pCgTRP1	<i>C. glabrata TRP1</i> in pUC19	28
YCplac33	<i>URA3 CEN4</i>	5
pK508	<i>PMS1 YNL083w END3 MKT1 YNL086w</i> and <i>YNL087w</i> sequence in YCp50	This study
YCplac33MKT1	<i>MKT1</i> sequence in YCplac33	This study
YCplac33MKT1-D32A	<i>MKT1-D32A</i> sequence in YCplac33	This study
YCpMYO4	<i>MYO4</i> sequence in YCp50 (<i>URA3 CEN4</i>)	This study
YCpSHE2	<i>SHE2</i> sequence in YCplac111 (<i>LEU2 CEN4</i>)	15
YCpSHE3	<i>SHE3</i> sequence in YCplac111 (<i>LEU2 CEN4</i>)	15
YCpSHE4	<i>SHE4</i> sequence in YCp50 (<i>URA3 CEN4</i>)	This study
YCpBNI1	<i>BNI1</i> sequence in YCplac111 (<i>LEU2 CEN4</i>)	15
pGAD-C1	<i>LEU2/2-μm GAL4-AD</i> sequence behind <i>ADH1</i> promoter	13
pGAD-PBP1	<i>GAL4-AD-PBP1</i> (amino acids 475–722) sequence in pGAD-C1	This study
pGBDU-C1	<i>URA3/2μm GAL4-BD</i> sequence behind <i>ADH1</i> promoter	13
pGBDU-MKT1	<i>BD-MKT1</i> sequence in pGBDU-C1	This study
pGBDU-MKT1 Δ C1	<i>BD-MKT1ΔC</i> (amino acids 1–760) sequence in pGBDU-C1	This study
pGBDU-MKT1 Δ C2	<i>BD-MKT1ΔC</i> (amino acids 1–570) sequence in pGBDU-C1	This study

GCGAGTCGC-3', incorporating an SmaI site, and the 3' primer 5'-GGAAACATAATGTTGACGGCTATATCCAGGGTACAATT-3'. The COOH-terminal portion and 3' UTR of *MKT1* were amplified by PCR with the 5' primer 5'-CTCCTCGAGTTCCTTTTCTTCTCTGCCGG-3', incorporating an XhoI site, and the 3' primer 5'-AATTGTACCTGGATATAGCCGTCAACCATTATGTTTCC-3'. The resulting two fragments were further amplified by PCR with the 5' primer 5'-CTCCCCGGCAATGAATTACGCGAGTCGC-3', incorporating an SmaI site, and the 3' primer 5'-CTCCTCGAGTTCCTTTTCTTCTTGCCGG-3', incorporating an XhoI site. The resulting fragment was inserted into the SmaI-SalI gap of YCplac33 vector to generate YCplac33MKT1(D32A).

Isolation of *mkt1* mutants. Yeast strain YKEN301 carrying the *Hop-ADE2* reporter and *GAL1p-KHD1* genes was treated with ethyl methanesulfonate to 30% survival. After ethyl methanesulfonate treatment, the cells were grown for two generations in YPD medium to allow for phenotypic lag. Cells were plated onto SC–Ade containing 10 mg of adenine per liter. After 3 days, about 3,000 red or pink colonies were selected and streaked onto both SG–Ade and SC–Ade plates. Out of about 3,000 colonies, 12 mutants grew well on SG–Ade but not on SC–Ade. These 12 mutants were transformed with YCpMYO4, YCpSHE2, YCpSHE3, YCpSHE4, and YCpBNI1. The adenine auxotrophy of nine of the mutants was complemented by YCpMYO4, and that of two was complemented by YCpSHE3. The adenine auxotrophy of the remaining mutant, YKEN9152, was not complemented by YCpMYO4, YCpSHE2, YCpSHE3, YCpSHE4, or YCpBNI1. The adenine auxotrophy of YKEN9152 segregated 2:2 in a cross to a strain having the parental genotype.

Cloning of *MKT1*. Strain YKEN9152 was transformed with a YCp50 yeast genomic library (27) and then replica plated to SC–UraAde plates. After 2 days of incubation, one transformant grew on SC–UraAde medium. The plasmid that complemented the adenine auxotrophy of YKEN9152, pK508, was recovered from *S. cerevisiae* by transformation into *E. coli*. The mutation site of *mkt1-19* was determined as follows. Chromosomal DNA from strain YKEN9152 was isolated as described previously (16). A 3.5-kb fragment encoding the *MKT1* 5' UTR (0.7 kb), the *MKT1* ORF, and the *MKT1* 3' UTR (0.3 kb) of YKEN9152 was amplified by PCR with the 5' primer 5'-CTCCCCGGCAATGAATTACGCGAGTCGC-3', incorporating an SmaI site, and the 3' primer 5'-CTCCTCGAGTTCCTTTTCTTCTTGCCGG-3', incorporating an XhoI site. Sequence analysis revealed that YKEN9152 contains a C-to-T transversion at position +385, producing a termination codon in place of the codon for Gln-129.

Gene deletions. Deletions of *MYO4*, *KHD1*, *MKT1*, *PBP1*, and *ASH1* were constructed by the PCR-based gene deletion method (1, 28, 30). Primer sets were designed such that 46 bases at the 5' end of the primers were complementary to those at the corresponding region of the target gene and 20 bases at their 3' end were complementary to the pUC19 sequence outside the polylinker region in plasmid pCgHIS3 containing the *Candida glabrata* *HIS3* gene, plasmid pCgTRP1 containing the *C. glabrata* *TRP1* gene, or plasmid pCgLEU2 containing the *C.*

glabrata *LEU2* gene as a selectable marker. Primer sets for PCR were designed to delete the ORF completely. The PCR products were used to transform the wild-type strain by selection for His⁺, Trp⁺, or Leu⁺. The disruption was verified by colony-PCR amplification (10) to confirm that replacement had occurred at the expected locus.

Construction of *MKT1-myc*, *MKT1 Δ C-myc*, *MKT1-HA*, and *PBP1-HA* strains. *MKT1-myc*, *MKT1 Δ C-myc*, *MKT1-HA*, and *PBP1-HA* strains were prepared by the method of Longtine et al. (20), with pFA6a-13Myc-kanMX6 and pFA6a-3HA-kanMX6.

Construction of *GAL1p-KHD1* strains. The *GAL1p-KHD1* strain was prepared by the method of Longtine et al. (20), with pFA6a-kanMX6-GAL1p-3HA.

Mating-type switching assays. Pedigree analysis was performed as described previously (33), with the homothallic strains TTC85 (*HO/HO*), TTC929 (*HO/HO mkt1 Δ /mkt1 Δ*), and TTC993 (*HO/HO pbp1 Δ /pbp1 Δ*).

β -Galactosidase assays. β -Galactosidase assays were performed as described previously (16).

Real-time quantitative RT-PCR. Primers and probes of the *Hop-ADE2*, *Hop-lacZ*, and *ACT1* mRNAs for TaqMan assays were designed by use of the Primer Express software program (ABI, Foster City, Calif.) and were purchased from ABI. TaqMan probes were labeled at the 5' end with 6-carboxyfluorescein and at the 3' end with the fluorescence quencher 6-carboxytetramethyl rhodamine. The level of *ACT1* mRNA was used as a control for the amount of cDNA. For real-time reverse transcriptase PCR (RT-PCR), 1 μ l of 0.3- μ g/ μ l DNase-treated total RNA was added to 50 μ l of PCR buffer, which contained the forward and reverse primers at 900 nM each and the fluorescence-labeled probe at 100 nM in TaqMan Universal master mixture reagent (ABI). Samples were incubated at 50°C for 5 min and at 95°C for 10 min, after which target amplification was carried out through 45 two-stage cycles with at 95°C for 15 s and at 60°C for 1 min in an ABI Prism 7700 Sequence Detection System (ABI). For each data point, duplicate samples were analyzed in parallel, and all assays were repeated with highly reproducible results. All of the results shown in this report represent the averages of these four measurements.

Localization of *ASH1* mRNA and Ash1 protein. In situ RNA hybridization with digoxigenin-labeled *ASH1* antisense probe was performed as described previously (35). Indirect immunofluorescence microscopy of an *ASH1-myc* strain with anti-Myc antibody 9E10 (Santa Cruz) was done as previously described (32).

Yeast two-hybrid assays. Two-hybrid screening with pGBDU-MKT1 and Y2HL yeast genomic two-hybrid libraries was done as previously described (13).

Immunoprecipitation. Strains were grown in YPD medium at 30°C, and the cells were harvested by centrifugation. All cells were washed twice in XT buffer (50 mM HEPES-KOH [pH 7.3], 20 mM potassium acetate, 2 mM EDTA, 0.1% Triton X-100, 5% glycerol). Cells were resuspended in the above-described buffer containing protease inhibitors. Glass beads were added, and the samples were vortexed 12 times for 45 s each time. The supernatants were removed and

centrifuged for 10 min at $4,000 \times g$. To immunoprecipitate Mkt1-Myc or MKT1 Δ C-Myc protein, extracts were incubated with anti-Myc antibody 9E10 coupled to protein G-agarose for 1 h at 4°C. Beads were washed three times in XT buffer and eluted in elution buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate [SDS]) for 10 min at 65°C. Western blot assays were performed with anti-Myc antibody 9E10 or anti-hemagglutinin (HA) antibody HA11 (Babco).

Polysome analysis. Yeast strains were grown in 500 ml of YPD medium at 30°C to an optical density at 600 nm (OD_{600}) of 0.8. Cells were pelleted by centrifugation, resuspended in 400 μ l of lysis buffer (20 mM HEPES [pH 7.4], 2 mM magnesium acetate, 100 mM potassium acetate, 100 μ g of cycloheximide per ml, 0.5 mM dithiothreitol) on ice, and transferred to 1.5-ml microcentrifuge tubes. After pelleting for 5 min at $4,000 \times g$, cells were resuspended in an equal volume of lysis buffer containing protease inhibitors and lysed in the presence of 1 volume of glass beads by vortexing six times for 20 s each time at 40-s intervals. Lysates were clarified briefly at $8,000 \times g$ for 5 min, followed by a 20-min centrifugation at $8,000 \times g$ to give the final lysate. Fifty A_{260} units of lysate were fractionated on 10 to 50% sucrose gradients as described previously (11). Gradients were centrifuged in an SW28 rotor (Beckman) at 27,000 rpm for 180 min at 4°C and analyzed by continuous monitoring of A_{260} . For protein analysis, each fraction from the gradient was precipitated with 10% trichloroacetic acid, washed with cold 70% ethanol, and resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer. For RNA analysis, total RNA was isolated from each fraction with an RNeasy kit (QIAGEN) and treated with RQ1 DNase (Promega). RT-PCR was performed with 1 μ l of RNA as the template and a QuickAccess RT-PCR kit (Promega) under the conditions suggested by the manufacturer. The following primers were used for amplification: *HO*-for, 5'-GGGAACCCGTATATTTTCAGC-3'; *HO*-rev, 5'-CTTTTATTACATACAACTTTTAAACTAATATACAC-3'; *ACT1*-for, 5'-GGAATCTGCCGGTATTGACC-3'; *ACT1*-rev, 5'-ACATACGCGCACAAAAGCAG-3'.

Western blotting. Cell extracts were fractionated by SDS-PAGE on 8% acrylamide gels, followed by electroblotting onto Hybond N⁺ membranes (Amersham). Blots were blocked by incubation for 15 min at room temperature in TBS-M (Tris-buffered saline [TBS] with 4% nonfat dry milk). Blots were then incubated with antibody in TBS-M overnight at 4°C. After three washes with TBS, blots were incubated for 2 h with a peroxidase-conjugated secondary antibody (Calbiochem) diluted 1:3,000 with TBS-M. After three final washes with TBS, blots were detected with an enhanced chemiluminescence detection kit (Amersham). The antibodies used were anti-HA antibody HA11 diluted 1:2,000, anti-Myc antibody 9E10 diluted 1:500, anti-tubulin antibody TAT-1 diluted 1:1,000, and anti- β -galactosidase polyclonal antibody (Sigma) diluted 1:1,000.

RESULTS

Isolation of mutants affecting *HO* expression. To monitor *HO* expression, we used an *HOp-ADE2* reporter system in which the *ho* ORF is replaced with the *ADE2* ORF at the *ho* locus (15, 34). Expression of the reporter can thus be assayed in an *ade2 Δ background by growth on medium lacking adenine (SC–Ade). Since asymmetric transcription of the *HO* gene results from the preferential accumulation of *ASH1* mRNA encoding a negative regulatory protein in daughter cells (18, 35), delocalization of *ASH1* mRNA in *she* mutants causes a reduction in *HO* expression (15). Consistent with this, *myo4*/*she1* mutants containing the *HOp-ADE2* reporter failed to grow on SC–Ade plates and disruption of the *ASH1* gene suppressed the growth defect associated with the *myo4 Δ mutation (Fig. 1A). We have previously shown that overexpression of *KHD1*, which encodes a protein carrying three KH RNA-binding motifs, inhibits translation of *ASH1* mRNA (12). Thus, overexpression of *KHD1* from the *GAL1* promoter suppressed the growth defect of *myo4 Δ mutants on plates containing galactose and lacking adenine (SG–Ade) (Fig. 1A). With this system, it was possible to isolate *she* mutations or mutations affecting *HO* expression itself by screening for mutants that exhibited reduced growth on SC–Ade and that were suppressed by overexpression of *KHD1*. Mutants suppressed by***

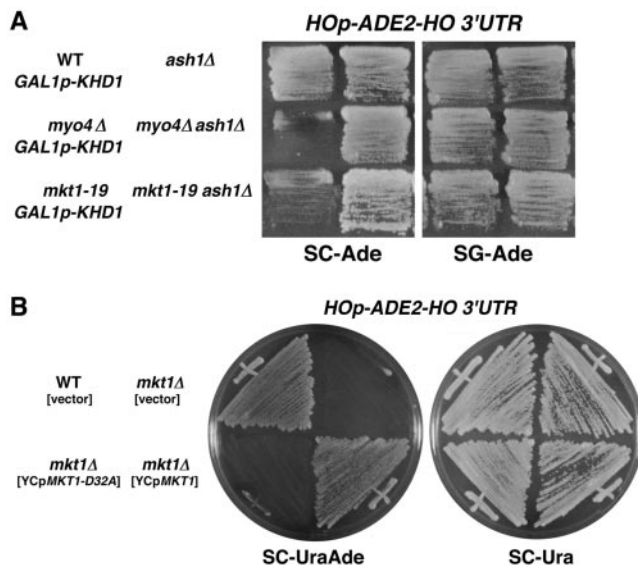


FIG. 1. Isolation of the *mkt1* mutant. (A) Effect of the *mkt1-19* mutation on expression of the *HOp-ADE2* reporter. Each yeast strain was streaked onto SC–Ade or SG–Ade plates and incubated for 3 days at 30°C. Yeast strains: YKEN301 (wild-type [WT] *GAL1p-KHD1 HOp-ADE2-HO 3'UTR*), YKEN303 (*myo4* Δ *GAL1p-KHD1 HOp-ADE2-HO 3'UTR*), YKEN9152 (*mkt1-19 GAL1p-KHD1 HOp-ADE2-HO 3'UTR*), YKEN304 (*ash1* Δ *HOp-ADE2-HO 3'UTR*), YKEN306 (*myo4* Δ *ash1* Δ *HOp-ADE2-HO 3'UTR*), and YKEN9152a (*mkt1-19 ash1* Δ *HOp-ADE2-HO 3'UTR*). (B) Effect of the *mkt1* Δ and *mkt1-D32A* mutations on expression of the *HOp-ADE2* reporter. Each yeast strain was streaked onto SC–UraAde or SC–Ura plates and incubated for 3 days at 30°C. Yeast strains: 10B (wild-type *HOp-ADE2-HO 3'UTR*) (YCplac33), TTC478 (*mkt1* Δ *HOp-ADE2-HO 3'UTR*) (YCplac33), TTC478 (*mkt1* Δ *HOp-ADE2-HO 3'UTR*) (YCpMKT1-D32A), and TTC478 (*mkt1* Δ *HOp-ADE2-HO 3'UTR*) (YCpMKT1).

KHD1 overexpression would also be expected to be suppressed by the loss of *Ash1*.

Starting with an *ade2 Δ strain carrying the *HOp-ADE2* reporter and the *GAL1p-KHD1* gene, mutants with reduced *HO* expression were selected as red or pink colonies on SC–Ade plates containing a limited amount of adenine. These mutants were then streaked onto SG–Ade plates to isolate mutants in which the adenine auxotrophy was suppressed by *KHD1* overexpression. Out of about 3,000 red or pink colonies on SC–Ade containing a limited amount of adenine, 12 mutants grew well on SG–Ade. We tested complementation with a series of low-copy-number plasmids carrying each of the *SHE* genes and found out that 9 of the 12 candidates could be complemented by the *MYO4* gene and 2 could be complemented by the *SHE3* gene. The remaining mutant, YKEN9152, contained a mutation at the *MKT1* locus (see below). Genetic analysis indicated that the *mkt1-19* mutation in YKEN9152 segregated 2:2 for the adenine auxotrophy when crossed to a strain with the parental genotype (data not shown). As expected, the reduced growth of the *mkt1-19* mutant on an SC–Ade plate was suppressed by disruption of the *ASH1* gene (Fig. 1A). We thus chose to characterize the *mkt1* mutation further.*

Cloning of the *MKT1* gene. We isolated the *MKT1* gene from a genomic library by virtue of its ability to complement the adenine auxotrophy of our mutant strain YKEN9152. The

TABLE 4. Effect of *mkt1Δ* mutation on localization of Ash1 protein

Genotype ^b	% of 100 cells ^a		
	Daughter only	Predominantly daughter	Mother and daughter
Wild type	92	6	2
<i>myo4Δ</i>	2	4	94
<i>mkt1Δ</i>	72	14	14

^a The percentage of cells that showed each pattern of Ash1 protein localization was determined by staining with anti-Myc antibody in cells expressing Ash1-Myc: daughter only = visible Ash1 only in the daughter; predominantly daughter = Ash1 staining predominantly in the daughter nucleus with an intermediate amount of staining in the mother nucleus; mother and daughter = equivalent levels of Ash1 in both mother and daughter nuclei.

^b Yeast strains: K5552 (wild-type *ASH1-myc*), YKEN451 (*myo4Δ ASH1-myc*), and TTC237 (*mkt1Δ ASH1-myc*).

4) (3, 18, 35). In contrast, the *mkt1Δ* mutation hardly affected the proper localization of *ASH1* mRNA and Ash1 protein, unlike the *myo4Δ* mutation (Tables 3 and 4). These results suggest that the reduction in *HO* expression caused by the *mkt1Δ* mutation is not the result of delocalization of *ASH1* mRNA or Ash1 protein. It is likely that Mkt1 does not affect *HO* expression by acting on *ASH1*, even though reduced *HO* expression in *mkt1Δ* mutants was suppressed by the *ash1Δ* mutation.

Mkt1 regulates *HO* expression at the posttranscriptional level via the *HO* 3' UTR. To determine at which stage Mkt1 regulates *HO* expression, we tested the effect of the *mkt1Δ* mutation on *ho* mRNA levels. In contrast to the *myo4Δ* mutation, which abolished transcription of the *ho* gene, the *mkt1Δ* mutation had no obvious effect on the concentration of *ho* mRNA (Fig. 3A). This suggests that Mkt1 may regulate *HO* expression posttranscriptionally.

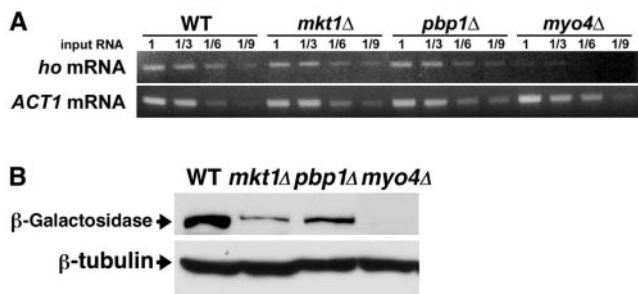


FIG. 3. Effect of the *mkt1Δ* and *pbp1Δ* mutations on levels of *ho* mRNA and β -galactosidase protein from the *Hop-lacZ* reporter. (A) Effects of the *mkt1Δ* and *pbp1Δ* mutations on *ho* mRNA levels. Each yeast strain was grown in YPD medium at 30°C and harvested. Total RNA samples were prepared from each strain and assayed by RT-PCR for *ho* mRNA levels (top) and *ACT1* mRNA levels (bottom) as a quantity control. The values above the lanes are template RNA dilution factors. Yeast strains: W303 (wild-type [WT] *ho*), TTC1313 (*mkt1Δ ho*), TTC1316 (*pbp1Δ ho*), and TTC1319 (*myo4Δ ho*). (B) Effects of the *mkt1Δ* and *pbp1Δ* mutations on β -galactosidase protein levels from the *Hop-lacZ* reporter. Each yeast strain was grown in YPD medium at 30°C and harvested. Western blot analysis was performed to quantitate the level of β -galactosidase protein (top) and tubulin protein (bottom) as a quantity control. Yeast strains: K1107 (wild-type *Hop-lacZ*), TTC1085 (*mkt1Δ Hop-lacZ*) TTC1089 (*pbp1Δ Hop-lacZ*), and TTC4 (*myo4Δ Hop-lacZ*).

TABLE 5. Effects of *mkt1Δ* and *pbp1Δ* mutations on expression of the *Hop-LacZ* reporter

Genotype ^a	β -Galactosidase activity (nmol/min/mg) ^b
Wild type	36.6
<i>myo4Δ</i>	4.7
<i>mkt1Δ</i>	13.4
<i>pbp1Δ</i>	12.1
<i>mkt1Δ pbp1Δ</i>	14.3

^a Yeast strains: K1107 (wild-type *Hop-LacZ*), TTC4 (*myo4Δ Hop-LacZ*), TTC1085 (*mkt1Δ Hop-LacZ*), TTC1089 (*pbp1Δ Hop-LacZ*), and TTC1163 (*mkt1Δ pbp1Δ Hop-LacZ*).

^b Yeast strains were grown in YPD medium and assayed for β -galactosidase activity. Values shown are averages of three experiments.

To test the effect of the *mkt1Δ* mutation on the translation of the *ho* gene, we used an *Hop-lacZ* reporter, which replaces the *ho* ORF with the *lacZ* ORF at the *ho* locus but retains the 5' and 3' UTRs of the *HO* mRNA (25, 34). We monitored the translation of the *Hop-lacZ* reporter with an antibody against β -galactosidase. First, we confirmed that the *mkt1Δ* mutation caused decreased expression of *Hop-lacZ* and observed an about threefold decrease in *HO* expression in *mkt1Δ* mutants compared with that in wild-type cells (Table 5). Western blotting analysis revealed that the *mkt1Δ* mutation reduced the levels of β -galactosidase protein (Fig. 3B). To eliminate the possibility that the reporter mRNA behaves differently from the authentic transcript, we examined whether the *mkt1Δ* mutation had an effect on the levels of *Hop-lacZ* and *Hop-ADE2* reporter mRNAs by real-time quantitative RT-PCR. The *mkt1Δ* mutation had no effect on the levels of the reporter mRNAs (105 and 93% of the *Hop-lacZ* and *Hop-ADE2* reporter mRNAs, respectively, in the *mkt1Δ* mutant cells compared with those in wild-type cells), while the *myo4Δ* mutation reduced the levels of those mRNAs (12 and 32% of the *Hop-lacZ* and *Hop-ADE2* reporter mRNAs, respectively, in the *myo4Δ* mutant cells compared with those in wild-type cells). Furthermore, Northern blotting also confirmed that the level and size of the *Hop-lacZ* reporter mRNA were not altered by the *mkt1Δ* mutation (data not shown). These results suggest that Mkt1 is involved in the translational control of *HO* mRNA.

To further localize the level of Mkt1 regulation, we examined the involvement of the *HO* 3' UTR in Mkt1-mediated regulation of *HO* expression. We used two reporters that differ only in their 3' UTRs, *Hop-ADE2-HO 3'UTR* and *Hop-ADE2-ADHI 3'UTR*. In the latter, the *HO* 3' UTR has been replaced with the *ADHI* 3' UTR (34). When the *HO-ADE2-HO 3'UTR* and *HO-ADE2-ADHI 3'UTR* reporters were expressed in *ade2Δ* strains, their mRNA levels were similar (data not shown). Consistent with this, *ade2Δ* strains harboring either *Hop-ADE2-HO 3'UTR* or *Hop-ADE2-ADHI 3'UTR* grew normally on SC–Ade plates, similar to the *ADE2* strain (Fig. 4A). However, the *mkt1Δ* mutant harboring *Hop-ADE2-HO 3'UTR* grew poorly on SC–Ade plates whereas the *mkt1Δ* mutant harboring *Hop-ADE2-ADHI 3'UTR* grew normally, similar to the wild-type strain harboring *Hop-ADE2-ADHI 3'UTR* (Fig. 4A). These results suggest that Mkt1 is

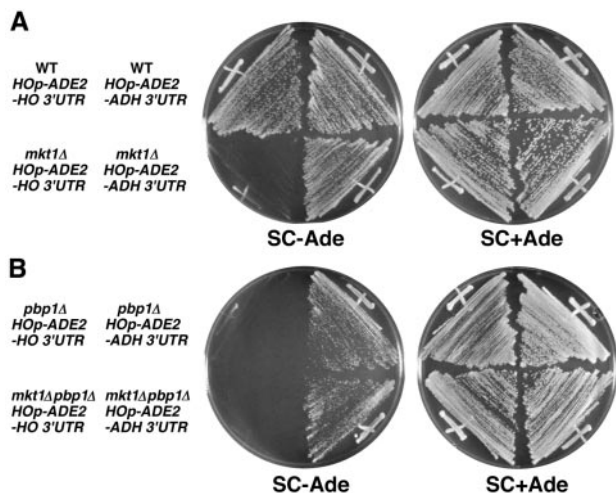


FIG. 4. The phenotype of *mkt1Δ* and *pbp1Δ* mutants is dependent on the *HO* 3' UTR. Each yeast strain was streaked onto SC–Ade or SC+Ade plates and incubated for 3 days at 30°C. (A) Yeast strains: 10B (wild-type [WT] *HOP-ADE2-HO* 3'UTR), TTC47 (wild-type *HOP-ADE2-ADH1* 3'UTR), TTC478 (*mkt1Δ HOP-ADE2-HO* 3'UTR), and TT1108 (*mkt1Δ HOP-ADE2-ADH1* 3'UTR). (B) Yeast strains: TTC798 (*pbp1Δ HOP-ADE2-HO* 3'UTR), TTC1334 (*pbp1Δ HOP-ADE2-ADH1* 3'UTR), TTC1257 (*mkt1Δ pbp1Δ HOP-ADE2-HO* 3'UTR), and TTC1333 (*mkt1Δ pbp1Δ HOP-ADE2-ADH1* 3'UTR).

involved in the regulation of *HO* expression through the *HO* 3' UTR.

Mkt1 interacts with the poly(A)-binding protein-binding protein Pbp1. To examine Mkt1-mediated regulation of *HO* expression further, we screened for an Mkt1-binding protein(s) by using the yeast two-hybrid screen with Mkt1 as bait. We screened 7×10^5 clones from a yeast genomic library and obtained 39 positive clones. All 39 clones encoded different COOH-terminal portions of the same protein, Pbp1 (aa 198 to 722, 357 to 722, and 475 to 722) (Fig. 5A; data not shown). Thus, the COOH-terminal half of the Pbp1 protein is able to interact with Mkt1. Pbp1 was previously identified as a protein that binds to Pab1, a poly(A)-binding protein (21). Two-hybrid analysis revealed that the COOH-terminal region of Mkt1 is required for its interaction with Pbp1 (Fig. 5A).

To confirm that Mkt1 binds Pbp1 in vivo, we performed immunoprecipitation analyses. We constructed strains harboring a Myc-tagged version of Mkt1, Mkt1-Myc, and an HA-tagged version of Pbp1, Pbp1-HA, as described in Materials and Methods. When Mkt1-Myc was immunoprecipitated from cell extracts with an anti-Myc antibody, Pbp1-HA was coimmunoprecipitated, as detected by anti-HA antibody in Western blot assays (Fig. 5B). When Mkt1ΔC-Myc lacking the COOH-terminal 230 aa was coexpressed with Pbp1-HA, coimmunoprecipitation analysis revealed that Mkt1ΔC-Myc failed to interact with Pbp1-HA (Fig. 5B). These results indicate that Mkt1 interacts with Pbp1 in vivo and that the COOH-terminal region of Mkt1 is required for its interaction with Pbp1.

Effect of the *pbp1Δ* mutation on *HO* expression. To examine whether Pbp1 also regulates *HO* expression in a manner similar to that of Mkt1, we disrupted the *PBP1* gene in a strain harboring the *HOP-ADE2-HO* 3'UTR or *HOP-ADE2-ADH1* 3'UTR reporter (see Materials and Methods). The *pbp1Δ* mu-

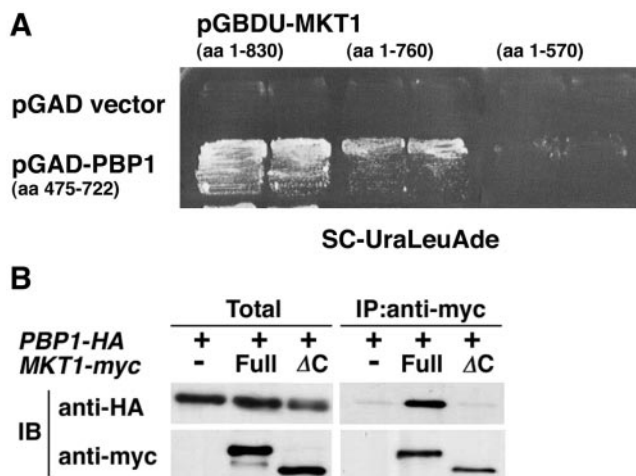


FIG. 5. Interaction of Mkt1 with Pbp1. (A) Two-hybrid analysis. Yeast strain PJ69-4A harboring *GAL2p-ADE2* and *GAL1p-HIS3* reporters was transformed with the indicated plasmids, and the transformants were streaked onto SC–UraLeuAde plates and incubated for 3 days at 30°C. The same results were obtained with the *HIS3* reporter assay (data not shown). Plasmids: pGAD-C1 (vector), pGAD-PBP1 (aa 475 to 722), pGBDU-MKT1-Full (aa 1 to 830), pGBDU-MKT1ΔC1 (aa 1 to 760), and pGBDU-MKT1ΔC2 (aa 1 to 570). (B) Coimmunoprecipitation (IP) analysis. Extracts prepared from strains TTC999 (*PBP1-HA*), TTC1049 (*PBP1-HA MKT1-myc*) and TTC1302 (*PBP1-HA MKT1ΔC-myc*) were incubated with anti-Myc antibody coupled to protein G-Sepharose. Each immunopellet was separated by SDS–10% PAGE, blotted, and probed with anti-HA or anti-Myc antibody for the presence of Pbp1-HA or Mkt1-Myc protein. Total extracts were also immunoblotted (IB) with anti-HA or anti-Myc antibody. Mkt1ΔC-Myc lacks the COOH-terminal 230 aa.

tant harboring *HOP-ADE2-HO* 3'UTR did not grow on SC–Ade plates, similar to the *mkt1Δ* mutant (Fig. 4B). In contrast, the *pbp1Δ* mutant harboring *HOP-ADE2-ADH1* 3'UTR grew normally, similar to the wild-type strain, suggesting that Pbp1 regulates *HO* expression via the *HO* 3' UTR. The *pbp1Δ* mutation decreased expression of the *HOP-lacZ* reporter (Table 5). Furthermore, an *mkt1Δ pbp1Δ* double mutant showed β-galactosidase activity similar to that of each single mutant, indicating that the effects of the *mkt1Δ* and *pbp1Δ* mutations on *HO* expression are nonadditive. Together with the observation that Mkt1 directly associates with Pbp1, our results suggest that Mkt1 and Pbp1 regulate *HO* expression at the same step. Consistent with this, the *pbp1Δ* mutation had no effect on *ho* mRNA levels (Fig. 3A) but caused a reduction in the levels of β-galactosidase protein in a strain carrying the *HOP-lacZ* reporter (Fig. 3B). Furthermore, the real-time quantitative RT-PCR analysis revealed that *HOP-lacZ* reporter mRNA levels were not affected by the *pbp1Δ* mutation (109% in *pbp1Δ* mutant cells compared with those in wild-type cells). Thus, the effect of Pbp1 on *HO* expression is similar to that of Mkt1. These results suggest that Mkt1 and Pbp1 are involved in the translational control of *HO* mRNA.

Mkt1 and Pbp1 cosediment with polysomes. It has been reported that Pbp1 cofractionates with polysomes (21). We therefore examined the subcellular localization of Mkt1. The subcellular localizations of Mkt1-HA and Pbp1-HA were assayed by fractionating cytoplasmic extracts on sucrose gradients and analyzing the fractions by Western blotting. As

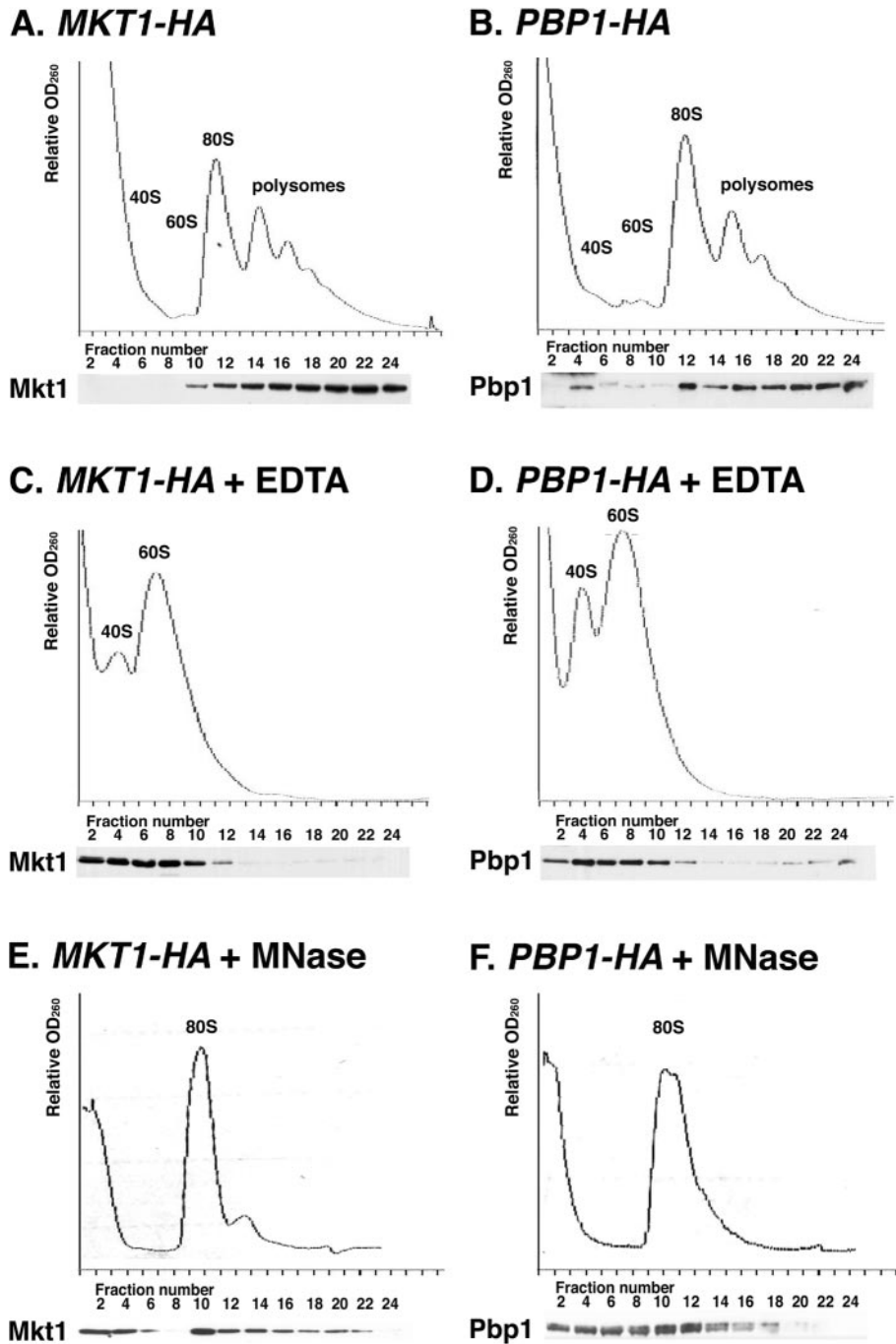


FIG. 6. Mkt1 and Pbp1 cofractionate with polysomes on sucrose gradients. Extracts prepared from strains expressing Mkt1-HA (A, C, and E) or Pbp1-HA (B, D, and F) were fractionated on 10 to 50% sucrose gradients. Extracts were pretreated with 30 mM EDTA (C and D) or 1 U of micrococcal nuclease (MNase) per μ l (E and F) for 20 min before being loaded onto the gradients. The upper part of each panel shows the OD₂₆₀ profile of the gradient. Peaks representing small (40S) and large (60S) ribosomal subunits, a single ribosome (80S), and polysomes (>80S) are indicated. The lower part of each panel shows an anti-HA Western blot of gradient fractions concentrated and subjected to SDS-8% PAGE. Yeast strains: TTC848 (*MKT1-HA*) and TTC996 (*PBP1-HA*).

observed previously (21), Pbp1-HA cosedimented with polysomes. We observed that Mkt1-HA and Pbp1-HA were distributed similarly in the gradient (Fig. 6A and B). Since the formation and maintenance of 80S ribosomes require the presence of Mg^{2+} ions, polysomes can be disrupted by adding

EDTA to the extracts. EDTA treatment caused a loss of polysomes and a large increase in the number of free 40S and 60S subunits (Fig. 6C and D). Concomitantly, there was a shift in the sedimentation pattern of Mkt1-HA and Pbp1-HA from polysomes to the lighter fractions of the gradient. Further-

more, when extracts were treated with micrococcal nuclease, which collapses polysomes into a single monosome peak (80S), the distribution of Mkt1-HA and Pbp1-HA was also disrupted (Fig. 6E and F). These results confirm that Mkt1 and Pbp1 cosediment with polysomes. Mkt1 and Pbp1 were still distributed in the ribosomal subunits or the monosome fraction after treatment with EDTA or micrococcal nuclease (Fig. 6C, D, E, and F), suggesting that the Mkt1-Pbp1 complex may associate with ribosome subunits.

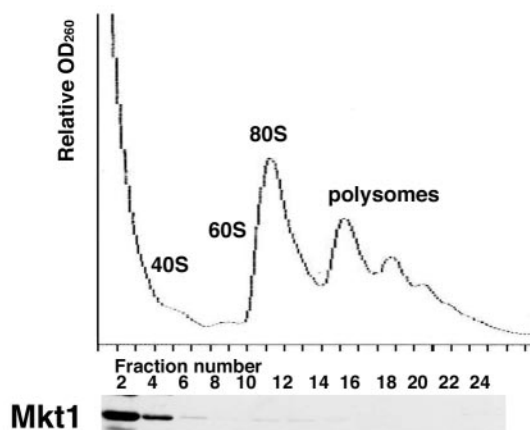
Since Mkt1 associates with Pbp1, we examined whether the association of Mkt1 and/or Pbp1 with polysomes depends on the presence of the other protein. Fractionation of extracts prepared from a *pbp1Δ* mutant expressing Mkt1-HA revealed that Mkt1 was not associated with polysomes (Fig. 7A). This indicates that the association of Mkt1 with polysomes is indirect and Pbp1 dependent. In contrast, Pbp1 was associated with polysomes even in the absence of Mkt1 (Fig. 7B). These results suggest that Pbp1 mediates the association of Mkt1 with polysomes. Finally, we examined the effects of the *mkt1Δ* and *pbp1Δ* mutations on the distribution of *HO* mRNA in the polysome fraction. *HO* mRNA was quantitatively incorporated into the polysome fractions (Fig. 8A). Neither the *mkt1Δ* nor the *pbp1Δ* mutation had any effect on *HO* mRNA polysome size distribution (Fig. 8B and C). These results suggest that Mkt1 may regulate the elongation or termination of translation, but not the initiation, of *HO* mRNA.

MKT1 and PBPI affect mating-type switching in mother cells. The *HO* gene encodes an endonuclease that stimulates mating-type switching in budding yeast (9). The ability of cells in a cell lineage to undergo mating-type switching has a very precise pattern in which mother cells switch efficiently (typically 70% of cell divisions) but daughter cells do not (<0.1% of cell divisions) (33). The basis for this difference is the presence of *HO* mRNA in mother cells but not in daughter cells (24). Since Mkt1 and Pbp1 are required for efficient *HO* expression, we tested whether the *mkt1Δ* or *pbp1Δ* mutation affects the regulation of mating-type switching. We introduced the *mkt1Δ* or *pbp1Δ* mutation into an *HO* background and measured its effect on mating-type switching by pedigree analysis (Table 6). In wild-type cells, we observed that 72% of the mother cells and none of the daughter cells switched mating type. Mutations in *MKT1* and *PBPI* reduced the frequency of mating-type switching in mother cells to 52 and 37%, respectively, but had no effect on daughter cells. Although substantial, the effects of these mutations on mother cell switching are not as strong as those observed in *swi* or *she* mutants. These results indicate that Mkt1 and Pbp1 are required for efficient mating-type switching in mother cells.

DISCUSSION

HO expression is regulated at the transcriptional level by a negative regulator, Ash1 (3, 32). Ash1 is a specific repressor of transcription that localizes asymmetrically to the daughter cell nucleus through the localization of *ASH1* mRNA to the distal tip of the daughter cell (18, 35). This localization depends on the actin cytoskeleton and five She proteins, one of which is a type V myosin motor, Myo4. Delocalization of *ASH1* mRNA in *she* mutants causes a reduction in *HO* expression (15). Recently, we have shown that an RNA-binding protein, Khd1

A. MKT1-HA *pbp1Δ*



B. PBP1-HA *mkt1Δ*

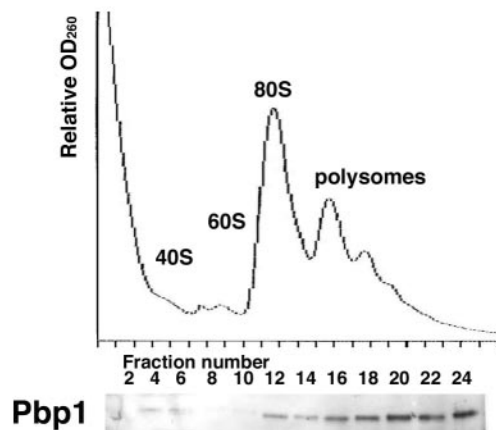
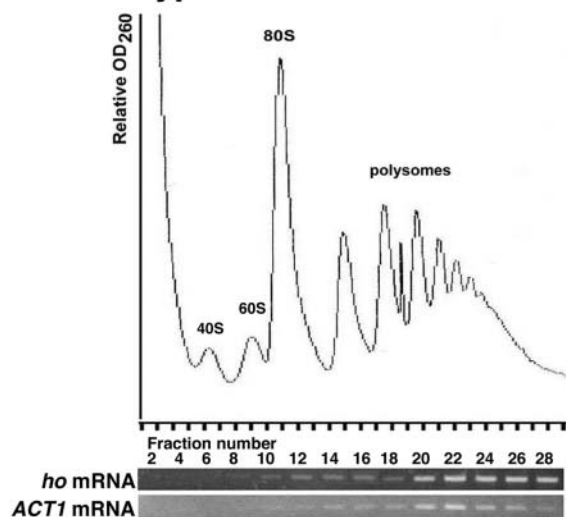


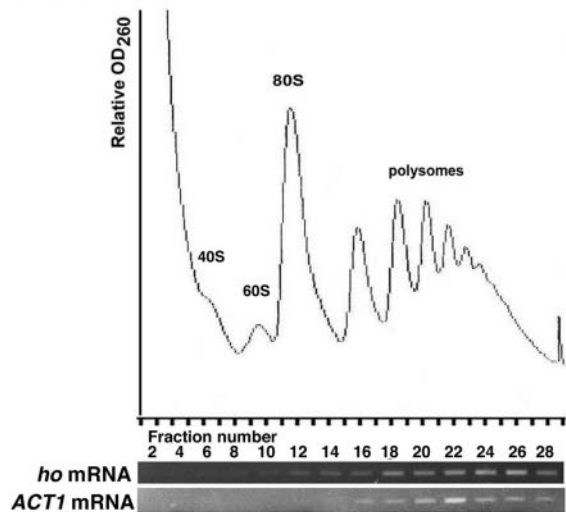
FIG. 7. Pbp1-dependent cofractionation of Mkt1 with polysomes. Extracts prepared from strains expressing Mkt1-HA (A) or Pbp1-HA (B) were fractionated on 10 to 50% sucrose gradients. The upper part of each panel shows the OD₂₆₀ profile of the gradient. Peaks representing small (40S) and large (60S) ribosomal subunits, a single ribosome (80S), and polysomes (>80S) are indicated. The lower part of each panel shows an anti-HA Western blot of gradient fractions concentrated and subjected to SDS-8% PAGE. Yeast strains: TTC1234 (*MKT1-HA pbp1Δ*) and TTC1233 (*PBP1-HA mkt1Δ*).

(KH domain protein 1), is also required for efficient localization of *ASH1* mRNA to the distal tip of the daughter cell, by associating with the N element of *ASH1* mRNA (12). Furthermore, overexpression of *KHD1* decreases the concentration of Ash1 protein and restores *HO* expression to *she* mutants. Therefore, we expected that by screening for mutants that had reduced expression of the *HOp-ADE2* reporter and that could be suppressed by *KHD1* overexpression, we could isolate *she* mutations or mutations affecting *HO* expression itself. Indeed, we isolated *myo4* and *she3* mutations by this screening method. Thus, screening with *KHD1* overexpression is useful for the isolation of *she* mutations. However, we failed to identify novel *SHE* genes. Since we did not isolate a *she2*, *she4*, or *she5*

A. Wild-type



B. *mkt1Δ*



C. *pbp1Δ*

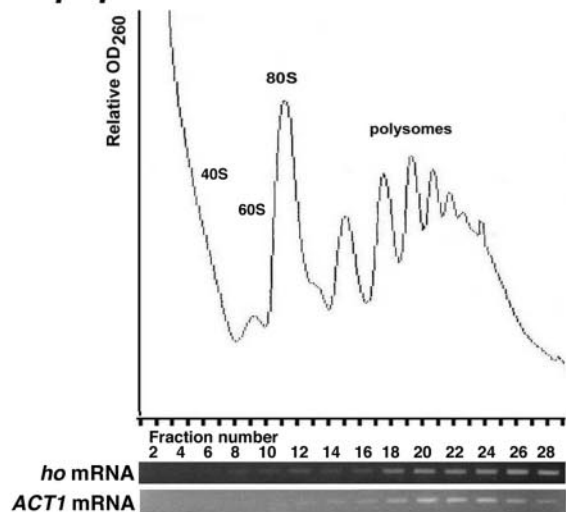


TABLE 6. Mating-type switching in *mkt1Δ* and *pbp1Δ* mutant cells

Genotype ^b	Mating-type switching frequency (%) ^a	
	Mother	Daughter
Wild type	72	0
<i>mkt1Δ</i>	52 ^c	0
<i>pbp1Δ</i>	37 ^d	0

^a Mean percentages of three independent experiments are indicated. More than 80 cell divisions were examined in each experiment. Note the significant differences between wild-type and mutant cells.

^b Yeast strains: TTC46 (wild-type *HO*), TTC929 (*mkt1Δ HO*), and TTC993 (*pbp1Δ HO*).

^c *P* < 0.002.

^d *P* < 0.0005.

mutation, our screening has not been saturated. Alternatively, such novel *SHE* genes, if present, might be essential for yeast cell growth.

In this study, we isolated *mkt1* as a mutation that caused reduced expression of an *HO**p*-*ADE2* reporter and that was in turn suppressed by overexpression of the *KHD1* gene. In contrast to *she* mutants, the *mkt1Δ* mutation had little effect on the localization of *ASH1* mRNA or Ash1 protein. This indicates that Mkt1 affects *HO* expression at a step different from that of the She proteins. In addition, we isolated the *PBP1* gene as an Mkt1-interacting protein. Pbp1 was previously identified as a protein that associates with the poly(A)-binding protein Pab1 (21). We have presented several pieces of evidence suggesting that Mkt1 regulates the translation of *HO* mRNA by forming a complex with Pbp1. First, loss of the *MKT1* or *PBP1* gene has little effect on *HO* mRNA levels but decreases *HO* protein levels. Second, regulation of *HO* expression by Mkt1 and Pbp1 is mediated by the *HO* 3' UTR. Third, Mkt1 binds to Pbp1 in vivo, and they cosediment with polysomes. Association of Mkt1 in the polysome fraction is dependent on Pbp1. Finally, both Mkt1 and Pbp1 are required for effective mating-type switching in mother cells. We thus propose that the Mkt1-Pbp1 complex regulates *HO* translation via the 3' UTR of *HO* mRNA. Reduced expression of *HO* in *mkt1* mutants is suppressed by *KHD1* overexpression or the *ash1Δ* mutation. This suggests that an increase in *HO* mRNA caused by inactivation of the Ash1 repressor can overcome inefficient translation caused by the *mkt1* mutation.

The observation that Mkt1 and Pbp1 cosediment with polysomes in similar distribution patterns in sucrose gradients is consistent with the idea that these two proteins associate. Furthermore, the association of Mkt1 with polysomes is dependent on Pbp1. These results suggest that Pbp1 interacts with and recruits Mkt1 to the polysomes. Pbp1 interacts with Pab1,

FIG. 8. Distribution of *ho* mRNA in the polysome fraction. Extracts prepared from wild-type (A), *mkt1Δ* (B), and *pbp1Δ* (C) strains were fractionated on 10 to 50% sucrose gradients. Total RNA samples were prepared from each fraction and assayed by RT-PCR for *ho* and *ACT1* mRNA levels. The upper part of each panel shows the OD₂₆₀ profile of the gradient. Peaks representing small (40S) and large (60S) ribosomal subunits, a single ribosome (80S), and polysomes (>80S) are indicated. The lower part of each panel shows the levels of *ho* and *ACT1* mRNAs. Yeast strains: W303 (wild-type *ho*), TTC1313 (*mkt1Δ ho*), and TTC1316 (*pbp1Δ ho*).

poly(A)-binding protein, and Pab1 also cosediments with polysomes (21). However, Pbp1 is still associated with polysomes in the absence of Pab1 (21). Furthermore, Pbp1 and Mkt1 are distributed in the monosome fractions even after micrococcal nuclease treatment. Thus, it is likely that the Mkt1-Pbp1 complex associates with ribosome subunits but not with mRNA. Consistent with this, we could not detect an interaction of Mkt1 with the 3' UTR of *HO* mRNA by coimmunoprecipitation and three-hybrid analyses (T. Tadauchi, T. Inada, K. Matsumoto, and K. Irie, unpublished data). The presence of Mkt1 and Pbp1 on polysomes suggests that the Mkt1-Pbp1 complex regulates the translation of *HO* mRNA through its interaction with polysomes. In fact, we observed that *HO* mRNA is quantitatively incorporated into the polysome fractions. The observation that neither the *mkt1Δ* nor the *pbp1Δ* mutation has any effect on the distribution of *HO* mRNA in the polysome fractions suggests that the Mkt1-Pbp1 complex may regulate the elongation or termination of translation but not the translational initiation or nuclear export of *HO* mRNA.

How does Mkt1 regulate *HO* expression at the translational level? We reexamined the amino acid sequence of the Mkt1 protein and found that Mkt1 has two domains, XPG-N and XPG-I, in its NH₂-terminal region. These domains are conserved within a family of nucleases that includes the XPG endonuclease (4, 22). The conserved residue essential for the nuclease activity in the XPG-N domain of Mkt1 is required for its ability to regulate *HO* expression. The XPG family members have exo- or endonuclease activities with different substrate specificities and are involved in repair, recombination, and replication (4, 22). Although we have not determined whether Mkt1 has nuclease activity, it might function in the processing of the *HO* mRNA. This processing, presumably involving the *HO* 3' UTR, would be required for efficient translation of *HO* mRNA at the elongation or termination step. The amino acid residues that are required for XPG nuclease activity, Asp-77 and Glu-791, are not conserved in Mkt1 (Asn-82 and Trp-193), suggesting that Mkt1 might have different activity or substrate specificity. One possibility is that Mkt1 accesses the 3' UTR of the *HO* mRNA in the polysome fraction through an interaction with Pbp1 and modification via the Mkt1 nuclease activity leads to the efficient elongation or termination of *HO* mRNA translation. There still remains the possibility that an unidentified factor that requires the Mkt1-Pbp1 complex for its function could be responsible for the control of translation.

Regulation of *HO* expression by Mkt1 and Pbp1 is mediated by the 3' UTR of the *HO* mRNA. The reduction in *HO* expression seen in *mkt1Δ* or *pbp1Δ* mutant cells is eliminated by replacement of the *HO* 3' UTR with the *ADH1* 3' UTR. *HO* expression is also regulated posttranscriptionally by the yeast Puf homolog Mpt5/Puf5 via the *HO* 3' UTR (34). Mpt5 directly binds to the 3' UTR of *HO* mRNA and represses *HO* expression. Analysis of *mpt5Δ mkt1Δ* double mutants revealed that the *mpt5Δ* mutation suppressed the defect in *HO* expression caused by the *mkt1Δ* mutation (Tadauchi et al., unpublished). This genetic analysis perhaps suggests that the Mkt1-Pbp1 complex could positively regulate *HO* expression by negatively regulating Mpt5 function. However, this possibility is unlikely because the mechanism by which *MPT5* overexpression affects *HO* mRNA is different from that by which deletion of the *MKT1* or *PBP1* gene affects *HO* mRNA. Overexpression

of the *MPT5* gene promotes degradation of the reporter mRNA containing the *HO* 3' UTR, an observation that suggests that Mpt5 may affect mRNA stability or turnover via binding to the *HO* 3' UTR (34). On the other hand, loss of the *MKT1* or *PBP1* gene has little effect on *HO* mRNA levels. Indeed, we observed that disruption or overexpression of the *MKT1* gene had no effect on the ability of Mpt5 to bind to the 3' UTR of the *HO* mRNA (Tadauchi et al., unpublished). Thus, it is likely that the Mkt1-Pbp1 complex and Mpt5 independently regulate *HO* expression posttranscriptionally via the *HO* mRNA 3' UTR. The observation that *mpt5Δ* suppresses the *mkt1Δ* defect might be explained by simple additivity, i.e., that an increase in *HO* mRNA caused by inactivation of Mpt5 may overcome the inefficient translation caused by the *mkt1Δ* mutation.

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