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The *Saccharomyces cerevisiae* Isw2p-Itc1p Complex Represses *INO1* Expression and Maintains Cell Morphology

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In the yeast *Saccharomyces cerevisiae*, *IRE1* encodes a bifunctional protein with transmembrane kinase and endoribonuclease activities. *HAC1* encodes a transcription factor which has a basic leucine zipper domain. Both gene products play a crucial role in the unfolded protein response. Mutants in which one of these genes is defective also show the inositol-auxotrophic (Ino$^-$) phenotype, but the reason for this has not been clear. To investigate the mechanism underlying the Ino$^-$ phenotype, we screened a multicopy suppressor gene which can suppress the Ino$^-$ phenotype of the Δ*hac1* strain. We obtained a truncated form of the *ITC1* gene that has a defect in its 3′ region. Although the truncated form of *ITC1* clearly suppressed the Ino$^-$ phenotype of the Δ*hac1* strain, the full-length *ITC1* had a moderate effect. The gene products of *ITC1* and *ISW2* are known to constitute a chromatin-remodeling complex (T. Tsukiyama, J. Palmer, C. C. Landel, J. Shiloach, and C. Wu, Genes Dev. 13:686–697, 1999). Surprisingly, the deletion of either *ITC1* or *ISW2* in the Δ*hac1* strain circumvented the inositol requirement and caused derepression of *INO1* even under repression conditions, i.e., in inositol-containing medium. These data indicate that the Isw2p-Itc1p complex usually represses *INO1* expression and that overexpression of the truncated form of *ITC1* functions in a dominant negative manner in *INO1* repression. It is conceivable that the repressor function of this complex is regulated by the C-terminal region of Itc1p.

It is well known that the accumulation of an unfolded protein in the endoplasmic reticulum (ER) initiates the unfolded protein response (UPR). The UPR induces the transcriptional upregulation of multiple ER resident proteins involved in protein folding (for reviews, see references 20, 23, and 44). BiP/GRP78 is an abundant protein residing in the ER and essential for protein folding and protein sorting as a molecular chaperone. The structure of BiP is highly conserved from higher eukaryotes to yeast. In the yeast *Saccharomyces cerevisiae*, the BiP protein is encoded by *KAR2*. As in mammalian cells, the expression of *KAR2* in yeast cells is induced by a variety of treatments, such as the addition of tunicamycin, which causes the accumulation of the unfolded protein in the ER. *IRE1* encodes a bifunctional protein with transmembrane kinase and endoribonuclease activities that transmits the stress signal from the ER to the nucleus. The accumulation of the unfolded protein triggers Ire1p oligomerization, thereby inducing autophosphorylation, resulting in subsequent elicitation of the kinase and RNase activities. Activated Ire1p, together with the tRNA ligase encoded by *RLG1* and Ada5p, causes unconventional splicing of *HAC1* mRNA. *HAC1* mRNA splicing allows efficient translation of Hac1p, which has a basic leucine zipper domain and functions as a transcriptional factor for genes regulated by the UPR, such as *KAR2*, *PDI1*, and *FKB2*. Since Hac1p is necessary for *IRE1*-mediated *KAR2* induction as a positive transcription factor, mutants having a defect in *ire1* or *hac1* are unable to induce the transcription of *KAR2*, resulting in an inability of yeast cells to grow under stress conditions such as with the addition of tunicamycin.

The *IRE1* gene was first identified as the gene for inositol prototrophy (Ino$^+$) of *S. cerevisiae* (29), and the *HAC1* gene was isolated as a multicopy suppressor gene for the *ire1* mutation (26). Mutants having a defect in *ire1* or *hac1* show inositol auxotrophy (Ino$^-$) due to an inability to fully induce the expression of the *INO1* gene, which encodes a rate-limiting enzyme for inositol synthesis (4, 24, 26). In *S. cerevisiae*, inositol is synthesized de novo in cells through the conversion of glucose 6-phosphate to inositol 1-phosphate, followed by dephosphorylation (5). The former reaction is mediated by inositol 1-phosphate synthase encoded by *INO1* (6). Inositol is also taken up into cells in a carrier-mediated manner. *S. cerevisiae* possesses two distinct inositol transport systems. The major transport system is encoded by *ITR1*, and the minor one is encoded by *ITR2* (30, 31). It is known that the expression of *INO1* and *ITR1*, as well as a number of genes for enzymes involved in the synthesis of phospholipids in *S. cerevisiae*, is repressed in cells grown in the presence of inositol and derepressed in cells grown in the absence of inositol (32). *INO1* and other coregulated genes of phospholipid biosynthesis contain one or two stretches of a conserved *cis*-acting promoter element, termed the inositol-choline-responsive element (ICRE). The *INO2* and *INO4* genes encode basic helix-loop-helix proteins that form a heterodimer and function as a transcriptional factor through binding to the ICRE (1, 41). Mutants having a defect in not only *ino1* but also *ino2* or *ino4* exhibit the Ino$^-$ phenotype (10, 12). Several other mutants also show the Ino$^-$ phenotype. For example, mutations in the large subunit of RNA polymerase II (40) and the TATA binding protein (2, 43) lead to the Ino$^-$ phenotype due to an inability to express the *INO1* gene. Depletion of the general transcription factor TFIIA also impairs *INO1* activation (21). Cells having defects

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in the *SWI1*, *SWI2*, and *SWI3* genes, which encode components of the SWI-SNF chromatin-remodeling complex, exhibit a derepression defect of *INO1* (33–35). Furthermore, deletion of the *INO80* gene, which is an *SNF2-SWI2* paralogue and encodes a component of the *INO80* chromatin-remodeling complex, prevents the efficient expression of *INO1* (7, 42). On the other hand, mutations in the *SIN3* and *UME6* genes lead to high-level *INO1* expression (15, 16). The *SIN3* and *UME6* gene products are components of a large complex that contains the *RPD3* gene product, a histone deacetylase (17, 18, 39). Deletion of the *RPD3* gene also leads to high-level *INO1* expression. Additionally, a mutation in the *OPI1* gene that encodes a protein containing leucine zipper and polyglutamine stretch motifs leads to an inositol overproduction phenotype (47).

Little is known about the mechanism by which defects of the *IRE1* or *HAC1* gene lead to a decrease in *INO1* expression or about the mechanism by which inositol regulates *INO1* expression. In this study, we attempted to isolate and characterize the yeast gene that can suppress the Ino$^-$ phenotype of the Δ*hac1* strain when present in multiple copies. Here, we show that multiple copies of truncated *ITC1* can suppress the Ino$^-$ phenotype of the Δ*ire1* and Δ*hac1* strains and that the Isw2p-Itc1p complex usually represses *INO1* expression.

MATERIALS AND METHODS

Yeast strains and culture. *S. cerevisiae* strains D452-2 (*MAT*α *leu2 his3 ura3*), as the wild-type strain, YF4 (*MAT*α *leu2 his3 ura3 ire1*::*URA3*), and HU1 (*MAT*α *leu2 his3 ura3 hac1*::*URA3*) were described previously (26). Yeast cells were cultured aerobically in either yeast-peptone-dextrose or synthetic minimal medium with shaking at 30°C. The compositions of the yeast-peptone-dextrose and inositol-free minimal medium were as described previously (48). Inositol was added to the minimal medium at a concentration of 20 μg/ml. When necessary, L-leucine, L-histidine, and uracil were each added to the culture media at 20 μg/ml. Tunicamycin was added to the culture media at 0.5 μg/ml.

Plasmid construction. YCp50 (38) and YCpL2 (22) are centromere-based vectors with the *URA3* and *LEU2* genes as selectable markers, respectively. YCpH2 is a centromere-based vector with the *HIS3* gene as a selectable marker and was constructed as follows. The 1.8-kbp *Bam*HI fragment of the *HIS3* gene was inserted into the *Bam*HI site of pUC19 to yield pUC-HIS3. The 2.3-kbp *Eco*RI/*Sma*I fragment of YCp50 was replaced with the 1.8-kbp *Eco*RI/*Hin*cII fragment of pUC-HIS3 to yield YCpH2. YEpM4 (28) and pHV-1 (37) are 2μm DNA-based vectors with the *LEU2* and *HIS3* genes as selectable markers, respectively. pADANS is a 2μm DNA-based vector with the yeast *ADH1* promoter, the following small part of the coding region, the *ADH1* terminator, and the yeast *LEU2* gene (3). The *LEU2* gene of pADANS was replaced with the *LEU2* gene of pGAD424 (Clontech). The *LEU2* gene in pADANSΔE thus obtained has no *Eco*RI site. Plasmid pIR42, harboring *HAC1*, was described previously (25). To construct a single-copy plasmid carrying the *HAC1* gene, pIR42 was digested with *Bam*HI and *Sma*I. An approximately 3.5-kbp fragment containing the *HAC1* gene was ligated between the *Bam*HI and *Sma*I sites of YCpL2 to yield YCpL2-42. Plasmid YEp133t containing a truncated form of *ITC1* was originally isolated from a yeast genomic library which was described previously (28). To construct a multicopy plasmid carrying a truncated form of *PCL10*, an approximately 1.8-kbp *Hin*dIII/*Sca*I fragment from YEp133t was ligated between the *Hin*dIII and *Sca*I sites of YEpM4 to yield YEpPCL10. To construct a single-copy plasmid containing the truncated form of *ITC1*, an approximately 4.2-kbp *Bam*HI/*Xba*I fragment from YEp133t was ligated between the *Bam*HI and *Xba*I sites of YCpL2 to yield YCpL2-133t.

To construct a multicopy plasmid containing full-length *ITC1*, *ITC1* was amplified by PCR with chromosomal DNA as a template. The PCR primers used were 5′-CAATGGTGTTATATAAAAGG-3′ and 5′-GTATGGTCCAATCTTG CGCG-3′. An approximately 3.9-kbp PCR product was inserted into vector pCR2.1-TOPO (Invitrogen) to yield pTA-ITC1. pTA-ITC1 was digested with *Nco*I and *Sac*I, and an approximately 1.7-kbp fragment containing the 3′ region of *ITC1* was separated by electrophoresis. YEp133t was digested with *Nco*I and *Sac*I and subjected to electrophoresis to remove the small fragment, and then the fragment containing the 3′ region of *ITC1* described above was inserted between the *Nco*I and *Sac*I sites of YEp133t to yield YEp133w. The sequence of the 3′ region of *ITC1* amplified by PCR was verified. To construct a single-copy plasmid containing full-length *ITC1*, YEp133w was digested with *Bam*HI and *Xba*I. An approximately 5.2-kbp fragment containing full-length *ITC1* was ligated between the *Bam*HI and *Xba*I sites of YCpL2 to yield YCpL2-133w. To construct a multicopy plasmid containing *ITC1* deletion derivatives, an approximately 3.6-kbp *Hin*dIII/*Eco*RV fragment of YEp133w was inserted between the *Hin*dIII and *Sma*I sites of YEpM4 to yield YEp133Δ1. An approximately 3.9-kbp *Hin*dIII fragment of YEp133w was inserted into the *Hin*dIII site of YEpM4 to yield YEp133Δ2. To construct single-copy plasmids containing the *INO1-lacZ* fusion gene, YEpINO1Z (13) was digested with *Hin*dIII and *Sma*I. An approximately 4-kbp *Hin*dIII/*Sma*I fragment containing the *INO1-lacZ* fusion gene was treated with Klenow large fragment and then inserted into the *Sma*I sites of YCpH2 and YCpL2 to yield YCpH2-INO1Z and YCpL2-INO1Z, respectively. To construct a single-copy plasmid containing the *ISW2* gene, pISW2w (see below) was digested with *Spe*I. An approximately 6.1-kbp fragment containing the *ISW2* gene was inserted into the *Xba*I site of YCpL2 to yield YCpL2-ISW2. To construct a multicopy plasmid that expresses Isw2p under the control of the *ADH1* promoter, the *ISW2* gene was amplified by PCR with chromosomal DNA as a template. The PCR primers used were 5′-TCATGACAGCCCAGCAAG-3′ and 5′-GCTTCTTGATCAATTTTG-3′. An approximately 3.3-kbp PCR product was inserted into pCR2.1-TOPO to yield pTA-ISW2. pTA-ISW2 was digested with *Spe*I and *Xho*I, and an approximately 3.3-kbp *Spe*I/*Xho*I fragment containing the *ISW2* gene was inserted between the *Spe*I and *Xho*I sites of pBluescript II KS(+) to yield pKS+ISW2. pKS+ISW2 was digested with *Not*I, and an approximately 3.3-kbp *Not*I fragment containing the *ISW2* gene was inserted into the *Not*I site of pADANSΔE to yield pAD-ISW2. pAD-ISW2 was digested with *Bam*HI, and an approximately 5.3-kbp *Bam*HI fragment containing the *ADH1-ISW2* fusion gene was inserted into the *Bam*HI site of pUC18 to yield pUC18-ISW2. An approximately 5.3-kbp *Sal*I/*Sma*I fragment of pUC18-ISW2 containing the

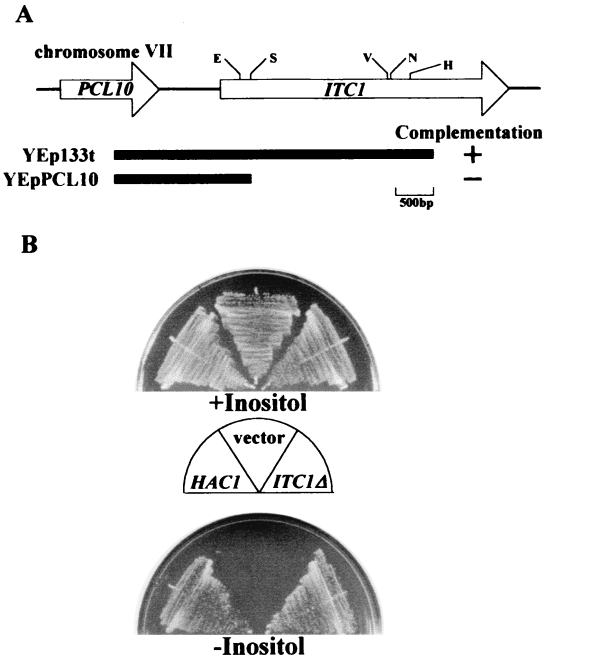

FIG. 1. Cloning of the suppressor gene. (A) Restriction map of the genomic region of *S. cerevisiae* containing the suppressor gene. The size and transcriptional orientation of each ORF are shown by arrows. Solid bars indicate the inserts of originally isolated plasmid YEp133t and its subclone, YEpPCL10. +, ability to complement the Ino$^-$ phenotype of the Δ*hac1* strain; −, inability to complement it. Abbreviations: E, *Eco*T22I; H, *Hin*dIII; N, *Nco*I; S, *Sca*I; V, *Eco*RV. (B) Suppression of the Ino$^-$ phenotype of the Δ*hac1* strain by *ITC1*Δ. The Δ*hac1* strain, HU1, harboring YCpL2-42 (*HAC1*), YEp133t (*ITC1*Δ), or vector YEpM4 alone was streaked onto minimal medium containing histidine with or without inositol. The transformants were grown at 30°C for 3 days.

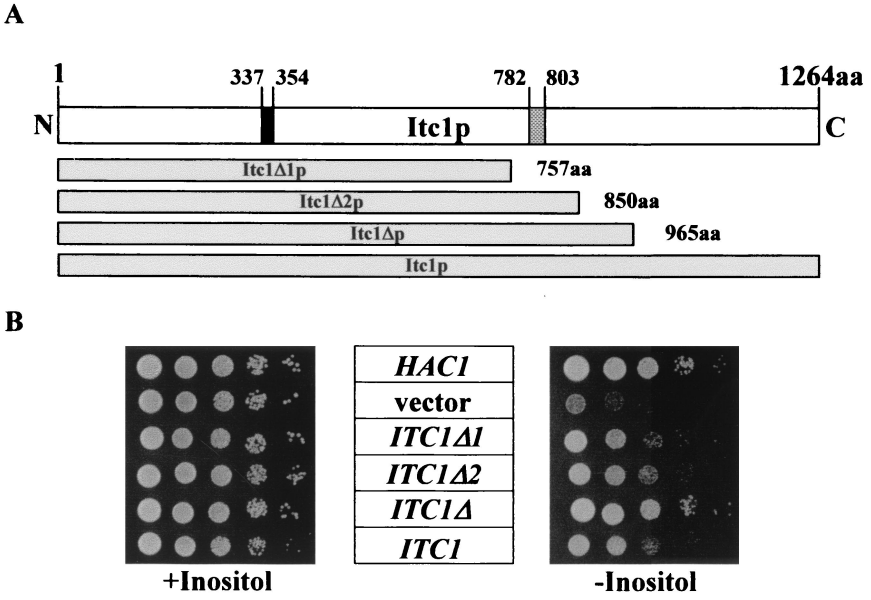

FIG. 2. Deletion analysis of *ITC1*. (A) Linear diagram of Itc1p with a schematic representation of the deletion derivatives. The nuclear localization signal and leucine zipper domain within Itc1p are shown as solid and hatched boxes, respectively. Amino acid numbers from the initiation methionine are shown at the top. The bars show the approximate sizes of the truncated forms of Itc1p, with the number of amino acids (aa) at the right of each bar. (B) Suppression of the Ino$^-$ phenotype of the Δ*hac1* strain by *ITC1* deletion derivatives. HU1 harboring YCpL2-42 (*HAC1*), YEp133Δ1 (*ITC1Δ1*), YEp133Δ2 (*ITC1Δ2*), YEp133t (*ITC1*Δ), YEp133w (*ITC1*), or vector YEpM4 alone was 10-fold serially diluted and then spotted onto minimal medium containing histidine with or without inositol. The transformants were grown at 30°C for 3 days.

ADH1-ISW2 fusion gene was inserted between the *Sal*I and *Sma*I sites of pHV1 to yield pHV-ADISW2.

Isolation of the wild-type *ISW2* gene. For isolation of the wild-type *ISW2* gene, genomic DNA from D452-2 was digested with *Bgl*II and *Xho*I, and then approximately 8-kbp fragments were separated by gel electrophoresis and inserted between the *Bam*HI and *Xho*I sites of pBluescript II KS(+). *Escherichia coli* cells were transformed with the ligation mixture. A transformant harboring the yeast *ISW2*-containing plasmid was detected by the PCR method using the synthetic primers described above, and the plasmid pISW2w was recovered from the transformant.

Construction of gene-disrupted strains. To construct the Δ*itc1*, Δ*isw2*, Δ*hac1* Δ*itc1*, and Δ*hac1* Δ*isw2* strains, *HIS3*-disrupted *ITC1* and *HIS3*-disrupted *ISW2* gene fragments were constructed by the method for the synthesis of marker-disrupted alleles of yeast genes (27). The PCR primers used for *ITC1* disruption were 5′-CAATGGTGTTATATAAAAGG-3′, 5′-GTGTCTCCTCACTATCCAG-3′, 5′-CTGGTTAGATAATTGGGG-3′, and 5′-CCTCGCGCCTGGCCTCTG-3′. The PCR primers used for *ISW2* disruption were 5′-TCATGACGACCCAGCAAG-3′, 5′-GTACGTATCGGACTTGTC-3′, 5′-GAGGCAGAAAATCGAACAG-3′, and 5′-GCTTCTTGATCAATTTTG-3′. The *HIS3*-disrupted gene fragments were used for the transformation of D452-2 and HU1. His$^+$ colonies were selected, and gene disruption was confirmed by PCR with their chromosomal DNA as a template. The Δ*itc1*, Δ*isw2*, Δ*hac1* Δ*itc1*, and Δ*hac1* Δ*isw2* strains thus obtained were designated IH-1, SH-1, HIW, and HSW, respectively.

β-Galactosidase assay. β-Galactosidase was assayed at 37°C by measuring the increase in the absorbance at 420 nm with *o*-nitrophenyl-β-D-galactoside as the substrate after cells had been permeabilized with chloroform and sodium dodecyl sulfate as described previously (14).

Northern blot analysis. For Northern blot analysis, total RNA was isolated from yeast cells as described by Kataoka et al. (19). Samples were subjected to electrophoresis in a 1% agarose gel containing formaldehyde, blotted onto a Biodyne A membrane (Pall BioSupport), and then hybridized. The probes used were ^{32}P-labeled DNA fragments (with BcaBEST Labeling Kit [Takara Biochemicals]) of the entire coding regions of *INO1* and *ACT1*, which were prepared by PCR. Hybridization and detection were carried out according to the manufacturer's manual.

Microscopic analysis. To investigate cell morphology, cells were grown to the early log phase in minimal medium containing inositol. Images were taken under a confocal laser scanning microscope (LSM510; Carl Zeiss).

RESULTS

Isolation of the suppressor gene. Disruption of *IRE1* or *HAC1* in *S. cerevisiae* results in the Ino$^-$ phenotype (4, 26). To elucidate the mechanism underlying the Ino$^-$ phenotype caused by *ire1* or *hac1* disruption, we attempted to isolate yeast suppressor genes that, when present in multiple copies, can suppress the Ino$^-$ phenotype of the Δ*hac1* strain. The Δ*hac1* strain, HU1, was transformed with a yeast genomic library constructed on a multicopy vector, with transformants that grew on inositol-free minimal medium being selected. Plasmids were isolated from the independent transformants. After retransformation of plasmids into yeast cells, four plasmids were found to be able to complement the Δ*hac1* strain. Restriction endonuclease analysis and sequence analysis revealed that all four plasmids were identical and contained 4.2-kbp inserts. We designated these plasmids YEp133t and used them for further analysis. As shown in Fig. 1A, the insert fragment in plasmid YEp133t was derived from chromosome VII and included two truncated open reading frames (ORFs), *PCL10* (*YGL132w*) and *ITC1* (*YGL133w*). However, the coding region of *PCL10* is incomplete and its promoter region is missing in plasmid YEp133t. The subcloning study indicated that the Δ*hac1* strain transformed with multicopy plasmid YEpPCL10 (Fig. 1A), which contains the 1.8-kbp *Hin*dIII/*Sca*I fragment derived from the insert fragment of YEp133t, exhibited the Ino$^-$ phenotype. Therefore, we concluded that *ITC1* but not *PCL10* suppresses the Ino$^-$ phenotype of the Δ*hac1* strain. The *ITC1* present in plasmid YEp133t is the truncated form and lacks the 3′ region of the ORF. We designated this truncated gene *ITC1*Δ. Plasmid YEp133t clearly suppressed the

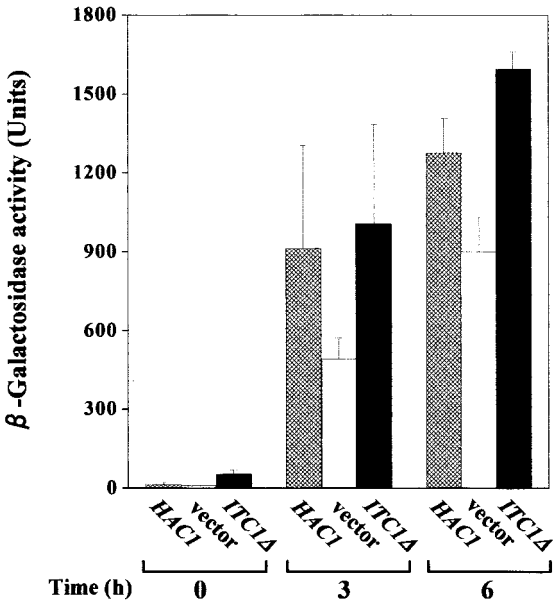

FIG. 3. Effect of *ITC1*Δ on *INO1* expression. The Δ*hac1* strain harboring YCpH2-INO1Z carrying the *INO1-lacZ* fusion gene together with YCpL2-42 (*HAC1*), YEp133t (*ITC1*Δ), or vector YEpM4 was precultured in minimal medium containing inositol for 12 h. The cells were then washed twice with water and cultured further in fresh minimal medium without inositol. At the indicated times, cells were removed from the cultures and β-galactosidase activity was measured. Data are means of three independent transformants. Error bars indicate standard deviations.

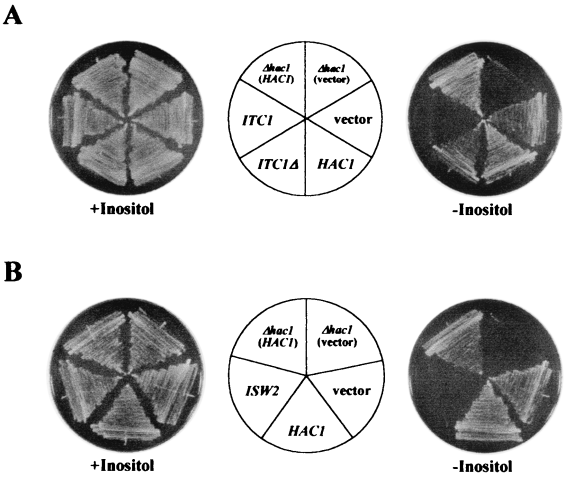

FIG. 4. Phenotypes of the Δ*hac1* Δ*itc1* and Δ*hac1* Δ*isw2* strains. (A) Ino$^+$ phenotype of the Δ*hac1* Δ*itc1* strain. The Δ*hac1* Δ*itc1* strain, HIW, harboring YCpL2-42 (*HAC1*), YCpL2-133t (*ITC1*Δ), YCpL2-133w (*ITC1*), or vector YCpL2 alone was grown on minimal medium with or without inositol at 30°C for 3 days. (B) Ino$^+$ phenotype of the Δ*hac1* Δ*isw2* strain. The Δ*hac1* Δ*isw2* strain, HSW, harboring YCpL2-42 (*HAC1*), YCpL2-ISW2 (*ISW2*), or vector YCpL2 alone was grown on minimal medium with or without inositol at 30°C for 3 days. In both panels, the Δ*hac1* strain, HU1, harboring vector YCpH2 together with YCpL2-42 or vector YCpL2 is also shown, indicated by Δ*hac1* (*HAC1*) and Δ*hac1* (vector), respectively.

Ino$^-$ phenotype of the Δ*hac1* strain on inositol-free minimal medium (Fig. 1B). Overexpression of *ITC1*Δ also suppressed the Ino$^-$ phenotype of the Δ*ire1* strain. However, overexpression of *ITC1*Δ could not restore the tunicamycin sensitivity of the Δ*ire1* and Δ*hac1* strains (data not shown). To determine whether a single copy of *ITC1*Δ could also suppress the Ino$^-$ phenotype of the Δ*ire1* and Δ*hac1* strains, a *CEN4*-based plasmid harboring *ITC1*Δ, YCpL2-133t, was constructed and introduced into the Δ*ire1* and Δ*hac1* strains. The transformants obtained showed the Ino$^-$ phenotype, indicating that the suppression of the Ino$^-$ phenotype of the Δ*ire1* and Δ*hac1* strains is caused by the gene dosage effect of *ITC1*Δ (data not shown).

Overexpression of full-length *ITC1* in a Δ*hac1* strain. *ITC1* encodes a protein of 1,264 amino acid residues. The *ITC1* product, Itc1p, contains a nuclear localization signal and a leucine zipper domain, a motif frequently found in DNA-binding proteins (8). The originally isolated *ITC1*Δ encodes a truncated protein, Itc1Δp, which lacks 299 amino acids at the C-terminal end (Fig. 2A). We next examined whether full-length *ITC1* can suppress the Ino$^-$ phenotype of the Δ*hac1* strain. For this purpose, we constructed multicopy plasmid YEp133w carrying full-length *ITC1* (see Materials and Methods) and introduced it into the Δ*hac1* strain, HU1. Interestingly, full-length *ITC1* suppressed the Ino$^-$ phenotype of the Δ*hac1* strain much more weakly than *ITC1*Δ (Fig. 2B). This revealed that deletion of the C-terminal region of Itc1p is important for efficient suppression of the Ino$^-$ phenotype of the Δ*hac1* strain.

Deletion analysis of *ITC1*. The C-terminal region of Itc1p missing in Itc1Δp does not contain any obvious amino acid motif. To investigate the functional significance of the C-terminal region of Itc1p in suppression of the Ino$^-$ phenotype of the Δ*hac1* strain, we next constructed multicopy plasmids carrying a series of *ITC1* deletion derivatives (Fig. 2A). Plasmids YEp133Δ1 and YEp133Δ2 carry *ITC1Δ1* and *ITC1Δ2*, respectively. *ITC1Δ1* and *ITC1Δ2* encode truncated proteins comprising amino acid residues 1 to 757 and 1 to 850, respectively. After transformation of these plasmids into the Δ*hac1* strain, HU1, the transformants were examined for their ability to suppress the Ino$^-$ phenotype of this strain. As shown Fig. 2B, all the transformants grew at approximately the same rate on inositol-containing medium. However, on inositol-free medium, only the transformant having multiple copies of *ITC1*Δ exhibited the Ino$^+$ phenotype similarly to the transformant having a single copy of *HAC1*. In contrast, *ITC1Δ1* and *ITC1Δ2* suppressed the Ino$^-$ phenotype of the Δ*hac1* strain much more weakly than *ITC1*Δ did. This indicates that the deletion of about 299 amino acids at the C-terminal end of Itc1p is critical for suppression of the Ino$^-$ phenotype of the Δ*hac1* strain, but further deletion abolished the ability of the suppression. Taken together, the above results suggest that the C-terminal region of Itc1p has an inhibitory effect on suppression of the Ino$^-$ phenotype of the Δ*hac1* strain and that the function of Itc1p might be regulated by its C-terminal region.

Effect of overexpression of *ITC1*Δ on *INO1* expression. We next determined the effect of multiple copies of *ITC1*Δ on *INO1* expression using the *INO1-lacZ* fusion gene as a reporter gene. Plasmid YCpH2-INO1Z carrying the *INO1-lacZ* fusion gene was introduced into the Δ*hac1* strain, HU1, together with a single copy of *HAC1*, multiple copies of *ITC1*Δ, or vector

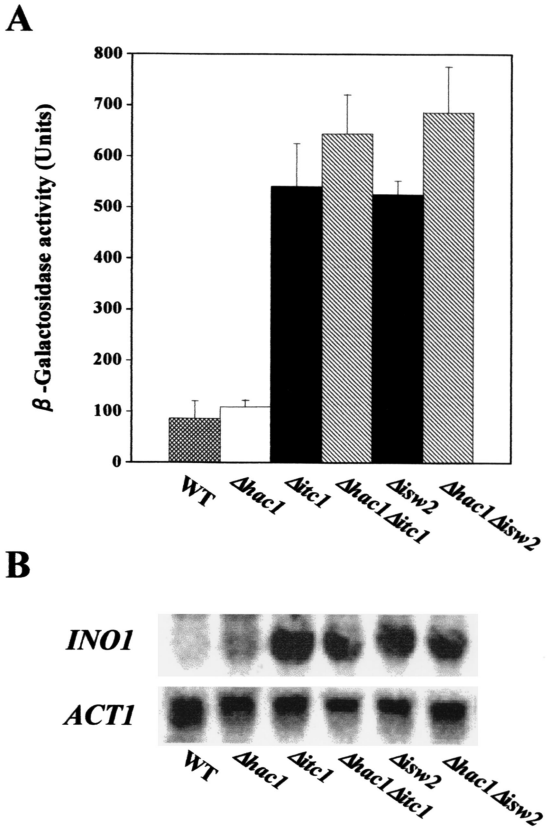

FIG. 5. *INO1* expression in Δ*itc1*, Δ*isw2*, Δ*hac1* Δ*itc1*, and Δ*hac1* Δ*isw2* strains. (A) Reporter gene analysis of *INO1* expression. Strains D452-2 (WT), HU1 (Δ*hac1*), IH-1 (Δ*itc1*), HIW (Δ*hac1* Δ*itc1*), SH-1 (Δ*isw2*), and HSW (Δ*hac1* Δ*isw2*) were transformed with YCpL2-INO1Z carrying the *INO1-lacZ* fusion gene. The transformants were precultured in minimal medium containing inositol for 12 h. The cells were then washed twice with water and cultured further in fresh minimal medium containing inositol. After the cells had reached the early log phase, β-galactosidase activity was measured. Data are means for four independent transformants. Error bars indicate standard deviations. (B) Northern blot analysis of *INO1* mRNA. Strains D452-2 (WT), HU1 (Δ*hac1*), IH-1 (Δ*itc1*), HIW (Δ*hac1* Δ*itc1*), SH-1 (Δ*isw2*), and HSW (Δ*hac1* Δ*isw2*) were grown to the early log phase in minimal medium with inositol. Total RNA was isolated and used for Northern blot analysis (25 μg per lane).

plasmid YEpM4. The transformants were grown in the absence of inositol, and then β-galactosidase activity was measured (Fig. 3). Consistent with a previous report (24), the level of β-galactosidase activity in the Δ*hac1* strain containing the vector alone was lower than that observed in the Δ*hac1* strain containing a single copy of *HAC1*. In contrast, the level of β-galactosidase activity in the Δ*hac1* strain transformed with multiple copies of *ITC1*Δ was higher than that observed in the Δ*hac1* strain transformed with the vector alone. These results indicate that the overexpression of *ITC1*Δ induces the transcriptional upregulation of *INO1*. This is why the Δ*hac1* strain transformed with YEp133t shows the Ino^+ phenotype.

Phenotype of the Δ*hac1* Δ*itc1* strain. We next determined the effect of the deletion of *ITC1* on cell growth. We first constructed a Δ*itc1* strain, IH-1, and its phenotype was examined. The Δ*itc1* strain did not show the Ino^- phenotype and, as reported previously (8), exhibited aberrant morphology (see below). We next constructed a Δ*hac1* Δ*itc1* strain, HIW. The Δ*hac1* Δ*itc1* strain also exhibited aberrant morphology (data not shown). Surprisingly, the Δ*hac1* Δ*itc1* strain did not show the Ino^- phenotype (Fig. 4A). To confirm this, a single copy of *ITC1* on a *CEN4*-based vector was introduced into the Δ*hac1* Δ*itc1* strain and then its phenotype was examined. The transformants clearly exhibited the Ino^- phenotype, similar to the Δ*hac1* strain (Fig. 4A). This indicates that the deletion of *ITC1* can suppress the Ino^- phenotype of the Δ*hac1* strain, suggesting that Itc1p is a negative regulator for the expression of *INO1*. However, the Δ*hac1* Δ*itc1* strain carrying a single copy of *ITC1*Δ did not exhibit the Ino^- phenotype (Fig. 4A), suggesting that only full-length *ITC1* can function as a negative regulator for the expression of *INO1*.

Phenotype of the Δ*hac1* Δ*isw2* strain. Recently, it was reported that Itc1p, together with a gene product of *ISW2* (45), constitutes a chromatin-remodeling complex (http://genome-www.stanford.edu/Saccharomyces/). Therefore, we next determined the effect of Isw2p on the expression of *INO1*. For this purpose, we constructed a Δ*hac1* Δ*isw2* strain, HSW. This strain exhibited aberrant morphology, similar to that of the Δ*hac1* Δ*itc1* strain (data not shown), and also exhibited the Ino^+ phenotype. To confirm this more exactly, the Δ*hac1* Δ*isw2* strain was transformed with a single copy of *HAC1*, a single copy of *ISW2*, or vector plasmid YCpL2. The transformants were grown on minimal medium with or without inositol, and then the phenotype was examined (Fig. 4B). The Δ*hac1* Δ*isw2* strain carrying the vector alone exhibited the Ino^+ phenotype. In contrast, the Ino^- phenotype reappeared when a single copy of *ISW2* was introduced into the Δ*hac1* Δ*isw2* strain. This clearly suggests that Itc1p, together with Isw2p, functions as a negative regulator of *INO1*. It should be noted that the Δ*isw2* strain we constructed also exhibited the Ino^+ phenotype and aberrant morphology, similar to the Δ*itc1* strain (see below).

The Isw2p-Itc1p complex represses *INO1* expression. The data presented above strongly suggest that the Isw2p-Itc1p chromatin-remodeling complex represses the expression of *INO1*. To examine this possibility further, we introduced single-copy plasmid YCpL2-INO1Z carrying the *INO1-lacZ* fusion gene into strains HU1 (Δ*hac1*), IH-1 (Δ*itc1*), HIW (Δ*hac1* Δ*itc1*), SH-1 (Δ*isw2*), HSW (Δ*hac1* Δ*isw2*), and D452-2 (wild type). The transformants were cultured in inositol-containing minimal medium, and then β-galactosidase activity was measured. As shown in Fig. 5A, the wild-type and Δ*hac1* cells exhibited low levels of β-galactosidase activity, indicating that the expression of *INO1* is repressed by inositol in the medium. In contrast, the levels of β-galactosidase activity in the Δ*itc1*, Δ*hac1* Δ*itc1*, Δ*isw2*, and Δ*hac1* Δ*isw2* cells were much higher than those observed in the wild-type and Δ*hac1* cells, strongly suggesting that the expression of *INO1* is derepressed in these cells even in the presence of inositol in the medium. To confirm this, we next determined the abundance of mRNA of *INO1* in disruptants by Northern blot analysis. The wild-type, Δ*hac1*, Δ*itc1*, Δ*hac1* Δ*itc1*, Δ*isw2*, and Δ*hac1* Δ*isw2* strains were grown to the early log phase in minimal medium containing inositol and then subjected to analysis (Fig. 5B). Consistent with the results obtained for the *INO1-lacZ* fusion gene, the amount of *INO1* mRNA was low in the wild-type and Δ*hac1*

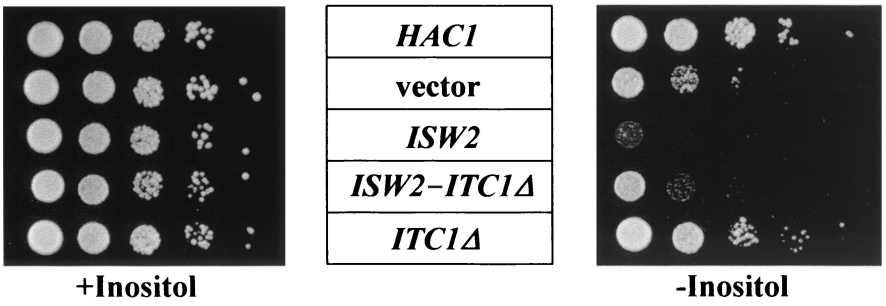

FIG. 6. Effects of overexpression of *ITC1* and *ISW2* on *INO1* expression. The Δ*hac1* strain, HU1, harboring pHV-ADISW2 plus vector YEpM4 (*ISW2*), vector pHV-1 plus YEp133t (*ITC1*Δ), pHV-ADISW2 plus YEp133t (*ISW2-ITC1*Δ), vector pHV-1 plus YCpL2-42 (*HAC1*), or vector pHV-1 plus vector YEpM4 (vector) was 10-fold serially diluted and then spotted onto minimal medium with or without inositol. The transformants were grown at 30°C for 3 days.

cells, whereas the Δ*itc1*, Δ*hac1* Δ*itc1*, Δ*isw2*, and Δ*hac1* Δ*isw2* cells showed high levels of *INO1* mRNA. These data indicate that the Isw2p-Itc1p chromatin-remodeling complex represses the *INO1* expression in the wild-type and Δ*hac1* cells, and the disruption of either *ITC1* or *ISW2* abolishes the function of the chromatin-remodeling complex, resulting in derepression of the *INO1* expression. This is why the Δ*hac1* Δ*itc1* and Δ*hac1* Δ*isw2* strains can grow on inositol-free medium.

Effects of overexpression of *ITC1*Δ and *ISW2* on the Ino⁻ phenotype. We next determined the effects of overexpression of *ITC1* and *ISW2* on the Ino⁻ phenotype of the Δ*hac1* strain. The Δ*hac1* strain was transformed with multiple copies of *ISW2*, multiple copies of *ITC1*Δ, or both. In the case of multiple copies of *ISW2*, we used a construct in which the expression of *ISW2* is under the control of the *ADH1* promoter. The transformants were grown on minimal medium with or without inositol (Fig. 6). As shown above, growth of the Δ*hac1* strain carrying multiple copies of *ITC1*Δ was similar to that of the strain having the *HAC1* gene. However, the Δ*hac1* strain carrying multiple copies of both *ISW2* and *ITC1*Δ showed the Ino⁻ phenotype, similar to the Δ*hac1* strain carrying vectors. Furthermore, the introduction of multiple copies of *ISW2* into the Δ*hac1* strain resulted in a stronger Ino⁻ phenotype, suggesting that the overexpression of *ISW2* causes severe repression of *INO1*. On the other hand, introduction of multiple copies of *ITC1* had little effect on the Ino⁻ phenotype of the Δ*hac1* strain, as described above. Multiple copies of *ITC1*, whose expression is under the control of the *ADH1* promoter, also had little effect (data not shown). It seems likely that the activity of the Isw2p-Itc1p complex is regulated by the amount of Isw2p.

Aberrant morphology of the Δ*itc1* and Δ*isw2* strains. It has been reported that the deletion of *ITC1* in α-type cells causes aberrant morphology resembling that of cells exposed to mating factors. This phenotype is not observed for *MAT***a** cells (8). This aberrant morphology is confirmed by the data shown in Fig. 7. Furthermore, we also found that the Δ*isw2* strain shows a similar aberrant morphology. We confirmed that a single copy of *ISW2* could reverse the aberrant morphology of the Δ*isw2* strain. This strongly suggests that the Isw2p-Itc1p chromatin-remodeling complex plays a critical role in maintenance of the morphology of cells. We next examined the effect of the truncated form of *ITC1* on cell morphology. The Δ*itc1* strain, IH-1, was transformed with a series of *ITC1* deletion derivatives constructed on a multicopy plasmid (Fig. 2), and the cell morphology of the transformants was observed. As shown in Fig. 7, the Δ*itc1* and Δ*isw2* cells exhibited aberrant morphology (29 and 26%, respectively). A small number of the Δ*itc1* cells exhibited the aberrant morphology when transformed with full-length *ITC1* (3%). On the other hand, the transformants of *ITC1*Δ*1* and *ITC1*Δ each included a moderate number of the aberrant cells (14 and 6%, respectively). It seems likely that the C-terminal region of Itc1p is critical for the repression of *INO1* but not for maintenance of morphology.

DISCUSSION

In this study, we isolated a truncated form of *ITC1*, *ITC1*Δ, as a suppressor gene for the Ino⁻ phenotype of the Δ*hac1* strain. From the results of the reporter gene assay (Fig. 3), multiple copies of *ITC1*Δ were found to be able to derepress *INO1* expression and circumvent the inositol requirement for growth of the Δ*hac1* strain (Fig. 2B). However, overexpression of the full-length *ITC1* had a moderate effect on the growth of the Δ*hac1* strain. *ITC1* encodes a protein of 1,264 amino acids which is a component of the chromatin-remodeling complex (45). The gene product of *ITC1*Δ we obtained lacks 299 amino acids at the C-terminal end. The truncated form further truncated at the C terminus suppressed the Ino⁻ phenotype of the Δ*hac1* strain more weakly than *ITC1*Δ, suggesting that the length of the C-terminal region of Itc1p is critical for the derepression of *INO1* expression. Furthermore, deletion of the chromosomal *ITC1* gene in the Δ*hac1* strain also suppressed the Ino⁻ phenotype of the Δ*hac1* strain (Fig. 4A). Introduction of a single copy of *ITC1* into the Δ*hac1* Δ*itc1* strain gave it the Ino⁻ phenotype again, but *ITC1*Δ did not. These results indicate that Itc1p is a negative regulator for *INO1* expression and that the truncated form of Itc1p, Itc1Δp, competes with the effect of full-length Itc1p.

Itc1p and Isw2p form a chromatin-remodeling complex (45). This complex possesses nucleosome-stimulated ATPase and ATP-dependent nucleosome spacing activities. Isw2p exhibits ATPase activity. Therefore, we predicted that the disruption of *ISW2* could also derepress the expression of *INO1* and suppress the Ino⁻ phenotype of the Δ*hac1* strain, and we found that this is the case. As shown in this study, deletion of not only

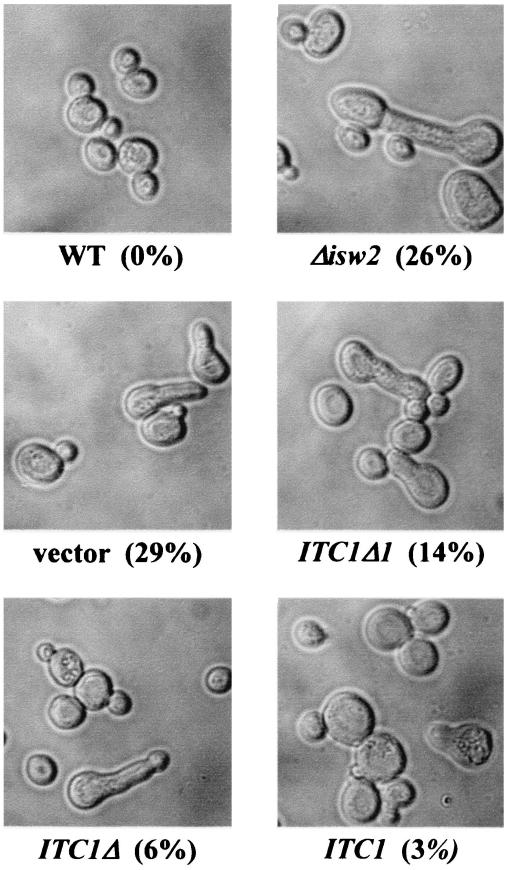

FIG. 7. Aberrant morphology of Δ*itc1* and Δ*isw2* strains. The Δ*itc1* strain, IH-1, transformed with either YEp133Δ1 (*ITC1Δ1*), YEp133t (*ITC1*Δ), YEp133w (*ITC1*), or vector YEpM4 alone and strains D452-2 (WT) and SH-1 (Δ*isw2*) were grown to the early log phase in minimal medium with inositol. Cell morphology was observed microscopically. Values in parentheses are percentages of morphologically aberrant cells determined under a microscope.

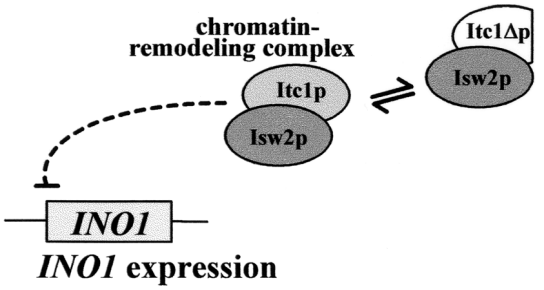

FIG. 8. Model of Itc1Δp-mediated *INO1* derepression. The Isw2p-Itc1p chromatin-remodeling complex represses the expression of *INO1*. However, overexpression of Itc1Δp causes depletion of the active form of Isw2p from the Isw2p-Itc1p complex responsible for the *INO1* repression, resulting in derepression of *INO1*. Although the Isw2p-Itc1Δp complex cannot repress the *INO1* expression, the Isw2p-Itc1p chromatin-remodeling complex with additionally overexpressed Isw2p restores the *INO1* repression. In this model, the C-terminal region of Itc1p plays a crucial role in the *INO1* repression.

ITC1 but also *ISW2* in the wild-type and Δ*hac1* strains caused the derepression of *INO1* expression even in the presence of inositol in the culture medium (Fig. 5). Inositol is well known to repress the expression of *INO1*. The Δ*hac1* Δ*isw2* strain can grow on inositol-free minimal medium (Fig. 4B). Conversely, introduction of multiple copies of *ISW2*, whose expression is under the control of the *ADH1* promoter, into the Δ*hac1* strain caused a severer Ino$^-$ phenotype. Multiple copies of *ITC1* of similar construction had no effect (data not shown). These results indicate that the amount of Isw2p limits the formation of the Isw2p-Itc1p complex.

During the preparation of this paper, data showing that the Isw2p-Itc1p chromatin-remodeling complex represses the expression of *INO1* were reported by Goldmark et al. (9). By determining the levels of *INO1* mRNA, they showed that the expression of *INO1* is partially derepressed in the Δ*isw2* strain. They also revealed that the Isw2p-Itc1p chromatin-remodeling complex represses early meiotic genes during mitotic growth in a pathway parallel to that of the Rpd3p-Sin3p histone deacetylase complex and that the repressor function of the Isw2p-Itc1p complex is largely dependent on Ume6p, a sequence-specific DNA-binding protein. Their findings that the Isw2p-Itc1p chromatin-remodeling complex represses *INO1* expression and that the disruption of *ISW2* abolishes the function of the remodeling complex are consistent with the results we obtained in this study.

Taken together, these results suggest that the Isw2p-Itc1p chromatin-remodeling complex usually represses *INO1* expression (Fig. 8). The amount of Isw2p is limited. Overexpression of *ITC1*Δ leads to the accumulation of the truncated form of Itc1p, Itc1Δp. Hence, a large amount of Itc1Δp deprives the Isw2-Itc1p chromatin-remodeling complex of Isw2p, resulting in derepression of the *INO1* expression. In this model, it is strongly suggested that the C-terminal region of Itc1p has a regulatory effect on the Itc1p function. Itc1Δp behaves in a dominant negative manner toward Itc1p. Consistent with this idea, Goldmark et al. noticed that there are two types of Itc1p in yeast cells and that the electrophoretically slow-migrating one preferentially interacts with Ume6p. These two species might be generated through modification of the C-terminal region of Itc1p. The truncated form of Itc1p encoded by *ITC1*Δ that we obtained might have no ability to bind to Ume6p, so the chromatin-remodeling function was lost. According to their data, the derepression of *INO1* caused by the deletion of *ISW2* is moderate compared to that caused by the deletion of *RPD3*, *SIN3*, or *UME6* (2.8-, 17-, 48-, and 117-fold, respectively). As described above, we showed that the disruption of not only *ISW2* but also *ITC1* restores the cell growth of the Δ*hac1* strain, which otherwise cannot grow on inositol-free medium. Therefore, our data clearly suggest that a decrease in the activity of the Isw2p-Itc1p chromatin-remodeling complex is sufficient to overcome the Ino$^-$ phenotype caused by the defect of *IRE1-HAC1*-mediated signaling. It is conceivable that the level of *INO1* expression is positively and negatively regulated through the *IRE1-HAC1*-mediated activation pathway and the Isw2p-Itc1p complex-mediated repression pathway, respectively. However, the relationship between these positive and negative pathways is still unknown. The expression of the *INO1* gene is known to be facilitated by the SWI-SNF and ADA-GCN5 complexes (35, 36). The gene product of *IRE1* interacts with some component of the ADA-GCN5 complex and thereby regulates the function of *HAC1* (20, 46). But so

far, the mechanisms by which these complexes regulate the *INO1* expression have not been elucidated precisely.

It has been reported that Δ*itc1* cells show aberrant morphology (8). We also found that the deletion of *ISW2* results in morphologic changes similar to those observed in the Δ*itc1* strain (Fig. 7). This clearly suggests that the Isw2p-Itc1p complex is responsible for maintaining the normal morphology of yeast cells. It has been reported that Δ*ino2* cells exhibit aberrant morphology, similar to Δ*itc1* and Δ*isw2* cells (11). The expression of *INO2* is known to be regulated similarly to that of *INO1*. The Isw2p-Itc1p complex might regulate *INO2* expression and maintain the morphology of the yeast cells via Ino2p. Judging from the results obtained here with the *ITC1* deletion derivatives, the morphologic abnormality was reversed upon the introduction of a series of the *ITC1* gene, depending upon its length. However, in contrast to the effect on the *INO1* expression, the overexpression of *ITC1*Δ, and even *ITC1Δ1*, partially but moderately suppressed the aberrant morphology of the Δ*itc1* strain. This suggests that the mechanism for the suppression of the aberrant morphology is different from that for the suppression of *INO1* expression and that the regulatory function of the C-terminal region of Itc1p is different in these two forms of suppression. As described above, early meiotic genes are known to be derepressed in the Δ*isw2* strain during mitotic growth. However, the relationship between the mating type-specific morphologic change observed here and the derepression of early meiotic genes is unknown.

We show here that the expression of *INO1* in Δ*isw2* or Δ*itc1* strains is partially derepressed even in the presence of inositol in the culture medium. Preliminary experiments revealed that the level of derepression increases further when inositol is removed from the culture medium. These results indicate that the repression of *INO1* by inositol is partly mediated through the Isw2p-Itc1p chromatin-remodeling complex but that there could still be a sensory machinery for detecting the level of inositol and for regulating the expression of genes which are regulated by inositol via the ICRE. To elucidate the nature of this machinery and the functions of the Hac1p, further detailed analyses with mutants of *ISW2* and *ITC1* are necessary.

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