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Hac1: A novel yeast bZIP protein binding to the CRE motif is a multicopy suppressor for *cdc10* mutant of *Schizosaccharomyces pombe*

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ABSTRACT

We cloned by phenotypic complementation a novel *Saccharomyces cerevisiae's* multicopy suppressor of the *Schizosaccharomyces pombe cdc10-129* mutant which we call *HAC1,* an acronym of 'homologous to ATF/CREB 1'. It encodes a bZIP (basic-leucine zipper) protein of 230 amino acids with close homology to the mammalian ATF/CREB transcription factor and gel-retardation assays showed that it binds specifically to the CRE motif. *HAC1* is not essential for viability. However, the *hac1* disruptant becomes caffeine sensitive, which is suppressed by multicopy expression of the yeast *PDE2* (Phosphodiesterase 2) gene. Although the mRNA level of *HAC1* is almost constitutive throughout the cell cycle, it fluctuates during meiosis. The upstream region of the *HAC1* gene contains a T_4C site, a URS (upstream repression sequence) and a TR (T-rich) box-like sequence, which reside upstream of many meiotic genes. These results suggest that *HAC1* may also be one of the meiotic genes.

INTRODUCTION

In the life cycle of both budding and fission yeasts, cells are confronted with two alternative pathways, one leading to vegetative growth (mitosis) and the other to the sexual phase (conjugation, meiosis). In the G1 phase of the cell cycle at a point called 'START', the cells assess the environment and enter the pathway best suited for survival. START may correspond to the restriction point of certain mammalian cells. In both the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast *Schizosaccharomyces pombe*, once cells have passed through START, they are committed to cell division and unable to initiate alternative developmental pathways such as conjugation until the cell cycle returns to G1. If the dividing *S.pombe* cells are confronted with a nutritional starvation such as the deprivation of nitrogen source, they cease proliferation, accumulate in G1 phase and proceed to conjugation and sporulation.

In the budding yeast, *SWI4* and *SWI6* genes together with *CDC28, CLN1* and *CLN2* genes are proposed to play an pivotal role in a autostimulatory feedback loop activated at START (1–3). Cells simultaneously disrupted for both *swi4* and *swi6* are inviable even though the single mutations are not lethal. These two genes are involved in the regulation of START by forming protein complexes. Swi4 and Swi6 constitute a transcription factor called SBF (Swi4/Swi6 cell cycle box binding factor) that activates G1 cyclin (*CLN1,CLN2* and *HCS26*) and *HO* endonuclease genes (4). Mbp1 (5) and Swi6 form a different but related transcription factor called MBF (MluI cell cycle box binding factor) that activates many DNA synthesis genes (6,7).

Of all the *cdc* mutants in fission yeast, only *cdc2* and *cdc10* arrest cells in G1 at a point still permissive for conjugation (8). It is suggested that the execution point of these gene products, $p34^{cdc2}$ and $p85^{cdc10}$, locates before or at START (8). Cdc10 (9) and Res1/Sct1 (10,11) constitutes MBF and activates the transcription of at least three genes in *S.pombe*; *cdc22*+, encoding a large subunit of ribonucleotide reductase (12), *cdc18*+ (13), and *cdt1*+ (14). The recently discovered Res2/Pct1 (15,16) also constitutes an MBF complex recognizing MCB, but not SCB sequences.

Using phenotypic complementation of one of these START mutants of *S.pombe* with a human cDNA library, human homologue of *cdc2*+ was isolated (17), indicating that a similar regulatory mechanism for cell-cycle progression is also used in mammalian cells. This technique is useful not only to isolate the homologues of other eukaryotic species but also to clone the genes for the factors that may interact with these homologues and regulate the cell cycle. We recently cloned novel human genes encoding RNA binding proteins which are multicopy suppressors of the *cdc2* mutant (18). We also prepared a cDNA library of

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S.cerevisiae to isolate novel multicopy suppressors of the *S.pombe cdc2* and *cdc10* mutants. Here we report a novel gene of *S.cerevisiae* encoding a transcription factor with a bZIP domain as a multicopy suppressor for the *S.pombe cdc10* mutant.

MATERIALS AND METHODS

Media and strains

The components of the rich and synthetic media supplemented with appropriate nutrients for plasmid maintenance were prepared according to standard protocols (19–21). The *S.pombe* complementation host genotype used in this study is h^{+N} *cdc10-129 leu1-32* (AS1). The genotypes of *S.cerevisiae* strains used in this study are as follows: S288C (*MATα mal gal2 mel SUC2 CUP1*), NCYC239 (prototrophic diploid), SLD101 (*MATa/MATα lys2/lys2 ho::LYS2/ho::LYS2 ura3/ura3 leu2::hisG/ leu2::hisG his4X/his4B trp1::hisG/trp1::hisG*), SLD109 (*MATa/MATα Δswi4::LEU2/Δswi4::LEU2*), SLD113 (*MATa/MATα Δswi6::TRP1/Δswi6::TRP1*), 1783/1784 (*MATa/MATα leu2-3,112/leu2-3,112 ura3-52/ ura3-52 trp1-1/ trp1-1 his4/his4 can1^r/can1^r*), HC6a (*MATa leu2-3,112 ura3-52 trp1-1 his4 can1^r*Δhac1::URA3), HC6b (*MATα leu2-3,112 ura3-52 trp1-1 his4 can1^r*), HC6c (*MATa leu2-3,112 ura3-52 trp1-1 his4 can1^r*), HC6d (*MATα leu2-3,112 ura3-52 trp1-1 his4 can1^r*Δ hac1::URA3).

Isolation of multicopy suppressors

The cDNA library of *S.cerevisiae* (from the S288C strain with a complexity of 4.5 million colony forming units in the pcD2 vector) was prepared as described (22) using mRNA obtained from the wild type yeast cells grown to mid-log phase. The plasmid DNA prepared from the cDNA library was used to transfect the *cdc10-129* mutant of *S.pombe* (AS1) with the transducing vector pAL19 as previously described (10,17,23,24). The transfected cells were spread on minimal medium agar (MMA) plates and incubated at 23°C for 24 h. The plates were shifted to the restrictive temperature (32°C), and incubated 3–5 days further to select surviving cells. The colonies that formed on MMA plates were isolated, and the plasmid cDNAs were recovered from yeast into *E.coli* (25). The complementation ability of the recovered cDNAs was tested by subsequent transformations into the host strains. The *HAC1* gene was isolated from a genomic library of *S.cerevisiae* (S288C) by colony hybridization with the DNA fragment of *HAC1* cDNA as a probe. The DNA sequence was determined by the dideoxynucleotide method (26).

Caffeine sensitivity

A null allele of *HAC1* was prepared as described (27) by replacing it with *URA3* gene derived from *YDp-U* (28). Haploid cells (HC6a, HC6b, HC6c, HC6d) microdissected from tetrads of the *HAC1/hac1::URA3* diploid (see Fig.3C) were streaked onto YPD plates with or without 10 mM caffeine and incubated at 30°C for 3–4 days. To assess the influence of overexpression, the *HAC1* cDNA was inserted to the pYES2 vector (Invitrogen, USA) containing the inducible *GAL1* promoter and the *TRP1* marker. Then, the plasmid DNA was transformed into the *hac1* disruptant (HC6d). Trp$^+$ cells were selected on SD-Trp plates, and a single colony was picked and streaked onto YPGal plate (a rich medium plate supplemented with galactose in place of dextrose). The selection was repeated once more to confirm the induction of the GAL promoter. Subsequently, the selected cells were streaked onto a YPgal plate containing 10 mM caffeine. The *hac1* disruptant (HC6d) and wild type haploids (HC6c) were transformed with the yeast *PDE1* (29) and *PDE2* (30) genes with *LEU2* markers and the Leu$^+$ cells were selected on SD-Leu plates. Single colonies were subsequently streaked on YPD plates containing 10 mM caffeine and incubated 2–3 days at 30°C. To investigate the growth rate of the *hac1* disruptant, the same number of cells cultured overnight were inoculated into rich medium (YPD) including various concentrations of caffeine, and incubated by shaking at 140 r.p.m. at 30°C. An aliquot of the cells were removed and the optical density at 600 nm was measured at appropriate intervals of the time course. For the experiments with 5 mM caffeine, the cells were collected at 10 h (shown by an arrow), filtered through a 0.22 μm bottle filter (Caster, USA), rinsed with YPD and the collected cells were resuspended in the original volume of YPD and the incubation was continued.

Preparation of Hac1–GST fusion protein

To obtain a fusion protein of full-size Hac1 with glutathione S-transferase (GST), a pair of oligonucleotides (CGTGGATCCCC-AATGGAAATGACTGATTTT and ACTGAATTCACTGTA-GTTTCCTGGT) containing the initiation and the termination sites of Hac1 was synthesized and used as primers for polymerase chain reaction (PCR) by *Pfu* DNA polymerase (Stratagene, USA) with the genomic DNA of *S.cerevisiae* as the substrate. The resulting 0.8-kb fragment was first inserted into the Bluescript II (SK+) vector, digested with *Bam*HI and *Eco*RI, and this 0.8-kb fragment was inserted into pGEX-4T-2 vector (Pharmacia, USA). To obtain a truncated form of a Hac1–GST fusion protein, the 0.4-kb *Sal*I fragment (see Fig. 1 for the restriction map) was inserted into the *Sal*I site of the pGST3 vector, a modified form of pGEX-2T vector we prepared (31). The *E.coli* cells (*PR745*) carrying these plasmid DNAs were grown to the midlog phase, the *lac* promoter of the vector was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and further incubated by moderate shaking (140 r.p.m.) at 20°C for 20 h. The cells were collected by centrifugation to prepare the cell extract and the GST–fusion protein was attached to and eluted from the glutathione–Sepharose 4B affinity purification system according to the manufacturer's protocol (Pharmacia, USA). The eluted protein was stored at −80°C in elution buffer (50mM Tris–HCl, pH.8.0) containing 20% glycerol before use.

Gel-retardation assays

A 148-bp DNA fragment of the 5′ upstream regulatory region of *S.cerevisiae SUC2* gene (32) containing one CRE motif was obtained by the polymerase chain reaction as described before (32; Sakai *et al.*,unpublished). The oligonucleotides for the CRE* (AAAAATGACGTCATTATG) or the TRE (AAAAATGAC-TCATTATG) motifs were synthesized and used as competitors. The oligonucleotides for CREB (AGAGATTGCCTGACGTCA-GAGAGCTAG) or AP1 (CGCTTGATGAGTCAGCC GGAA) recognition sites were purchased (Promega, USA). These CREB or AP1 oligonucleotides were radiolabelled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase, separated from the unincorporated nucleotides by the Suprec filter (Takara-shuzo, Japan) and used as probes. The purified GST-fusion proteins or the cell extracts were incubated with the probe (10,000 c.p.m.) in 10mM Tris–HCl (pH.7.5), 50mM NaCl, 0.5mM

dithiothreitol (DTT), 0.5 mM ethylendiamine tetraacetate (EDTA), 1mM $MgCl_2$, 4% glycerol, 80 μg/ml calf thymus DNA, 75 μg/ml poly(dI:dC) carrier DNA (Pharmacia LKB) in a total volume of 20 μl and incubated at 20°C for 20 min. The competitor oligonucleotides were premixed with the GST-fusion proteins or the extract in the same assay buffer and incubated at 20°C for 20 min before addition of the probe. The protein−DNA complexes were separated on non-denaturing 5% or 7.5 % polyacrylamide gels run at 20°C for 1−2 h at 12−18 V/cm in 20mM Tris−HCl (pH.7.5) and 4% glycerol.

Synchronization and Northern analysis

S.cerevisiae cells (NCYC239) were synchronized by passage through a Beckman JEIOX elutriator rotor as described before (33). Cells at each time point were collected by centrifugation and RNA was extracted by the standard phenol-extraction protocol (21). The same amount of RNA (20 μg) was loaded onto each lane in the agarose gel electrophoresis and the Northern blots were performed by the glyoxal method as described (34). The blotted nylon membranes (Biodyne; PALL) were hybridized in 5× SSC, 10× Denhardt's solution, 0.1% SDS, 0.1 mg/ml of denatured salmon sperm DNA and 50% (v/v) deionized formamide at 42°C for 16 h with the relevant ^{32}P-labeled probes. The membranes were washed twice in 1× SSC at 25°C for 10 min, and once in 0.1× SSC at 50°C for 15 min in the presence of 0.1% SDS. The same membrane was used for reprobing as follows; first, the membrane was hybridized with the *HAC1* probe, stripped and reprobed separately with *URA3*, *PR1/H2A* and *Pol1* probes in that order.

The isogenic **a** and α strain of wild type (WT) or strains lacking *SWI4* or *SWI6* in the SK1 background were prepared (Leem *et al.*, unpublished). After these strains were mated, the diploid strain was selected by the ability to sporulate. Diploid cells were returned to vegetative growth by spreading onto YPD plates. Single colonies of the diploid cells were transferred to sporulation medium (21), an aliquot of the sporulating cells were withdrawn at an appropriate interval during the time course of meiotic process to obtain RNA by the standard phenol-extraction protocol (21). The RNA samples were subjected to Northern blots (34), which was probed by a ^{32}P-labeled *Spe*I−*Hin*dIII fragment (0.8-kb) of *HAC1* cDNA.

RESULTS

Isolation of novel multicopy suppressors of cdc10

To search for novel multicopy suppressors of the *cdc10* mutant of *S.pombe* (AS1; *h^{+N} cdc10-129 leu1-32*), a cDNA library of *S.cerevisiae* whose cDNA inserts are transcribed by the SV40 promoter in *S.pombe* cells (10,17,24) was transformed into these mutant cells, incubated at the restrictive temperature and the plasmid DNA was recovered from the surviving yeast cells. Out of approximately 200,000 Leu$^+$ colonies, we selected several clones that suppressed the temperature sensitivity of the *cdc10* mutant. One of the clones, tentatively named 10C29 was further analyzed.

A novel suppressor encodes a protein homologous to ATF/CREB

DNA sequencing of 10C29 revealed that it contained a cDNA insert with 1,424 nucleotides encoding a protein of 230 amino acids (Fig. 1A). Homology search in the SWISSPROT and PIR databases revealed that the protein harbors a bZIP (basic-leucine zipper) domain (35) and belongs to a family of ATF (activating transcription factor)/ CREB (cyclic AMP response element binding) proteins (36). Hence we named the cDNA clone (10C29) *HAC1*. The alignment of amino acid sequences around the bZIP region of Hac1, human CREB and Sko1/Acr1 (37,38), another *S.cerevisiae* protein that belongs to the ATF/CREB family, is depicted in Figure 1B. *HAC1* is distinct from *SKO1/ACR1* (38) not only by the amino acid sequences but also by the overall size and the location of the bZIP domain. Although the human CREB possesses four leucine residues to constitute a leucine zipper, both *HAC1* and *SKO1/ACR1* carry only two leucine residues. Tyrosine for *SKO1/ACR1* or valine for *HAC1* instead of the second or the third leucine may not be suitable to form a zipper (34) and thus, the zipper would be of weak strength. A potential phosphorylation site (RKCS; residues 69−72) for a cAMP-dependent protein kinase (39) is found in the zipper region.

The genomic DNA fragment of *HAC1* was also cloned by a colony hybridization of *S.cerevisiae* (S288C) genomic library with a *HAC1* cDNA probe, and the DNA sequence of the upstream regulatory region was determined (Fig. 1A). No introns were detected when compared with the cDNA structure. In the 5′ regulatory region of the *HAC1* gene, there were four TATA boxes but no MCB was found at least up to −595. Therefore, *HAC1* gene is probably not under the control of the MCB. Instead, a T_4C site (TTTTCXXCG or CGXXGAAAA), a stretch of conserved nucleotide sequences widely distributed among early meiotic genes (40), was found at −347. The core sequence for the upstream repression sequence (URS), that is required for meiosis specific expression (41), was also found at +60 (caaCCGCCac). The notable nucleotide sequences are T-CTTTTGTTC at −138, CTTCTTGTCT at −30 and TCTT-CTTGTTT at −23, which are homologous to the TR (T-rich) box of *S.pombe*, an essential *cis*-acting element found in the upstream region of the genes inducible by nitrogen starvation (42). The existence of these consensus sequences suggests that *HAC1* is involved in the meiotic process.

Complementation of *cdc10* mutation

To assay complementation efficiency of *S.pombe cdc10* by the *S.cerevisiae HAC1*, an equal amount of plasmid DNAs of several relevant clones were transformed and the photographs of the plates incubated at the restrictive temperature (32°C) are shown in Figure 2A. *HAC1* complements several times less efficiently than other *S.pombe* multicopy suppressors such as *res1$^+$* (10), *rep1$^+$* (24) and *cdc10$^+$* itself (9). Cells rescued by *HAC1* grew more slowly than the cells rescued by *res1$^+$*, *rep1$^+$* or *cdc10$^+$*, requiring one extra day of incubation to form colonies at the restrictive temperature. The promoter of *S.cerevisiae*'s *HAC1* also works in *S.pombe* cells as judged by the fact that not only the cDNA form of *HAC1* transcribed by the SV40 promoter but also the *HAC1* gene driven with its own promoter complemented the *cdc10* mutation at an equal efficiency. *HAC1* expression in wild type *S.pombe* cells yields an equal proportion of small and large colonies (data not shown).

The overall shape and size of *cdc10* mutant cells complemented by *HAC1* (both cDNA form and the gene) as observed under a microscope (Fig. 2B) is similar to *cdc10* mutants rescued by *S.pombe* plasmid suppressors such as *res1$^+$*, *rep1$^+$* and *cdc10$^+$* itself. The transformation of the vector (pcD2-neo) alone showed no effect on the elongated *cdc*-phenotype. The result indicates

Figure 1. Nucleotide and deduced amino acid sequences of the *HAC1* gene. (A) The amino acid sequence is aligned below the DNA sequence as a single-letter code. Nucleotide numbering starts at the 5′ end of the cDNA insert. The initiation (ATG) and the termination (TGA) codons are denoted by overhead lines (==). The underline or overhead lines are to indicate the following sites: a cluster of basic amino acids (.....), possible leucine-zipper forming residues (____), the recognition sequences for relevant restriction enzymes (____), the polyadenylation site (= =), TATA box-like sequences (....), T_4C site (__), TR box-like sequence (____) and URS (‾‾). A potential phosphorylation site for a cAMP-dependent protein kinase is shown by a wavy underline. (B) Amino acid homology among human CREB, *S.cerevisiae* Skol/Acrl and Hacl proteins. Identical (:) and homologous (*) amino acids, basic amino acids (encircled), and leucine-zipper forming residues (#) are denoted. Schematic drawings shown below are to depict the relative location of these motifs. The accession number of *HAC1* in the DDBJ/EMBL/GenBank is D26506.

that the ability of *HAC1* to rescue *cdc10* mutant cells from elongated *cdc*-phenotype is almost equal to *res1*$^+$, *rep1*$^+$ and *cdc10*$^+$, although the complementation efficiency of *HAC1* as judged by the viability was lower than others.

HAC1 disruptant is viable

To investigate the physiological role of *HAC1* , a null allele was created by one-step gene replacement (27). Of a *HAC1* genomic DNA fragment, the 0.86-kb *Sal*I–*Hin*dIII fragment containing most of the coding region of *HAC1* was replaced with the 1.1-kb fragment of *URA3* gene (Fig. 3A). The construct containing the disrupted *hacl* gene was transformed to a diploid strain (1783/1784), and the genomic DNA from stable Ura$^+$ transformants was analysed by a Southern blotting (34) to confirm a successful disruption of *HAC1* (Fig. 3B). The *HAC1/hacl::URA3* diploid was sporulated and the tetrads were microdissected onto a YPD plate. Out of ten tetrads analyzed, all spores grew not only at 30°C but also at a lower (18°C) and a higher (37°C) temperature (data not shown). DNA from one set of tetrads were extracted, digested with *Sal*I/*Eco*RI, and subjected to a Southern analysis to confirm that only two spores viable on a SD-Ura plate showed a 1.2-kb band (Fig. 3C; HC6a and HC6d). No abnormality was observed in the growth rate and the mating hormone sensitivity of these disruptants (data not shown).

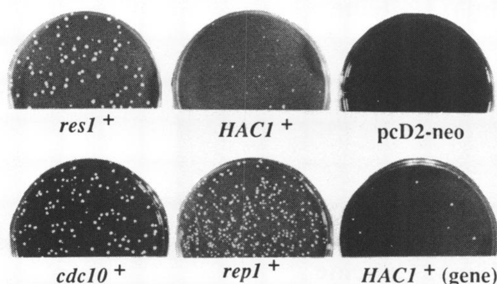
To examine whether the deletion of *Sal*I–*Hin*dIII fragment actually lacked complementation activity of AS1 and assess the essential portion of the molecule, plasmid DNAs with a set of deletions of the *HAC1* coding region were prepared. All of the constructs including the deletion of *Sal*I–*Hin*dIII fragment failed to complement AS1 (data not shown), indicating that the entire structure of this small molecule is necessary for complementation.

HAC1 disruptant is caffeine sensitive

When the germinated spores from tetrad were streaked on YPD plates containing 10 mM caffeine, only the disruptants showed poor growth (Fig. 4A; HC6a and HC6d) as compared to the wild-type (Fig. 4A; HC6b and HC6c). To confirm that the caffeine sensitivity is actually due to the lack of *HAC1* activity, the intact *HAC1* cDNA was inserted into the pYES2 vector downstream of the *GAL1* promoter (Invitrogen, USA), introduced into the wild (HC6c) or the *hacl* disruptant (HC6d) cells and overexpressed by spreading on the rich medium plate supplemented with galactose (YPGal) and 10 mM caffeine. As expected, *HAC1* overexpression suppressed the caffeine sensitivity of the *hacl* disruptant (Fig. 4B-i). As a negative control, the vector without an insert was transfected separately, which showed no suppression (Fig. 4B-ii).

Since caffeine is a potent inhibitor of cAMP phosphodiesterase (*PDE*), it is expected that the *PDE* gene would complement the caffeine sensitivity when it is introduced as a multicopy suppressor

A Host : *h* $^{+N}$ *cdc10-129* leu1-32 (32 °C)



B

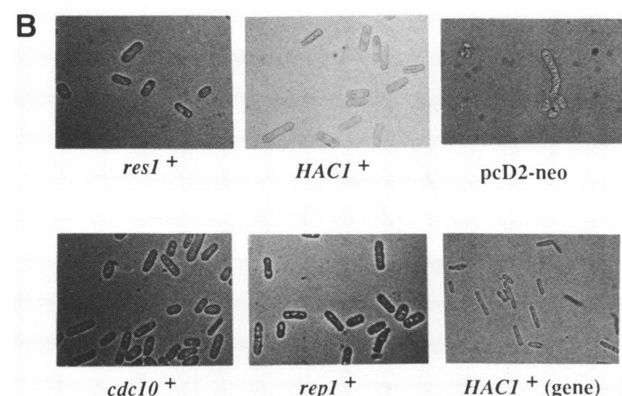


Figure 2. Either cDNA or the genomic form of *HAC1* suppresses the *cdc10* mutation of *S.pombe*. **(A)** Complementation of *cdc10-129* mutant of *S.pombe* by the cDNA and the gene of *HAC1*. Plasmid DNA harbouring *res1*$^+$,*rep1*$^+$, or *cdc10*$^+$cDNAs of *S.pombe* or the plasmid without cDNA insert (pcD2-neo) were used as the positive or the negative controls. The *cdc10* mutant cells were transformed with the indicated plasmids, spread on MMA plates and incubated at the restrictive temperature (32°C) for 3–5 days. **(B)** Morphologies of the complemented mutant cells with indicated plasmid DNAs were introduced into *cdc10-129* cells. Yeast cells picked up from single colonies on MMA plates at the restrictive temperatures (32°C) were dispersed into a spot of water on glass slides and observed under a microscope. Since no colony was formed in the case of pcD2-neo transformation, plates were rinsed with minimum medium, spun down by centrifugation and the precipitated cells were viewed under a microscope.

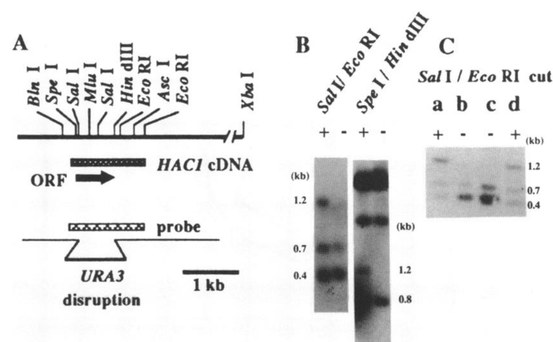


Figure 3. Disruption of the *HAC1* gene and deletion analysis. **(A)** Restriction map of the *HAC1* gene and *HAC1* cDNA. The open reading frame encoded by the *HAC1* gene (ORF), the 0.8-kb *Spe*I–*Hin*dIII fragment of *HAC1* used as a probe for Southern hybridization, and the schematic drawing for the construct of *hacl:URA3* are shown below the map. **(B)** Detection of the disrupted chromosomal *hacl* (+) gene of diploid cells as compared with the wild type (−) by Southern hybridization as digested by *Sal*I/*Eco*RI or *Spe*I/*Hin*dIII. **(C)** Southern blot analysis of the *Sal*I/*Eco*RI-digested chromosomal DNA from haploid cells microdissected from tetrads of the *HAC1/hacl::URA3* diploid.

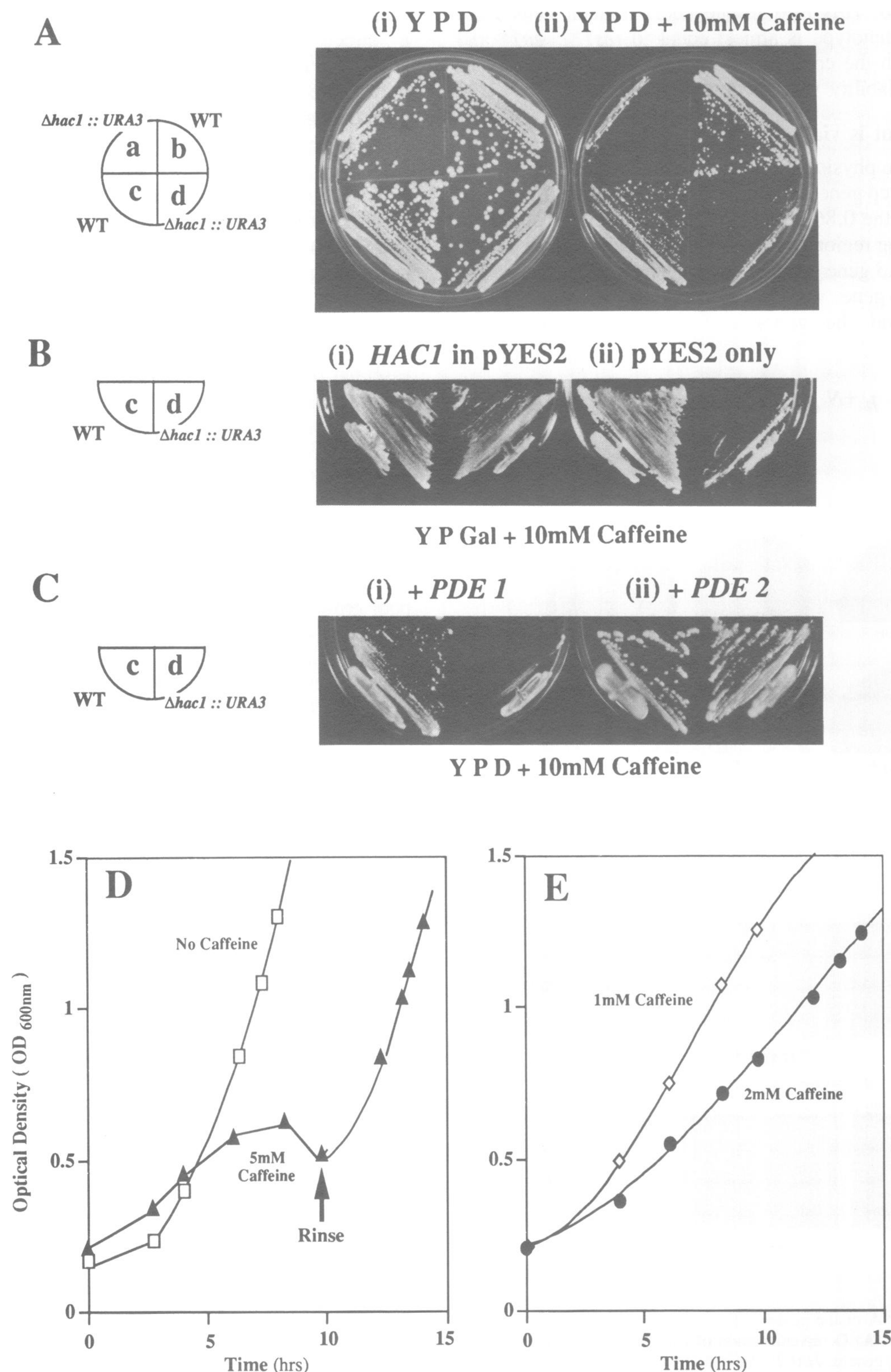


Figure 4. Caffeine sensitive growth phenotypes of *hac1* disruptants. **(A)** Haploid cells microdissected from tetrads of the *HAC1/hac1::URA3* diploid (see Fig. 2C) were streaked on YPD plates with (right) or without (left) 10 mM caffeine and incubated at 30°C. **(B)** *HAC1* cDNA was inserted downstream of the *GAL1* promoter (pYES2 vector), introduced into the haploid cells (HC6c or HC6d) and streaked on YPGal plates. Single colonies were picked up and restreaked on a YPGal plate to confirm the induction of *HAC1* expression. Then, single colonies were streaked (left) on YPGal plates containing 10 mM caffeine. Cells transformed with the YES2 vector alone were also shown as negative controls (right). **(C)** *PDE1* (i) or *PDE2* (ii) genes of *S.cerevisiae* were introduced into the microdissected haploid cells (HC6c and HC6d) and were streaked on YPD plates containing 10 mM caffeine. **(D,E)** The growth curve of the *hac1::URA3* haploid cells in YPD medium containing 0 and 5 mM (D) or 1 and 2 mM (E) caffeine. The optical density at 600 nm for the removed aliquots of the cells was measured during the time course. The cells incubated in the presence of 5 mM caffeine (D; Δ)were rinsed (see Experimental Procedures) at 10 h (shown by an arrow) and the incubation was continued.

into the disruptant cells. As shown in Fig. 4C, the caffeine sensitivity was actually suppressed by introduction of multicopy *PDE2* (28), the high-affinity cAMP phosphodiesterase gene of *S.cerevisiae*. However, no suppression was observed by introduction of multicopy *PDE1* (30), the low-affinity cAMP phosphodiesterase gene of *S.cerevisiae*. These results suggest that the caffeine sensitivity of the *hac1* disruptant is due to an increase in the intracellular cAMP level by inhibition of *PDE2*, but not *PDE1* (see Discussion).

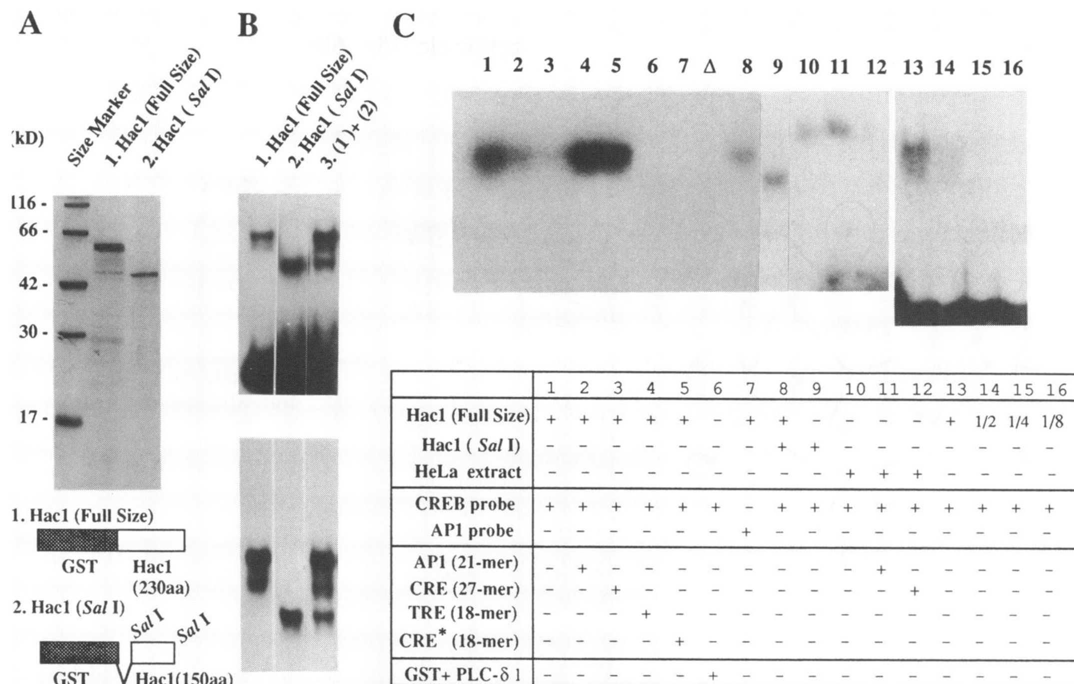
The growth rate of the *hac1* disruptant was inversely proportional to the concentration of caffeine added to the medium as shown in Figure 4D and 4E. We detected no conspicuous difference in the shape and size of the growing cells under the microscope (data not shown). When the cells cultured in rich medium containing 5mM caffeine were rinsed to remove caffeine (Fig. 4D), the growth rate recovered to that of the culture which originally contained no caffeine. This indicates that the growth inhibition by caffeine in the *hac1* disruptant was a reversible phenomenon.

Hac1 binds to a CRE motif

We constructed a plasmid to obtain two kinds of GST-fusion proteins of Hac1. One is the full length and the other is the truncated form (*Sal*I fragment) of Hac1 as schematically depicted in the lower panel of Fig. 5A. The purified fusion proteins migrated primarily as a single band in the SDS–polyacrylamide gel electrophoresis (Fig.5A).

When these two proteins were independently incubated with the 148-bp DNA fragment of the 5′ upstream regulatory region of the *S.cerevisiae SUC2* gene (32), which contain one CRE motif, each protein generated a protein–DNA complex of distinct electrophoretic mobility (Fig.5B, lanes 1,2). Although we expected to observe an extra band due to a heterodimeric complex migrating an intermediate distance if both of these proteins were present in the assay mixture, a weak middle band originally present for full-size protein hindered detection of this putative heterodimeric complex. Hence the dimerization is still unproven.

To further demonstrate that Hac1 binds to a ATF/CRE site in a specific manner, we incubated the fusion proteins with a ^{32}P-labeled 27-mer oligonucleotide containing a CRE motif (AGAGATTGCCTGACGTCAGAGAGCTAG). The full-size Hac1 fusion protein strongly bound to the oligonucleotide (Fig.5C, lane 1). This band disappeared when the nonradioactive oligonucleotide was premixed with the protein (lane 3). The protein complex with the 27-mer oligonucleotide seems to be highly favored since only a very weak competition was observed if 21-mer AP1 (CGCTTGATGA GTCAGCC GGAA), 18-mer CRE* (AAAAATGACGTCATTATG) or 18-mer TRE (AAAAATGAC TCATTATG) oligonucleotides were premixed with the protein (lanes 2, 4 and 5). No protein–DNA complex was detected when radiolabeled AP1 oligonucleotide was used as a probe (lane 7). The band represents a specific interaction of the Hac1 portion of the fusion protein with the oligonucleotide, since a GST-fusion protein with phospholipase C-δ1 (31), which



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Hac1 (Full Size)	+	+	+	+	+	–	+	+	–	–	–	–	+	1/2	1/4	1/8
Hac1 (*Sal* I)	–	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–
HeLa extract	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–
CREB probe	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+
AP1 probe	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
AP1 (21-mer)	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
CRE (27-mer)	–	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–
TRE (18-mer)	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
CRE* (18-mer)	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
GST+ PLC-δ 1	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–

Figure 5. Gel-retardation assay. (**A**) Quality of the affinity purified GST–Hac1 fusion proteins used for the gel-retardation assay as observed in 12.5% polyacrylamide–SDS gel electrophoresis. The schematic representation of the construct for the full size (1) and the *Sal*I fragment of *HAC1* cDNA is shown underneath. (**B**) The full size and the truncated form (*Sal*I fragment) of Hac1p both bind to the ^{32}P-labeled 148-bp DNA fragment of the 5′ upstream regulatory region of *S.cerevisiae SUC2* gene. The purified GST–Hac1 fusion proteins (150 ng each) were incubated independently (lanes 1 and 2) or as a mixture (lane 3) with the probe (10,000 c.p.m.) and the DNA–protein complex were analyzed in nondenaturing 8% (upper panel) or 5% (lower panel) polyacrylamide gel electrophoresis. (**C**) The gel-retardation assay with the ^{32}P-labeled CREB oligonucleotide (lanes 1–6, 7–16) or AP1 oligonucleotide (lane 8) as probes. The combination of the components in the assay mixture such as the protein source, the probe and the competitor is summarized in the table shown underneath. A blank lane (**D**) is included. Plus (+) means 150 ng for GST-Hac1 fusion proteins, 2 μg for affinity purified GST-PLC-δ1 fusion protein, 20 μg for HeLa cell extract, 1.75 pmole of CRE or AP1 oligonucleotides and 7 pmole of TRE or CRE* oligonucleotides.

does not bind DNA, displayed no gel shift (lane 6). The band for a truncated form of Hac1 migrated faster (lane 9), but the oligonucleotide preferred to form a complex with the full-size Hac1 fusion protein if both fusion proteins were mixed with oligonucleotide (lane 8). HeLa cell extract was also incubated with the probe as a positive control to show that the CRE oligonucleotide could indeed bind a CREB in a specific manner as the expected gel-shift band (lane 10) was eliminated by premixing with the CRE (lane 12) but not with the AP1 (lane 11) oligonucleotide. It was also confirmed that the intensity of the band observed with the full-size Hac1 decreased in a stoichiometric manner (lanes 13−16).

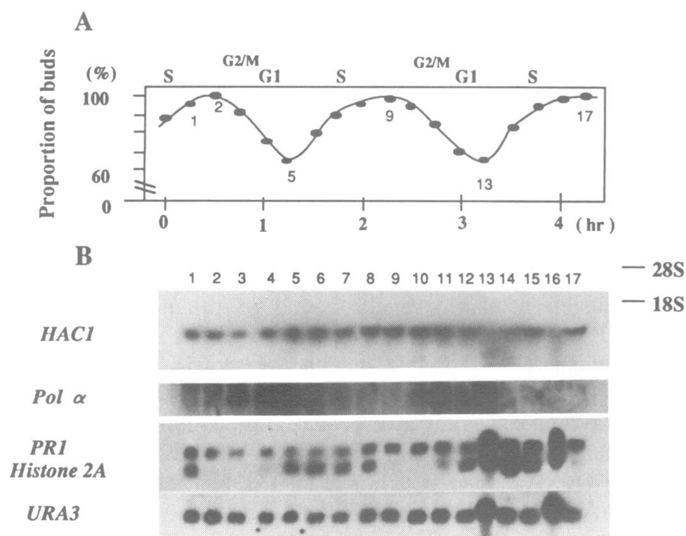


Figure 6. Periodic or constitutive expressions of *HAC1, Pol1, PR1, H2A and URA3* mRNAs in a yeast cell culture synchronized by elutriation. (**A**) The budding profile of the culture. Proportion of the cells with buds are counted under a microscope. Numbers beside the dots show the sample number for the benefit of comparison with the figures shown below. (**B**) Northern blots for 20 μg total RNA extracted from each sample were hybridized with the ^{32}P-labeled DNA fragments from the *S.cerevisiae* genes shown on the left and the suitably exposed autoradiographs are presented.

Expression pattern of the HAC1 gene during the cell cycle

To investigate whether *HAC1* displays a periodic transcription during the cell cycle, wild type yeast cells (NCYC239) were synchronized by elutriation and the RNA extracted from cells in the time course were subjected to Northern analysis (Fig. 6). Typical periodic genes of *S.cerevisiae* such as DNA polymerase α (*POL1*) and *histone2A (H2A)* were utilized as molecular measures to assess the synchrony achieved. *H2A* is known to be periodically transcribed, reaching a peak in mid S-phase, while the transcription level of an adjacent gene with an unknown function called *Protein 1* (*PR1*) remains at a constant level (43,44). *POL1*, which is under the regulation of MCB sequences (7), is also known to be periodically transcribed with a peak at the G1/S phase. The *URA3* gene, whose transcript level is known to be constant during the cell cycle, was also used as a loading control. A profile for the proportion of cells with buds counted under a microscope is also presented in Figure 6A in parallel with the Northern blots. Northern analysis of the *HAC1* transcript indicates that its expression is constitutive throughout the mitotic cell cycle (Fig. 6B).

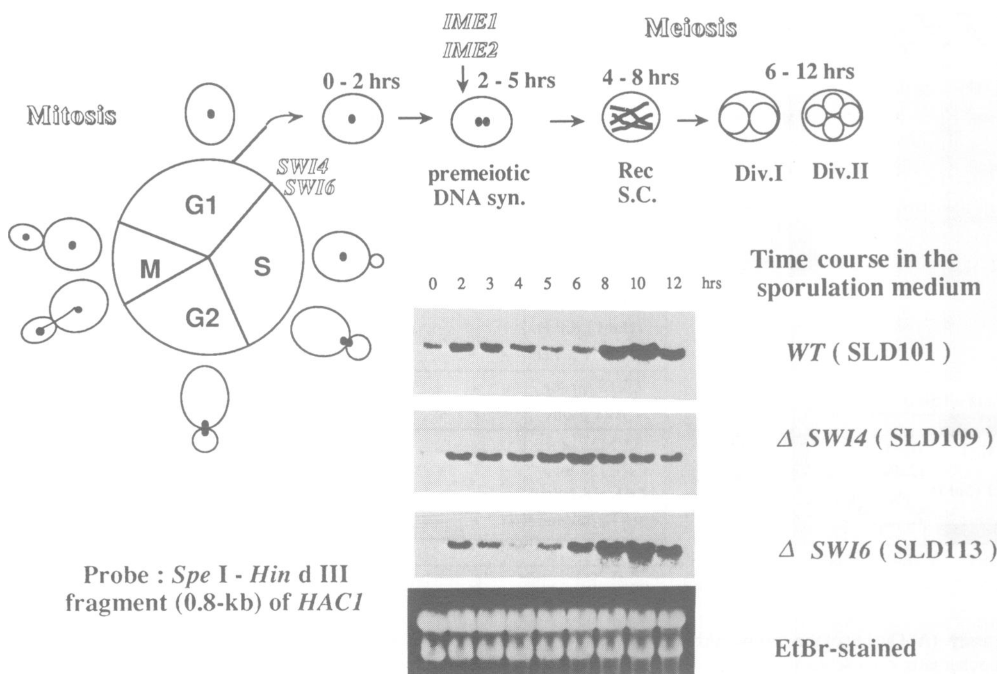


Figure 7. Expression of *HAC1* mRNA in the meiotic process. The isogenic **a** and α strains of *swi4* or *swi6* disruptants or of the wild type (WT) were transferred to the sporulation medium and the RNA extracted from each sample during the time course was analyzed by Northern blots by probing with the *HAC1*. The ethidium bromide-stained gel before transfer was shown underneath. The nucleotide sequence reported here (*HAC1*) have been submitted to the DDBJ/EMBL/GenBank databases under accesion accession number D26506.

Northern analysis in meiosis

The existence of a T_4C site (40) and a URS (41) in the 5′ upstream regulatory region of *HAC1* gene suggests that it may be involved in early meiosis. To examine whether *HAC1* transcription is regulated in meiosis, RNAs extracted from the *S.cerevisiae* cells undergoing sporulation were subjected to Northern analysis by probing with *HAC1*. As shown in Figure 7, the level of *HAC1* transcription oscillates in meiosis displaying two peaks, one at the early stage concurrent with premeiotic DNA synthesis and the other at a later stage, concurrent with meiotic division. The oscillation is retained in the *SWI6* disruptant (SLD109), but it is abolished in the *SWI4* disruptant (SLD113), indicating that the oscillation is under the control of the *SWI4* gene, but not the *SWI6* gene.

DISCUSSION

By complementation cloning of an *S.pombe cdc10* mutant with a *S.pombe* cDNA library, we have successfully obtained two transcription factors, *res1*$^+$ (10) encoding Swi4-like protein with two ankyrin repeats (45), and *rep1*$^+$ (24), encoding a novel zinc finger protein. Using an *S.cerevisiae* cDNA library, we report the isolation of *HAC1*, encoding a novel bZIP-type transcription factor that specifically binds to the CRE motif. As *S.pombe* Cdc10 protein is a transcription factor, it is notable that all of these multicopy suppressors of *cdc10* mutation also encode transcription factors. Thus, a major mechanism for rescue of *S.pombe cdc10* may be through interactions with other transcription factors that stabilize the mutant Cdc10 protein.

The caffeine sensitivity of the *HAC1* disruptant suggests that it may be involved in a signal transduction pathway using cAMP as a second messenger. The existence of a potential phosphorylation site by cAMP-dependent protein kinase in the bZIP region strongly supports this notion. Suppression of caffeine sensitivity by the high-affinity *PDE2* gene, but not by the low-affinity *PDE1* gene, suggests that unidentified distinct mechanisms between the signal pathways involving these two separate genes exist and that *HAC1* is linked only to the pathway containing *PDE2* gene. Considering that *HAC1* is a multicopy suppressor of the *cdc10* mutation and feasibly involved in the onset of an early meiotic process, the detailed investigation of the target gene of the putative Hac1 transcription factor may lead to understanding of the mechanism that modulates the cAMP signal at the junction of mitosis and meiosis.

The existence of a T_4C site and URS motif in the 5′ upstream regulatory region of *HAC1* suggests that it is one of the early meiotic genes (40). The existence of three stretches of TR box-like sequences in the 5′ upstream region which are meiosis-specific elements in *S.pombe* (42) also suggests that the *HAC1* gene product is involved in meiosis and these motifs govern *HA-C1* transcripion. Oscillation of *HAC1* mRNA transcription as revealed by a Northern analysis supports this idea. In that sense, *HAC1* resembles *rep1*$^+$ (24), a multicopy suppressor of the *cdc10* mutation we recently cloned from a cDNA library of *S.pombe*. It encodes a zinc finger protein, harbours two TR boxes in the 5′ upstream region and transcription is induced abruptly right after the onset of meiosis by nitrogen starvation.

The disappearance of *HAC1* transcript oscillation during meiosis in the *swi4* disruptant (Fig. 7) indicates that the transcription of *HAC1* is somehow under the control of a complex involving Swi4 as a cofactor. Considering the absence of SCB upstream of *HAC1*, it connotes that an unrecognized motif for the Swi4 complex formation might be present upstream of *HAC1*. The fact that Hac1 specifically binds to a CRE motif and contains a potential phosphorylation site for cAMP-dependent kinase implies that it transduces an intracellular cAMP signal. The physiological activity of Hac1 as a multicopy suppressor of *cdc10* mutation suggests that a Cdc10-like protein of *S.cerevisiae* also participates in this signal transduction pathway. It remains to be investigated whether a gene encoding a Cdc10-like protein besides Swi4, Swi6 or Mbp1 exists in the genome of *S.cerevisiae*. It is quite tempting to speculate that such a Cdc10-like protein functions as a sorter of the intracellular signal regulating the molecular switch at the junction of meiosis and vegetative growth. In *S.pombe*, nutritional starvation, particularly for nitrogen, induces meiosis (19,46) and the resultant decrease of the intracellular cAMP level plays a pivotal role as an ignition signal for the meiotic process (42,47). The property of *HAC1* as reported here implicates that a similar mechanism might also apply to the meiotic process of *S.cerevisiae*.

One of the immediate targets of our research is to search for a homologue of *HAC1* in the genome of *S.pombe*. A preliminary total genomic Southern analysis under a very nonstringent hybridization condition disclosed a faint band when the genomic DNA of *S.pombe* was digested with relevant restriction enzymes and was probed with the ^{32}P-labeled *HAC1* DNA fragment (data not shown). The result suggests that such a *HAC1* homologue actually exists in the genome of *S.pombe*.

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