Identification and characterization of novel *cdc* genes that regulate G1/S transition in fission yeast *Schizosaccharomyces pombe*

分裂酵母 *Schizosaccharomyces pombe* の G1/S 期移行を制御する新規細胞周期調節遺伝子の発見と解析

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平成5年4月　転入学

第3種博士課程

第二基礎医学専攻

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I. Introduction

Virtually all eukaryotic cells proliferate through a specific process called the cell cycle. This process is divided into four characteristic phases named G1 (gap 1), S (DNA replication), G2 (gap 2) and M (mitosis), and major cell cycle control points have been defined. One of these is the G2/M boundary, at which the timing of the onset of mitosis is regulated by intracellular signals, such as completion of DNA replication, repair of DNA damage and cell size. The other is G1 phase in which the extracellular signals (e.g., nutritional conditions, growth and differentiation factors, hormones, etc.) make commitment to the start of the cell cycle or differentiation or falling in a stationary phase. Thus, in G1, a switch mechanism operates to determine whether cells should grow, arrest or differentiate. Once cells have committed to enter S phase, they are unable to initiate alternative developmental pathways until they return to G1. Such a control system occurs in various eukaryotic cells from unicellular species such as yeast to multicellular species such as mammals (reviewed by Cross *et al*, 1989) and underlies some important pathological processes, such as immortalization, transformation and cell death.

The process of G1 to S progression is better understood with two kinds of yeast, *Saccharomyces cerevisiae* (also called budding yeast) and *Schizosaccharomyces pombe* (also called fission yeast). These yeasts are excellent organisms for classical genetic and molecular genetic analyses, which are invaluable for investigating the complex regulation of the cell cycle and effectively complement biochemical approaches. Despite its simplicity, yeast has most of the basic biological features of higher eukaryotes. Although these two yeasts are evolutionary diversed from one another, recent studies indicate that basic mechanisms underlying cell cycle regulation are well conserved (reviewed by Forsburg and Nurse, 1991). Moreover, *S. pombe* more resembles higher eukaryotes in general aspects than *S. cerevisiae* such does. In fact, the G2 control system of *S. pombe* is strikingly similar to that of mammals (Okayama *et al.*, 1992). These facts led us to use the *S. pombe* as a model organism to understand the G1/S regulatory system of mammalian cells.

1. *Schizosaccharomyces pombe*

S. pombe cells are rod-shaped, grow in the longitudinal direction, and divide by septation and medial fission. They grow in a haploid state with a very short G1 phase when cells are rapidly growing in rich medium. If nitrogen source is depleted, however, cells come to cell cycle arrest in G1 phase. When cells of opposite mating types (h^+ and h^-) come close, they conjugate to form diploid cells, which subsequently undergo meiosis and form haploid spores (Figure 1). Mating pheromones (h^+ cells produce P-factor and h^- cells produce M-factor), which are produced responding to nitrogen

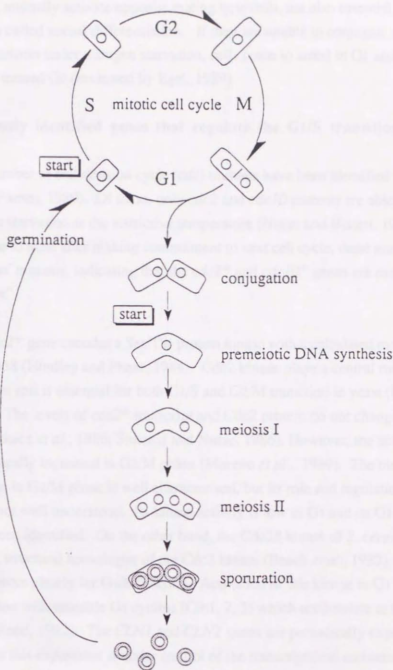

Fig.1. A schematic of the life cycle of *S. pombe*. It is not shown in this figure, if newly formed zygotes are transferred to nitrogen-rich medium, some of them restart the diploid vegetative cell cycle instead of meiosis.

starvation and mutually activate opposite mating type cells, are also essential for the start of this process called sexual differentiation. If they are unable to conjugate with opposite mating type partners under nitrogen starvation, cells come to arrest in G1 and enter a dormant state termed G0 (reviewed by Egel, 1989).

2. Previously identified genes that regulate the G1/S transition in *S. pombe*

The number of cell division cycle (*cdc*) mutants have been identified in *S. pombe* (reviewed by Fantes, 1989). Of these, only *cdc2* and *cdc10* mutants are able to conjugate under nitrogen starvation at the restrictive temperature (Nurse and Bissett, 1981). Since cells are unable to mate after making commitment to next cell cycle, these mutants are defined as 'start' mutants, indicating that the $cdc2^+$ and $cdc10^+$ genes are essential for cell cycle "start".

A. $cdc2^+$

The $cdc2^+$ gene encodes a Ser/Thr protein kinase with a calculated molecular weight of 34,358 (Hindley and Phear, 1984). Cdc2 kinase plays a central role in cell cycle regulation and is essential for both G1/S and G2/M transition in yeast (Forsburg and Nurse, 1991). The levels of $cdc2^+$ transcript and Cdc2 protein do not change during the cell cycle (Durkacz *et al.*, 1986; Simanis and Nurse, 1986). However, the activity of Cdc2 is periodically increased in G2/M phase (Moreno *et al.*, 1989). The mechanism of Cdc2 activation in G2/M phase is well characterized, but its role and regulation in G1/S transition are not well understood. Its kinase activity is low in G1 and its G1 substrates have not yet been identified. On the other hand, the Cdc28 kinase of *S. cerevisiae*, the functional and structural homologue of the Cdc2 kinase (Beach *et al.*, 1982), has been characterized more clearly for G1/S function. Activation of this kinase in G1 is governed by its association with unstable G1 cyclins (Cln1, 2, 3) which accumulate at G1/S phase (reviewed by Reed, 1992). The *CLN1* and *CLN2* genes are periodically expressed in G1/S phase and this expression is under control of the transcriptional activator complex Swi4-Swi6 (reviewed by Koch and Nasmyth, 1994). In turn, the activity of the Swi4-Swi6 complex depends on Cdc28 kinase. This dependency can be resolved by invoking a positive feedback loop whereby low levels of active Cdc28 kinase would allow some Swi4-Swi6 activation, leading to increased *CLN* expression and more active Cdc28 kinase (Figure 2) (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). This positive feedback circuit mechanism can lead to a very rapid activation of the cell cycle "start" system in response to environmental conditions.

B. $cdc10^+$

The other "start" gene in *S. pombe* is $cdc10^+$ and its function is required for entry into S phase and premeiotic S phase in vegetative growth and in the meiotic process, respectively (Nasmyth and Nurse, 1981; Beach *et al*, 1985; Grallert and Sipiczki, 1991).

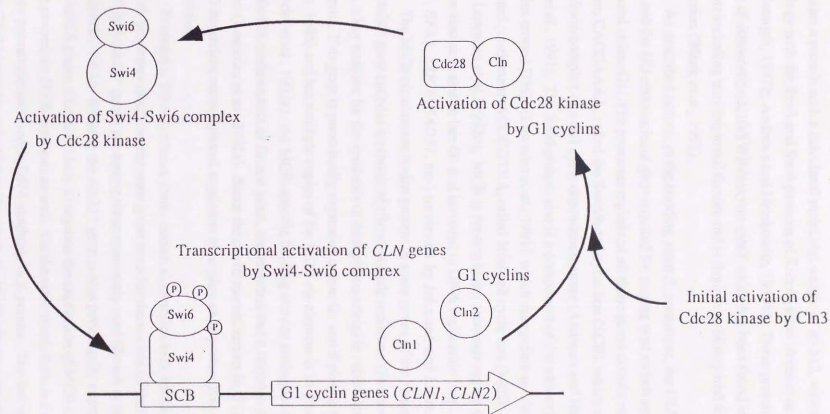

Fig.2. Cdc28 kinase of *S. cerevisiae* is regulated by positive feed-back loop in G1/S transition. The expression of *CLN1* and *CLN2* genes specifically appears around "start", but *CLN3* expression and protein levels do not change during cell cycle. Although Cln1, Cln2 and Cln3 play some overlapping functions in "start", recent experiments suggest that Cln3 is essential for the activation of *CLN1* and *CLN2* expression as a initial activator of Cdc28 kinase (Tyers *et al.*, 1993).

It encodes a protein with a calculated molecular weight of 85,512, which has significant homology with the Swi4 and Swi6 proteins of *S. cerevisiae* (Aves *et al.*, 1985; Breeden and Nasmyth, 1987b; Andrews and Herskowitz, 1989b). These proteins contain two copies of conserved cdc10/SWI6/ankyrin motif which has been found in a variety of proteins including transcriptional factors and is thought to be required for protein-protein interaction (Blank *et al.*, 1992).

As described before, in the budding yeast *S. cerevisiae*, the *CLN1* and *CLN2* genes and the *HO* endonuclease gene required for mating type switch are periodically expressed in late G1. The promoter regions of all these genes contain sets of a sequence element, CACGAAAA, called the Swi4/6 cell cycle box (SCB), which is activated by a Swi4-Swi6 complex in a cell cycle dependent manner (Andrews and Herskowitz, 1989a; Taba *et al.*, 1991). The Swi6 protein also is a component of the transcriptional activator complex termed DSC1 (Lowndes *et al.*, 1991), which recognizes another cell cycle regulated *cis*-element, ACGCGTNA, called *Mlu*I cell cycle box (MCB) (Dirick *et al.*, 1992; Lowndes *et al.*, 1992b). MCB is present in the promoter region of many genes that are also activated in late G1 and involved in DNA replication (e.g., *POL1*, *PRI1*, *RNR1*, *RFA1*, *CDC6*, *RAD51*, etc.) (reviewed by Johnston and Lowndes, 1992).

The MCB *cis*-element is also present in some genes of the fission yeast *S. pombe*. The *cdc22*$^+$ gene encodes a subunit of ribonucleotide reductase (Fernandez-Sarabia *et al.*, 1993), a key enzyme for the synthesis of deoxyribonucleotide substrates for DNA synthesis. This gene is periodically expressed in late G1 and S phases (Gordon and Fantes, 1986) and has multiple copies of the MCB *cis*-element in its promoter region (Lowndes *et al.*, 1992a). An MCB-specific binding activity analogous to DSC1 has been identified in crude extract of fission yeast, and reported that it contains the *cdc10*$^+$ gene product (Lowndes *et al.*, 1992a). Since the *cdc10* mutant arrests in G1 before "start", Cdc10-dependent transcriptional regulation must play an important role in G1/S progression.

Premeiotic DNA synthesis shares certain similarities with mitotic DNA synthesis, and both processes often use the same genes even for the control of their initiation. The *cdc10*$^+$ and *cdc22*$^+$ genes are among those commonly used (Beach *et al.*, 1985; Grallert and Sipiczki, 1991). Because the *cdc22*$^+$ gene is also periodically expressed during premeiotic S phase, Cdc10 is likely to regulate the expression of MCB-containing genes during premeiotic DNA synthesis as well. On the other hand, there is a clear dissimilarity between premeiotic and mitotic DNA synthesis in *S. pombe*. The former does not require Cdc2 kinase for its initiation (Beach *et al.*, 1985) whereas the latter does not require Mei2 (Bresch *et al.*, 1968). It is still largely unknown how the onset of meiosis is controlled following the activation of Mei2 (see below) and what other genes play key roles in its control.

C. *pat1*$^+$

Another interesting mutant that seems to be related to G1/S regulation has been isolated. In the *S. pombe*, the initiation of meiosis is committed by the action of Mei2 which is activated in a chain reaction following conjugation. In the mitotic cell cycle, the expression of the *mei2*$^+$ gene is repressed and its product is inactivated by the *pat1*$^+$ gene product, a Ser/Thr protein kinase (McLeod and Beach, 1986). When cells with opposite mating type are induced to conjugate by nitrogen starvation, the *mei3*$^+$ gene is turned on and inhibits the Pat1 kinase, resulting in the activation of Mei2. This leads to the initiation of premeiotic DNA synthesis (reviewed by Egel *et al.*, 1990). A temperature-sensitive *pat1* (*pat1*^{ts}) mutant was originally isolated as a suppressor mutant of *mei1* mutation which is defective in meiosis since it can not induce *mei3*$^+$ (Iino and Yamamoto, 1985), or as a mutant that is able to conjugate even in the rich medium (Nurse, 1985). At the restriction temperature, *pat1*^{ts} mutant cells arrest in G1 and undergo meiosis even if they are heterothallic haploid cells (Iino and Yamamoto, 1985). Thus, the *pat1*$^+$ gene seems to promote G1/S progression by inhibiting the Mei2 activity that causes G1 arrest of the cell cycle and initiates meiosis.

To understand more in detail how the cell cycle "start" of *S. pombe* is regulated and what genes play key role in its regulation, we screened for extragenic suppressors of temperature sensitive mutants of the G1/S phase related genes, *cdc2*$^+$, *cdc10*$^+$ and *pat1*$^+$. In this report, I describe the identification and characterization of three novel genes, *res1*$^+$, *res2*$^+$ and *rep1*$^+$.

The *res1*$^+$ gene have been isolated as a multicopy dual suppressor of the *pat1*^{ts} and *cdc10*^{ts} mutants (Tanaka *et al.*, 1992). It specifies a protein similar to Cdc10, Swi4 and Swi6, and is able to rescue a *cdc10* null mutant. Strikingly, Res1 and Swi4 are uniquely homologous in their amino-terminal region which has been shown to be the DNA binding domain of *SWI4* gene product (Primig *et al.*, 1992), whereas Cdc10 and Swi6 are uniquely so in their carboxy-terminal region (Breeden and Nasmyth, 1987b). The structural and functional similarities of Res1 to Swi4 and Cdc10 to Swi6 suggest that Res1 might form a complex with Cdc10 and act as a transcriptional factor, possibly for MCB, in a close analogy with the relationship between Swi4 and Swi6.

Although cells deleted for the *res1*$^+$ gene had a severe growth defect with heat- and cold-sensitivity, they were viable: they were not able to grow at all at 36°C or 23°C, but could grow at 30°C (Tanaka *et al.*, 1992). We considered that the ability of the *res1* null (*Δres1*) mutant to grow at 30°C was indicative of the presence of another gene functionally similar to *res1*$^+$. We therefore screened an *S. pombe* genomic library for multicopy suppressors of the heat- and cold-sensitive phenotype of the *Δres1* cells and identified one such gene, *res2*$^+$ (Miyamoto *et al.*, 1994). *res2*$^+$ encodes a protein which shares the highest sequence and structure similarity with the *res1*$^+$. *res2*$^+$ is also largely

redundant in function with *res1*$^+$ and is required for the initiation of mitotic and premeiotic DNA synthesis, but has an additional role in meiotic division. We concluded that the fission yeast contains two functionally overlapping parallel "start" systems, Res1-Cdc10 and Res2-Cdc10, the former of which plays a major role in mitotic cycle whereas the letter in meiotic cycle.

The *rep1*$^+$ gene was isolated as an extragenic suppressor of *cdc10*^{ts} mutant and could also effectively suppress the *Δres1* mutant (Sugiyama *et al.*, 1994). It encodes a protein with one zinc finger motif in its carboxy-terminal. *rep1*$^+$ transcript is highly induced during conjugation and an early phase of meiosis, and *Δrep1* mutant cells are unable to initiate premeiotic DNA synthesis when meiosis is induced after complete growth arrest. These results suggest that the *rep1*$^+$ gene product interacts with the Res-Cdc10 complex, thereby promoting the initiation of premeiotic DNA synthesis.

II. Results

Section 1

Fission yeast *Schizosaccharomyces pombe* contains two functionally overlapping parallel cell cycle start-specific transcriptional regulatory complexes, Res1-Cdc10 and Res2-Cdc10, which act in both mitotic and meiotic cycles

Isolation of *res1*$^{+}$ gene

The temperature-sensitive *pat1-114* mutation induces unconditional growth arrest and subsequent meiotic cell division even for heterothallic haploid cells upon shifting to the restriction temperature (Iino and Yamamoto, 1985). We screened an *S. pombe* expression cDNA library for suppressors of this temperature-sensitive mutant as described previously (Okazaki *et al.*, 1990), and isolated 9 distinct active cDNA clones. Among the 9 clones, two were *pat1*$^{+}$ (McLeod and Beach, 1986) and *pac2*$^{+}$ (Watanabe and Yamamoto, 1990) cDNAs, and the remaining 7 clones were new genes. To our surprise, one named *res1*$^{+}$ (required for entry into S phase, see below), suppressed a *cdc10*^{ts} mutation, and therefore, was further characterized. In a separate experiment, we re-isolated a *res1*$^{+}$ cDNA, as an extragenic suppressor of a *cdc10*^{ts} mutation (see Section 3; Sugiyama *et al.*, 1994).

The *res1*$^{+}$ cDNA suppressed the *pat1*^{ts} mutation, but only partially and at modest non-permissive temperatures up to 33°C (Figure 3). The colonies contained spores and sporulating cells. In contrast, the *res1*$^{+}$ gene suppressed the *cdc10*^{ts} mutation almost completely: suppression occurred even at 36°C (Figure 3), yielding cells with a normal size. Furthermore, not only the *res1*$^{+}$ cDNA expressed from the strong SV40 promoter, but also a *res1*$^{+}$ genomic DNA in a multicopy plasmid effectively rescued the *pat1*^{ts} and *cdc10*^{ts} mutants. Interestingly, both *pat1*$^{+}$ and *cdc10*$^{+}$ had no reciprocally suppressing activity to either mutant (Figure 3).

The *res1*$^{+}$ gene encodes a protein homologous with Cdc10 and Swi4

The nucleotide and deduced amino acid sequences of the 2.8 kb *res1*$^{+}$ cDNA are shown in Figure 4. The *res1*$^{+}$ cDNA contains a long 763 bp 5'-noncoding sequence followed by an open reading frame encoding a 637 amino acid protein with a calculated molecular weight of 72,441. The function of the long 5'-noncoding region is unclear, but its elimination increased the *cdc10*^{ts} mutation-complementing activity (see Figure 6). The size of the *res1*$^{+}$ mRNA was approximately 2.8 kb as estimated by Northern blot hybridization (see Figure 19D), indicating that this cDNA clone is full length or nearly full length. A homology search using the Swiss Prot Data Base detected the Cdc10

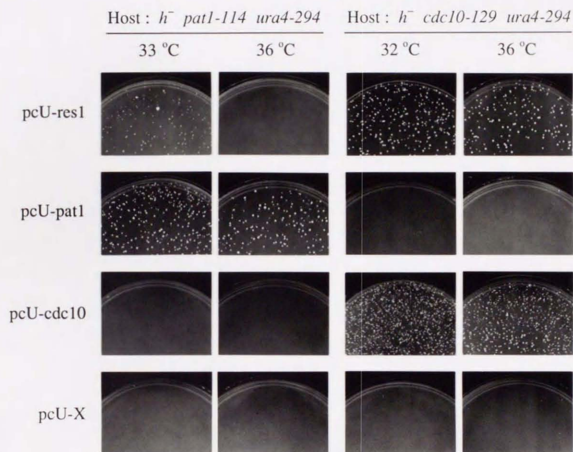

Fig.3. Suppression of *pat1*^{ts} and *cdc10*^{ts} mutants. The *pat1-114* (SO5) or *cdc10-129* (AS2) mutant cells were transformed with the indicated plasmids, spread on MMA plates and incubated at the indicated temperatures. pcU-X is a pcU vector plasmid with no insert and used as a negative control.

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-790                     GGGGGGGGGGGGGGGGGGGGGGGGGGGAGTATTTATCGTTTACCACTAACTCACCATAAGAGAGGGTGAA
-720  AGATTTAGTTCTGTTTGTGTGAGATCTTAAAAAAGCAAGCAAATTTCCTTTTTATCAAAAAAAGGATCCGCATTTCCTGAAGCACTTTAC
-630  TGTGATTTTAGGGATTTTTTTGGTGAGTAAGAAGCAGTGTTTTTCGATTTTTCTGAGATCTCGCTGTTGAAAAAATCCGGACTAAATCGG
-540  CTCCGTAGTCGATAAACCCAAATCTACAGCTTTTTGATTTAAATTATACAAATCGTGATCTGGAAAGTTTCGAAAGGTTTCTGTCCTTAC
-450  ATATTAAAGTTCTTGGTAGTTGAGTATCTTACATCAGCTGGTAAGATACGTGGATTTTTAGGGATAGCAGAGGTCTTTAAAAACATCTGT
-360  TTTTAACTCAAAACATAGAGTTAAATACTTTTTAATTGAAAAAATCTTTGAGGCAAACGCCGCTGAGAGAATATATCCTTTCTTTAGTGC
-270  ATGCAGTTAGTATCATATGTATTTCCGACTTTATCTGCTATAAATACTCGGTTTTCAAAAACTATCGATATTTTGTAGAGGAAATTTCAA
-180  TTTGCTTCTTCTTTCTCATTTGCTTCGTTTTTTTTTTTATATGATATTAATAGCTGGCGCTGTGGAGTTGAGACATTAAATTATTACAAA
 -90  CATTTAGAGAGACGAAACGTTGTTATTGCATCAAGTTTTGAAGCTCTTCCTTCCTTCCTTTTTTTTAAATTGCTTGCCAATAGTGCCGTT
   1  ATGTATAACGACCAAATACATAAAATCACCTATTCAGGAGTGGAAGTATTTGAGTATACGATCAATGGCTTTCCTTTAATGAAACGTTGT
   1   M  Y  N  D  Q  I  H  K  I  T  Y  S  G  V  E  V  F  E  Y  T  I  N  G  F  P  L  M  K  R  C
  91  CATGATAACTGGCTTAATGCCACGCAAATCCTTAAAATTGCAGAGCTCGATAAACCCAGGAGGACAAGGATTCTGGAGAAATTCGCTCAA
  31   H  D  N  W  L  N  A  T  Q  I  L  K  I  A  E  L  D  K  P  R  R  T  R  I  L  E  K  F  A  Q
 181  AAGGGACTTCATGAAAAAATTCAAGGTGGTTGTGGAAAATATCAAGGAACTTGGGTACCCTCTGAAAGAGCTGTTGAACTTGCTCATGAA
  61   K  G  L  H  E  K  I  Q  G  G  C  G  K  Y  Q  G  T  W  V  P  S  E  R  A  V  E  L  A  H  E
 271  TATAATGTTTTTGATTTAATACAACCTCTGATTGAATACTCTGGCTCTGCGTTCATGCCAATGTCTACGTTCACTCCTCAGAGCAATCGT
  91   Y  N  V  F  D  L  I  Q  P  L  I  E  Y  S  G  S  A  F  M  P  M  S  T  F  T  P  Q  S  N  R
 361  AAGCCTACAGAAGCATATCGACGAAACAGTCCAGTAAAAAAGTCATTTTCCCGGCCTTCGCATTCTTTACTCTACCCTTACACATCTTCC
 121   K  P  T  E  A  Y  R  R  N  S  P  V  K  K  S  F  S  R  P  S  H  S  L  L  Y  P  Y  T  S  S
 451  AATAATATGACATCTACTTCTAGAATGTCAGGAATCCATGATGCTTTGTCTCTTCAATCTGATTTCACGCGCTCTCCCGATATGCCTTCT
 151   N  N  M  T  S  T  S  R  M  S  G  I  H  D  A  L  S  L  Q  S  D  F  T  R  S  P  D  M  P  S
 541  GATTCCTTTACGGGATCTTTGCACGATATCAAAGCTTCTCCGTTTTCTTCAAACAATTATGCGCAGTCTCTCTTGGATTACTTTTTATTA
 181   D  S  F  T  G  S  L  H  D  I  K  A  S  P  F  S  S  N  N  Y  A  Q  S  L  L  D  Y  F  L  L
 631  CCAAATACTACACAACCTCCTGATTTCGTATATGATCGGCCATCAGATTGGGATGTTAATGCTGGCATTGATGAGGATGGTCATACAGCA
 211   P  N  T  T  Q  P  P  D  F  V  Y  D  R  P  S  D  W  D  V  N  A  G  I  D  E  D  G  H  T  A
 721  CTTCATTGGGCTGCTGCAATGGGAAATTTGGAAATGATGCATGCACTATTGCAAGCAGGCGCCAACGTAGTTGCTGTCAATTACCTTCAA
 241   L  H  W  A  A  A  M  G  N  L  E  M  M  H  A  L  L  Q  A  G  A  N  V  V  A  V  N  Y  L  Q
 811  CAAACATCTTTGATGCGCTGTGTAATGTTTACCATGAACTATGATTTACAGACTTTTGAGGTAGTTTCGGAGTTGCTTCAATCTGCTATC
 271   Q  T  S  L  M  R  C  V  M  F  T  M  N  Y  D  L  Q  T  F  E  V  V  S  E  L  L  Q  S  A  I
 901  TGCATGAACGACTCTTTTGGCCAAACCGTCTTTCACCACATCGCATTATTAGCTTCTTCTAAATCAAAGATGGAAGCCGCTCGTTATTAT
 301   C  M  N  D  S  F  G  Q  T  V  F  H  H  I  A  L  L  A  S  S  K  S  K  M  E  A  A  R  Y  Y
 991  ATGGATATCTTATTGCAAAATTTAACTGCAACACAATCCGTTGATGTCGCTGCCCAAATTATAAACCTTCAGGATGATCATGGGGATACC
 331   M  D  I  L  L  Q  N  L  T  A  T  Q  S  V  D  V  A  A  Q  I  I  N  L  Q  D  D  H  G  D  T
1081  GCTCTTTTGATCTGCGCTAGAAATGGTGCAAAGAAATGCGCGAGATTATTATTATCCTTTTATGCATCATCCTCCATACCAAATAATCAG
 361   A  L  L  I  C  A  R  N  G  A  K  K  C  A  R  L  L  L  S  F  Y  A  S  S  S  I  P  N  N  Q
1171  GGCCAATACCCAACAGATTTTTTGTCTTCGAAAGACATGTCTTTTCCCGAAAATGATGATTCCCCCTTAAATTCCAAAATCGAGGATAAT
 391   G  Q  Y  P  T  D  F  L  S  S  K  D  M  S  F  P  E  N  D  D  S  P  L  N  S  K  I  E  D  N
1261  TTGATAGATAACTTAAAATATCCGCAATCACTTGATGACCACTTATCGTCCAAAAAACCTATTTCTTACTTTTCTAATAAACTTACTCAC
 421   L  I  D  N  L  K  Y  P  Q  S  L  D  D  H  L  S  S  K  K  P  I  S  Y  F  S  N  K  L  T  H
1351  CAAACCCTTCCCAATGTCTTCACTCAGCTGAGCGAACTTTCAAAATGCCATGAAGCATCTCTTGCTGAAAAACAACTAACCTATAATTTG
 451   Q  T  L  P  N  V  F  T  Q  L  S  E  L  S  K  C  H  E  A  S  L  A  E  K  Q  L  T  Y  N  L
1441  GCAATGGAGGCGTTGGAGCAAACAGTTCGGGAAACTGAAACTTGCCAAAGATTATGGAATGAACGTACCAATAATGACGAGAACTACTTG
 481   A  M  E  A  L  E  Q  T  V  R  E  T  E  T  C  Q  R  L  W  N  E  R  T  N  N  D  E  N  Y  L
1531  GTAAATCAGCGGGAGGATTTGATTCACCAGTGTAAAAAATTTCTGCATACATTGAAAACTGCTAGATATTACCTTGAAACAGTTCAGCTT
 511   V  N  Q  R  E  D  L  I  H  Q  C  K  K  F  L  H  T  L  K  T  A  R  Y  Y  L  E  T  V  Q  L
1621  CATCAATTAAAAAAATACGTAACTTACTTTTCTCAAATATGGAGTACTGATGAGTTAGCTGATATCTCGGAGACCAAAAACCTTGTTGGT
 541   H  Q  L  K  K  Y  V  T  Y  F  S  Q  I  W  S  T  D  E  L  A  D  I  S  E  T  K  N  L  V  G
1711  CATGATACTAAAACGAACCGTTCGTCTTTAAGCTCAAAACACGAAGTTGATCTTTTTACAGCTGAAAACGAAGCTGCTCGTGAAAAGCTC
 571   H  D  T  K  T  N  R  S  S  L  S  S  K  H  E  V  D  L  F  T  A  E  N  E  A  A  R  E  K  L
1801  GTTGAGCAATTATGCTCATTGCAAGCGCAACGGAAACAGAAAATTAACGAAATTTTGAACTTGCTTTCTATGGGAATGTATAATACGATT
 601   V  E  Q  L  C  S  L  Q  A  Q  R  K  Q  K  I  N  E  I  L  N  L  L  S  M  G  M  Y  N  T  I
1891  AATACAGATCAAAGTGGATCTTAATTTTTTTGGTTTTAAATTATTATTCAAAATAATTTTTTCTTTCGTTATCTTATTTTTTGCATTCTT
 631   N  T  D  Q  S  G  S  *
1981  TATGTTTTTTATATAAATACCAACTTGAACTTATAGATATTTAACAATCTTTTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig.4. Nucleotide and predicted amino acid sequences of the *res1*$^{+}$ cDNA (EMBL sequence database accession number X68789). The amino acid sequence is shown below the DNA sequence in a single-letter code. Nucleotide numbering starts at the initiating ATG of the predicted *res1*$^{+}$ translation product.

protein of *S. pombe* as the one with the highest homology: 27 % of the amino acids are identical in the entire protein, and 40 % are identical in the central 161 amino acid region (Figure 5A). It is also significantly homologous with Swi4 and Swi6 of *S. cerevisiae*, which are required for the expression of the *HO* endonuclease gene (Stern *et al.*, 1984; Breeden and Nasmyth, 1987a) and the G1 cyclin genes (*CLN1*, *CLN2*) (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). Within these four protein molecules, the highest homology is found in the two 33-amino acid repeats generally called the cdc10/SWI6 motif or ankyrin repeat (50 % identity between Res1 and Cdc10, Figure 5C) and in their surrounding region. Interestingly, an additional similarity resides uniquely in the amino-terminal region of Res1 and Swi4 (the amino-terminal 150 amino acids are 40 % identical), and in the carboxy-terminal region of the Cdc10 and Swi6 proteins (the carboxy-terminal 141 amino acids are 40 % identical) (Figure 5B, D, E). Thus, in structure, Res1 resembles Swi4 whereas Cdc10 resembles Swi6.

The amino-terminal region is essential for Res1 function

To define the functional domain of Res1, deletion mutants were constructed and their ability to suppress the *cdc10*^{ts} mutant was determined. As shown in Figure 6, the entire carboxy terminal region after the second cdc10/SWI6/ankyrin motif was totally dispensable for the function, and Res1's truncated in the carboxy-terminal region retained a nearly full activity. On the contrary, the amino-terminal region that is homologous with Swi4 was absolutely essential for the Res1 activity. Even a deletion of only 35 amino acids within this region completely knocked off the suppression activity. This result was also confirmed by using a *Δres1* mutant as a host.

res1^{+} is required for G1/S progression

To investigate the physiological role of *res1*$^{+}$, a null allele of *res1*$^{+}$ was constructed by one-step gene replacement. A *res1*$^{+}$ genomic DNA fragment was isolated, and the 1.7 kb *Sac*I-*Sca*I fragment containing more than 80 % of the *res1*$^{+}$ coding region (including the two cdc10/SWI6/ankyrin motifs and two thirds the region homologous with Swi4) was replaced with the 1.8 kb *ura4*$^{+}$ gene fragment (pUC-BEres1::ura4) (Figure 7A). Inactivation of *res1*$^{+}$ in the construct was confirmed by its inability to rescue the *cdc10*^{ts} mutant.

A diploid strain (DP2) was transformed to ura^{+} with the *res1::ura4*$^{+}$ DNA excised from pUC-BEres1::ura4 with *Bam*HI and *Eco*RI endonucleases. Southern blot hybridization of genomic DNAs prepared from 4 representative transformants displaying a stable ura^{+} phenotype revealed that *res1*$^{+}$ was successfully disrupted in 3 transformants (Figure 7B). Tetrad analysis of the disruptants showed a 2:2 segregation of ura^{-} and presumed ura^{+} progeny. However, haploid segregants containing disrupted *res1*$^{+}$ (ura^{+} segregants) displayed a low viability, and many of ura^{+} progeny were unable to grow.

A

```
Cdc10   (58aa)-LRDGRPVELYAVECSGMKYMELSCGDNVALRRCPDSYFNISQILRLAGTSSSENAKELDDIIESGDYENVDSKHPQIDGVWVPYDRAISIAKRYGVYEILQPLISFNLDLF--
Res1              MYNDQIHKITYSGVEVFEYTINGFPLMKRCHDNWLNATQILKIAELDKPRRTRILEKFAQKGLHEKIQGGCGKYQGTWVPSERAVELAHEYNVFDLIQPLIEYSGSAFMP
Res2            MAPRSSAVHVAVYSGVEVYECFIKGVSVMRRRRDSWLNATQILKVADFDKPQRTRVLERQVQIGAHEKVQGGYGKYQGTWVPFQRGVDLATKYKVDGIMSPILSLDIDEGKA
```

homologous with Swi4 DNA binding domain

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Cdc10   -PKFSKQQQIESSSI-SKNLNTSSFNTRSPLRNHNFSNPSKS-SKNGVHTINNMQSSPSPSSSFLL-PLTQIDSQNV-(42aa)-SLFPDTPRLNHSLSFVSLVSSLPPLDQNIMQDYHT
Res1    MSTFTPQ------SN-RKPTEAYRRN--SPVKKS-FSRPSHS-LLYPYTSSNNMTST-SRMSGIHD-ALSL---QSDF-------TRSPDMPSD-------SFTGSLHDIKASPFSSNNY
Res2    IAPKKKQ------TKQKKPSVRGRRG-RKPSSLS--SSTLHSVNEKQPNSSISPTIESSMNKVNLPGAEEQV-SATPLPAS----PNALLSPND-------NTIKPVEELGMLEAPLDKY
```

```
Cdc10   SKDILTSIFLDVNFADSSALEAKLSDSLDIDVPIDELGHAALHWAAAVAKMPLLQALIHKGANPLRGNLTGETALMRSVLVTNHLNQNSFGDLLDLLYASLPCTDRAGRTVVHHICLTAG
Res1    AQSLL-DYFLLPNTTQPPDFVYDRPSDWDVNAGIDEDGHTALHWAAAMGNLEMMHALLQAGANVVAVNYLQQTSLMRCVMFTMNYDLQTFEVVSELLQSAICMNDSFGQTVFHHIALLAS
Res2    EESLL-DFFLHPEEGRIPSFLYSPPPDFQVNSVIDDDGHTSLHWACSMGHIEMIKLLLRANADIGVCNRLSQTPLMRSVIFTNNYDCQTFGQVLELLQSTIYAVDTNGQSIFHHIVQSTS
```

cdc10/SWI6/ankylin motif I

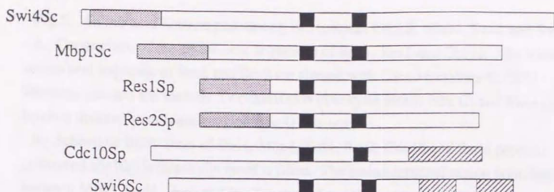
```
Cdc10   IKGRGSASRYYLETLLNWAKKHASGNNGYMLKDFINYLNHQDKNGDTALNIAARIGNKNIVEVLMQAGASAYIPNRAGLSVANFGIFVENALKQPEDSK--QTKV--SLM---------
Res1    SKSKMEAARYYMDILL--QNLTATQSVDV--AAQI--INLQDDHGDTALLICARNGAKKCARLLLSFYASSSIPNNQGQYPTDFLSSKDMSFPENDDSPL-NSKIEDNLIDNLKYPQSLD
Res2    TPSKVAAAKYYLDCIL--EKLISIQPFEN--VVRL--VNLQDSNGDTSLLIAARNGAMDCVNSLLSYNANPSIPNRQRRTASEYLLEADKKPHSLLQSNSNASHSAFSFSGISPAIISPS
```

cdc10/SWI6/ankylin motif II

```
Cdc10   ---------SENLSSKEKTAVPPRQKSRDIIASVTDVISSLDKDFQDEMAAKQSMIDSAYTQLRESTKKLSDLREQLHVSETQ-RTLFL-----ELRQRCKNLMTSIEEQKSELSNLYES
Res1    DH----------LSSKKPISYFSNKLTHQTLPNVFTQLSELSKCHEASLAEKQLTYNLAMEAL-EQTVRETETCQRLWNERTNNDENYLVNQREDLIHQCKKFLHTLKTARYYL-ETVQL
Res2    CSSHAFVKAIPSISSKFSQLAEEYESQLREKEEDLIRANRLKQDTLNEISRTYQELTFLQKNNPTYSQSMENLIREAQETYQQLSKRLLIWLEARQIFDLERSLKPHTSLSISFPSDFLK
```

```
Cdc10   FDPNGIHDSLSLDADAPFTVNENNNKNLSIAELKFQVAAYERNEARLNELANKLWQRNSNIKSKCRRVVSLCTGVDESR-VDSLLESLLQAVESDGQQGEVDMGRVAGFLRVVKEHQA
Res1    HQLKKYVTYFSQIWSTDELADISETKNLVGHDTKT---------------------NRSSLSSKHEVDLFTAENEAAREKLVEQLCSLQA-QRKQKINEILNLLSMGMYNTINTDQSGS
Res2    KEDGLSLNNDFKKPACNNVTNSDEYEQLINKLTSL---------------------QASRKKDTLYIRKLYEELGIDDTVNSYRR--LIA-MSCGINPEDLSLEILDAVEEALTREK
```

B



C

```
Swi4-1   NFEIDDQGHTPLHWATAMANIPLIKMLITLNAN     Swi4-2   LNYQDNIGNTPLHLSALNLNFEVYNRLVYLGAS
Mbp1-1   DAPIDPELHTAFHWACSMGNLPIAEALYEAGTS     Mbp1-2   LNTQDKNGDTALHIASKNGDVVFFNTLVKMGAL
Res1-1   NAGIDEDGHTALHWAAAMGNLEMMHALLQAGAN     Res1-2   INLQDDHGDTALLICARNGAKKCARLLLSFYAS
Res2-1   NSVIDDDGHTSLHWACSMGHIEMIKLLLRANAD     Res2-2   VNLQDSNGDTSLLIAARNGAMDCVNSLLSYNAN
Cdc10-1  DVPIDELGHAALHWAAAVAKMPLLQALIHKGAN     Cdc10-2  LNHQDKNGDTALNIAARIGNKNIVEVLMQAGAS
Swi6-1   NIPVDEHGNTPLHWLTSIANLELVKHLVKHGSN     Swi6-2   LNAQDSNGDTCLNIAARLGNISIVDALLDYGAD
```

D

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Swi4   (31aa)-SNHPVIEIATYSETNVYECYIRGFETKIVMRRTKDDWINITQVFKIAQFSKTKRTKILEKESNDMQHEKVQGGYGRFQGTWIPL
Mbp1          MSNQIYSARYSGVDVYEFIHSTG---SIMKRKKDDWVNATHILKAANFAKAKRTRILEKEVLKETHEKVQGGFGKYQGTWVPL
Res1         MYNDQIHKITYSGVEVFEYTINGF---PLMKRCHDNWLNATQILKIAELDKPRRTRILEKFAQKGLHEKIQGGCGKYQGTWVPS
Res2        MAPRSSAVHVAVYSGVEVYECFIKGV---SVMRRRRDSWLNATQILKVADFDKPQRTRVLERQVQIGAHEKVQGGYGKYQGTWVPF

Swi4   DSAKFLVNKYEIIDPVVNSILTFQFDPNNPPPKRSKNSILRKTSPGTKI-TSPSSYNKTRT----KKN------SSS---STS-(909aa)
Mbp1   NIAKQLAEKFSVYDQLKPLFDFTQTDGSASPPPAPKHHHASKVDRKKAI-RSASTSAIMETKRNNKKAEENQFQSSKILGNPT-(671aa)
Res1   ERAVELAHEYNVFDLIQPLIEYS---GSAFMPM-STFTPQSNRKPTEAY-RRNSPVKKSFSRPSHSLLYPY--TSSNNMTSTS-(480aa)
Res2   QRGVDLATKYKVDGIMSPILSLDIDEGKAIAPK-KKQTKQKKPSVRGRRGRKPSSLSSS-TLHSVNEKQPN---SSISPTIES-(496aa)
```

E

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Cdc10  (608aa)-LREQLHVSETQRTLFLELRQRCK-NLMTSIEEQK-SEL--SN-LYESFDPN-GIH-DSLSLDADAPFTVNENNNKNLSI
Swi6   (601aa)-SREQL-ANVKQLKDEYSLMQEQLTNLKAGIEEEEESFREESKKLGIIADESSGIDWDSSEYDADEPFKVEF-(49aa)-

Cdc10  AELKFQVAAYERNEARLNELANKLWQRNSNIKSKCRRVVSLCTGVDESRVDSLLESLLQAVESDGQQGEVDMGRVAGFLRVVKEHQA
Swi6   VLLKARINAYKRNDHKLTNVLDTISTKQSELENKFRRVLSLCLKIDENKVDNMLDGLLQAISSEDPQ-DIDTDEMQDFL---KKHAS
```

Fig.5. Amino acid homologies among Res1, Res2, Cdc10, Mbp1, Swi4 and Swi6.

A. Comparison of the amino acid sequences of Res1, Res2 and Cdc10. The whole amino acid sequence of Res1 and Res2 are aliened with Cdc10 (residues 62-767). Identical matches are shaded. Two cdc10/SWI6/ankylin motifs (see C) and Swi4 DNA binding domain homologous region (see **D**) are boxed.

B. Schematic illustration of Swi4, Mbp1, Res1, Res2, Cdc10 and Swi6 proteins. The conserved cdc10/SWI6/ankylin motif is filled. The amino-terminal region homologous between Mbp1, Swi4, Res1 and Res2 is shaded and the carboxy-terminal region homologous between Cdc10 and Swi6 is hatched.

C. Homology in the region of the conserved cdc10/SWI6/ankylin motif between Swi4 (amino acid 514-546, 635-667), Mbp1 (388-420, 506-538), Res1 (230-262, 351-383), Res2 (241-273, 362-394), Cdc10 (350-382, 477-509), and Swi6 (311-343, 462-494) proteins. Two copies of this motif are present in the central region of these proteins. The numbers shown after the names indicate either the first or second copy. Identical amino acids with Res1 are boxed.

D. Alignment of the amino-terminal sequences of Swi4 (amino acid 32-184), Mbp1 (1-162), Res1 (1-157) and Res2 (1-161). Identical amino acids with Res1 are boxed.

E. Alignment of the carboxy-terminal sequences of Cdc10 (top line, amino acid 610-767) and Swi6 (bottom line, 603-803 except for a 53 amino acid gap from 670 to 722). This homology was reported by Breeden and Nasmyth (1987b), but is shown here again for comparison to the amino-terminal homology of Res1 and Swi4. Identical amino acids are boxed.

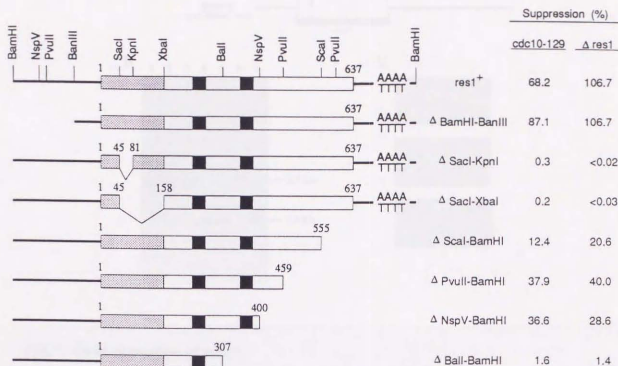


Fig.6. Deletion analysis of the functional domain of Res1. The intact *res1*+ cDNA is shown at the top. The protein coding region is boxed, the cdc10/SWI6/ankylin motif is filled and the region homologous with Swi4 is shaded. The amino acid numbers are shown at the each protein coding region. Extra 7 (Δ*Bal*I-*Bam*HI) or 23 (Δ*Sca*I-*Bam*HI, Δ*Pvu*II-*Bam*HI and Δ*Nsp*V-*Bam*HI) amino acids from pcL vector were added in carboxy-terminal of each deletion mutants. The suppression activities of deletion mutants are indicated in the right column as percentages of suppressed cells per stably transfected cells.

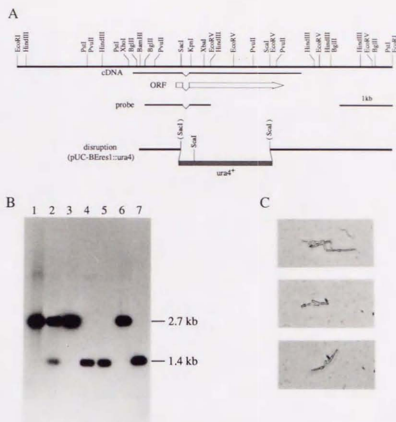


Fig.7. Gene disruption of *res1*$^{+}$.

A. Restriction map of the *res1*$^{+}$ gene. The *res1*$^{+}$ cDNA, the open reading frame encoded by the *res1*$^{+}$ gene, the 1.1 kb *BglII*-*Eco*RV fragment used as a probe for Southern hybridization, and construction of pUC-BEres1::ura4 are shown below the map. The coding region of the *res1*$^{+}$ gene is interrupted by a 127 bp intron which was confirmed by DNA sequencing. The junctions of this intron contain typical consensus sequences for S. pombe introns (5' splice site, **GT**TTGT; branch site, CTA**A**C; 3' splice site, C**AG**). The *res1*$^{+}$ gene may contain additional small intron(s) which escaped our detection.

B. Southern blot detection of the disrupted chromosomal *res1*$^{+}$ gene. The *res1*$^{+}$ gene was disrupted as described in Materials and methods. Genomic DNA was prepared from each strains, digested with *Xho*I and *Sca*I, separated by agarose gel electrophoresis and blotted to a nitrocellulose filter. Lane 1, wild type (L972); Lane 2, diploid strain carrying the *res1::ura4*$^{+}$ on one chromosome (D1787); Lane 3-6, a set of haploid progenies derived from D1787 (D1787-5A, 5B, 5C, 5D); Lane 7, pUC-EEres1::ura4 used as a marker for disrupted gene. The *res1*$^{+}$ specific hybridization probe used is shown in the panel A. pUC-EEres1::ura4 was constructed by inserting the *Eco*RI-*Bam*HI fragment of the *res1*$^{+}$ genomic DNA (5' upstream) into pUC-BEres1::ura4. The 2.7 kb band corresponds to the intact *res1*$^{+}$ allele and the 1.4 kb band to the disrupted allele.

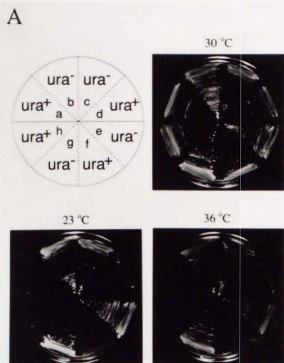
C. Terminal phenotype of *Δres1* mutant cells that unable to grow. Cells were germinated on YEA plate at 30°C and photographed under a microscope.

The percentage colony formation at 30°C of the ura⁻ and ura⁺ progenies of these three strains was 72:20, 87:20 and 70:23, respectively. In microscopic examination, *Δres1* spores actually germinated, divided once or twice, and then often died with an extremely elongated cell shape (Figure 7C). These results indicate that the *res1*⁺ gene is vital for normal cell growth.

The *Δres1* cells that grew at 30°C were back-crossed with the wild type strain to eliminate possible second mutations, and used for further characterization. At 30°C, *Δres1* cells (K123-14D) grew with a long lag and a slightly longer doubling time of 180 min versus 144 min for the wild type strain (K137-10). The *Δres1* cells growing in a log phase at 30°C displayed an increased cell size (Figure 8B). Indeed, the size at division of *Δres1* cells (K123-14D, 17.8 μm) growing at this temperature was approximately 24 % longer than that of the wild type cells (K137-10, 14.3 μm) growing under the identical conditions. Furthermore, *Δres1* cells possessed severe heat- and cold-sensitivities with complete growth inhibition at 23°C and 36°C (Figure 8A). At these non-permissive temperatures, *Δres1* cells underwent extreme elongation, typical of a *cdc* phenotype (Figure 8B). Although both the *res1*⁺ cDNA and the *res1*⁺ gene could rescue these heat- and cold-sensitive phenotypes effectively, Δ*Sac*I-*Kpn*I and Δ*Sac*I-*Xba*I deletion mutants had no complementation activities (see Figure 6). Flow cytometry of *Δres1* cells revealed that they had a distinctive cell cycle distribution pattern. Unlike the wild type cells, a significant number of growing *Δres1* cells (K123-14D) had a 1n DNA content (Figure 9B). Upon shifting to the non-permissive temperatures, *Δres1* cells were arrested exclusively in G1 (Figure 9C, D). On the other hand, *Δres1* cells were able to arrest in G1 upon nitrogen starvation (Figure 9E).

To confirm the G1 arrest of *Δres1* cells at non-permissive temperature, we assayed their conjugation ability. The *Δres1* cells (K123-14D) were cultured at 30°C to a mid-log phase in nitrogen-rich minimal medium, transferred to the non-permissive temperature (18°C), and incubated further to induce G1 arrest. The cells were harvested, resuspended in nitrogen-free minimal medium, and mixed with cells of an opposite mating type (SO7) to allow conjugation to take place. After a 48 hr incubation at the non-permissive temperature, the cell suspension was spread on a plate to determine the number of diploid cells formed by conjugation. The same experiments were performed with the wild type cells as a positive control. The conjugation frequencies of *Δres1* cells relative to those of the wild type cells, obtained in two separate experiments, were 90 and 240 %. Thus, G1-arrested *Δres1* cells conjugate at least as efficiently as the wild type strain. These results taken together led us to conclude that the arrest point of *Δres1* cells was indeed in G1, and thus, *res1*⁺ is required for the "start" of S phase.

The *res1*⁺ gene is also required for meiosis. Diploid strains homozygous (K135) or heterozygous (K134) for *Δres1* were constructed and assayed for their ability to sporulate at permissive (30°C) and non-permissive (23°C) temperatures. The



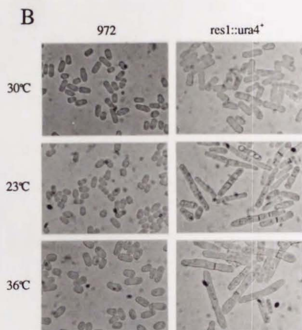


Fig.8. Phenotypes of *Δres1* mutant.

A. Heat- and cold-sensitive growth phenotypes associated with the null allele of the *res1*$^+$ gene. Two set of haploid progenies (upper half: a, b, c, d; lower half; e, f, g, h) derived from diploid cells that carry the null allele of the *res1*$^+$ gene on one chromosome was inoculated on MMA supplemented with adenine, uracil and leucine, and then incubated at the indicated temperatures. a, K123-14A; b, K123-14B; c, K123-14C; d, K123-14D; e, K130-1A; f, K130-1B; g, K130-1C; h, K130-1D

B. Cell morphologies of the wild type cell (L972) and a *Δres1* mutant (K123-14D). Cells were inoculated on MMA containing adenine and leucine, and incubated overnight at the indicated temperatures and photographed under a microscope. Bar = 10μm.

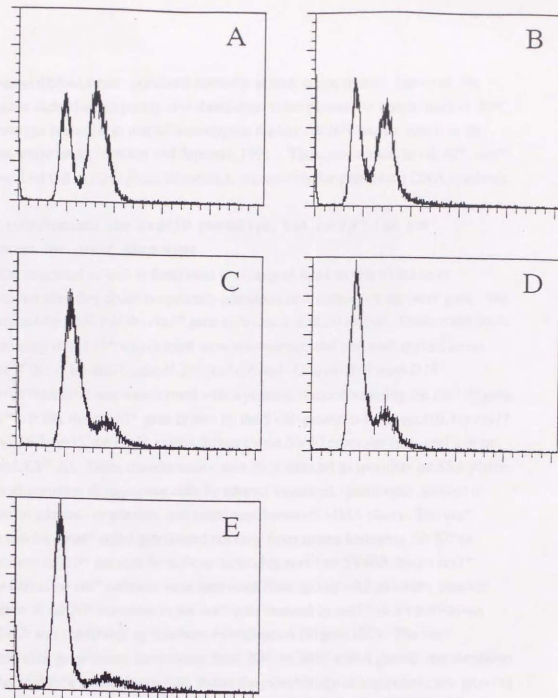


Fig.9. Flow cytometry of cells with a null allele of the *res1*$^+$ gene. Flow cytometry of the indicated strains was performed as described in Materials and methods. The panels show frequency histograms of the number of cells in each condition. All cells were cultured in minimal medium containing adenine, uracil and leucine, or in nitrogen-free minimal medium.

A. The *cdc10-129* mutant incubated at a restriction temperature (37°C) for 2 hr. At this time point, the *cdc10*^{ts} cells were in the middle of arresting in G1.

B. The *Δres1* mutant growing at the permissive temperature (30°C). The cell concentration was about 5 x 10^6 cells/ml.

C. The *Δres1* mutant incubated at 37°C for 2 hr.

D. The *Δres1* mutant incubated at 18°C for 8 hr.

E. The *Δres1* mutant incubated at 30°C for overnight in nitrogen-free minimal medium.

heterozygous diploid strain sporulated normally at both temperatures. However, the homozygous diploid strain poorly sporulated even at the permissive temperature of 30°C. This phenotype is similar to that of homozygous diploid *cdc10*ts mutant strains at the restrictive temperature (Grallert and Sipiczki, 1991). Thus, just similar to *cdc10*+, *res1*+ is also required for an early phase of meiosis, presumably for premeiotic DNA synthesis.

res1+ complements the *Δcdc10* phenotype, but *cdc10*+ can not complement the *Δres1* phenotype

The structural as well as functional similarity of Res1 to Cdc10 led us to investigate whether they could reciprocally complement mutations of the other gene. We first examined the ability of the *res1*+ gene to rescue a *Δcdc10* mutant. Diploid strains in which one copy of *cdc10*+ was deleted were constructed, and one such diploid strain DC107 (*h+N/h-S ade6-M216/ade6M-210 leu1-32/leu1-32 ura4-D18/ura4-D18 cdc10::ura4+/cdc10+*) was transformed with a plasmid vector containing the *cdc10*+ gene (pALSK+-cdc10), the *cdc10*+ gene driven by the SV40 promoter (pcL-cdc10), the *res1*+ gene (pALSK+-res1), the *res1*+ cDNA driven by the SV40 promoter (pcL-res1), or no insert (pALKS+-X). These transformants were then induced to sporulate on SSA plates, and, after elimination of vegetative cells by ethanol treatment, spores were allowed to germinate on adenine- or adenine- and uracil-supplemented MMA plates. The ura+ colonies (*cdc10::ura4*+ cells) germinated not only from spores harboring *cdc10*+ or SV40P-driven *cdc10*+ but also from those harboring *res1*+ or SV40P-driven *res1*+ cDNA, whereas no ura+ colonies were recovered from spores with an empty plasmid. The absence of *cdc10*+ sequence in the ura+ cells rescued by *res1*+ or SV40P-driven *res1*+ cDNA was confirmed by Southern hybridization (Figure 10C). The ura+ germinated cells grew at any temperature from 20°C to 36°C with a growth rate similar to that of the wild type cells. Figure 10A shows the morphology of logarithmically growing cells rescued by SV40P-driven *cdc10*+ or SV40P-driven *res1*+ cDNA. The rescued cells were similar in size to those rescued by *cdc10*+, indicating that *res1*+ overexpression almost completely cures the defects caused by the *Δcdc10* mutation.

In contrast, *cdc10*+ is unable to rescue the heat- and cold-sensitive growth phenotypes of *Δres1* cells. A *Δres1* strain K123-14D was similarly transformed with the *cdc10*+ gene, the *cdc10*+ gene driven by the SV40 promoter, the *res1*+ gene, the *res1*+ cDNA driven by the SV40 promoter, or empty plasmids, and then determined for its growth ability at 23°C and 36°C. Whereas *Δres1* cells transformed with the *res1*+ cDNA or the *res1*+ gene could grow at these temperatures, those transformed with the *cdc10*+ gene failed to grow at both temperatures (Figure 10B), irrespective of whether *cdc10*+ was expressed from its own promoter or from the SV40 promoter.

These results indicate that Res1 and Cdc10 proteins functionally overlap each other. Under certain limited conditions, such as 30°C or Res1 overproduction, either

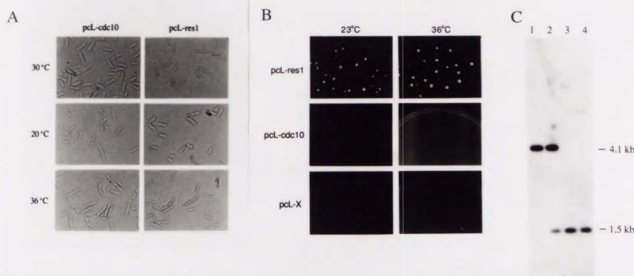


Fig.10. The *res1*$^+$ gene can rescue the *Δcdc10* mutation, but the *cdc10*$^+$ gene can not rescue the *Δres1* mutation.

A. Suppression of the *Δcdc10* mutation by *res1*$^+$. The *Δcdc10* mutant strains suppressed by the indicated plasmids were cultured at the indicated temperatures. Cells were logarithmically grown in minimal medium containing adenine.

B. *Δres1* mutation is not suppressed by *cdc10*$^+$. The *Δres1* mutant (K123-14D) cells were transformed with the indicated plasmids, spread on MMA plates containing adenine and incubated at the indicated temperatures. pcL-X is a pcL vector plasmid with no insert and used as a negative control.

C. Southern blot detection of the disrupted chromosomal *cdc10*$^+$ gene of *Δcdc10* mutant cells that suppressed by *res1*$^+$. The *cdc10*$^+$ gene was disrupted as described in Materials and methods. Genomic DNA was prepared from each strains, digested with *Hin*dIII, separated by agarose gel electrophoresis and blotted to a nitrocellulose filter. Lane 1, wild type (L972); Lane 2, diploid strain carrying the *cdc10::ura4*$^+$ on one chromosome (DC107); Lane 3, *Δcdc10* mutant suppressed by pcL-res1; Lane 4, *Δcdc10* mutant suppressed by pAL-res1. The 4.1 kb *Hin*dIII fragment is used as a *cdc10*$^+$ specific hybridization probe. The 4.1 kb band corresponds to the intact *cdc10*$^+$ allele and the 1.5 kb band to the disrupted allele.

Res1 or Cdc10 is dispensable for growth. However, Res1 can replace Cdc10 but not vice versa. This strikingly resembles the fact that whereas the *SWI4* gene suppresses the *swi6* mutation, the *SWI6* gene can not suppress the *swi4* mutation (Breeden and Nasmyth, 1987b; Andrews and Herskowitz, 1989b), and, therefore, provides further indication for the similarity of Res1 to Swi4 and Cdc10 to Swi6.

Isolation of *res2⁺* gene

Since the *res1⁺* gene could rescue the *Δcdc10* mutant but not vice versa, the function crucial for the "start" of the cell cycle resides in Res1 but not Cdc10. However, cells deleted for the *res1⁺* gene could grow at 30°C, though poorly. We considered that this viability was indicative of the presence of another gene functionally similar to *res1⁺*. To search for such putative *res1⁺*-related genes acting during the G1/S transition in mitotic cell cycle, we screened an *S. pombe* genomic library for those which were able to suppress the cold-sensitivity of a *Δres1* mutant, and one gene did not hybridize with *res1⁺* and was chosen for further analyses. This gene suppressed both the cold- and heat-sensitivities of *Δres1* mutant cells as nearly as the *res1⁺* gene (Miyamoto *et al.*, 1994). The new gene was capable of encoding a 657 amino acid protein which had two copies of the cdc10/SWI6/ankyrin motif and homology with the Cdc10/Swi family members, particularly with Res1, throughout the entire region (see Figure 5A). Therefore, this new gene was named *res2⁺*. Res2 retains a well conserved sequence commonly found in the amino-terminal region of Res1, Swi4 and recently identified Mbp1 (Koch *et al.*, 1993), but not of Cdc10 or Swi6 (see Figure 5D). This motif is essential for the function of Res1 and Swi4 (see Figure 6; Priming *et al.*, 1992).

res2⁺ is functionally similar to *res1⁺* and is required for the initiation of mitotic cycle

To identify the role of the *res2⁺* gene, we constructed a *Δres2* mutant by one step gene replacement. The *Δres2* mutant cells had no apparent mitotic growth defect at any temperature between 21°C and 36°C, with a morphology similar to that of wild type cells. To obtain the genetical relationship of *res2⁺* with other "start" genes, *res1⁺* and *cdc10⁺*, we constructed double mutants and examined its growth properties. First, we constructed a *Δres1 Δres2* double mutant. As previously described, *Δres2* cells grow at 30°C without any apparent defect and *Δres1* cells also grow at this temperature, though poorly. On the contrary, *Δres1 Δres2* double disrupted cells were unable to grow even after a prolonged incubation (Miyamoto *et al.*, 1994). The efficient suppression of the *Δres1* phenotype by *res2⁺* and the synthetic lethality with *Δres1* mutant strongly indicate that *res2⁺* is functionally redundant with *res1⁺* and acts as a "start" gene for mitotic cell cycle. This indication is further supported by the following data.

The temperature-sensitive *cdc10-129* mutant has no visible growth defect at permissive temperatures around 25°C. We crossed this mutant with a *Δres2* mutant. The resulting *cdc10-129 Δres2* double mutant cells were, however, unable to grow at 25°C. Thus cells with the *cdc10-129* mutation unconditionally require *res2*$^+$ for the mitotic growth, perhaps as a result of less efficient interaction of the mutated Cdc10 with the Res1 protein even below the restriction temperature. As described before, Res1 protein has been implicated in acting in association with the Cdc10 protein and the recent biochemical data support this implication (see Figure 11; Caligiuri and Beach, 1993). We examined the ability of *res2*$^+$ to rescue temperature-sensitive *cdc10-129* and *Δcdc10* mutants. The *res2*$^+$ gene efficiently suppressed the *cdc10-129* mutant even at 36°C, but very inefficiently a *Δcdc10* mutant (Miyamoto *et al.*, 1994). This indicates that like Res1, Res2 acts in association with Cdc10, but unlike Res1, Res2 highly depends on Cdc10 for its function.

We therefore, analyzed the association of Res1 and Res2 with Cdc10 *in vivo* by using the *GAL4* two-hybrid system (Fields and Song, 1989). Full-length Res1 and Res2 were fused with the DNA binding domain of Gal4 (GBT9-res1, GBT9-res2), and full-length Cdc10 was fused with the activator domain of Gal4 (GAD424-cdc10). These plasmids were then transfected into the *S. cerevisiae* reporter strain SFY526, and β-galactosidase activity was assayed with o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate. Compared with a background β-galactosidase activity obtained by co-transfection of GBT9-res2 with GAD424-X (no insert), co-transfection with GAD424-cdc10 yielded more than a 180-fold stimulation in activity (Figure 11). On the other hand, only an about 3.8-fold stimulation was observed when GBT9-res1 was co-transfected with GAD424-cdc10. This high stimulation by a combination of Res2 with Cdc10 indicates that Res2 binds Cdc10 with a high-affinity. This is consistent with the fact that unlike Res1, Res2 almost absolutely requires Cdc10 for its function. From these data, we conclude that Res2 is functionally redundant with Res1 and constitutes another parallel cell cycle "start" system with Cdc10.

res2$^+$ is also a "start" gene for meiosis

The *res2*$^+$ gene acts in mitotic cycle, but plays a relatively minor role. We therefore wondered whether *res2*$^+$ might have other roles. To investigate this possibility, we examined its expression under various conditions by Northern blot analysis. The *res2*$^+$ transcript was highly induced during nitrogen starvation only in h^{90} homothallic haploid and diploid cells but not in heterothallic haploid cells (Miyamoto *et al.*, 1994). This is in sharp contrast to the behavior of *res1*$^+$, which undergoes no significant changes in transcription during conjugation and meiosis (Obara-Ishihara and Okayama, 1994). These results suggest that *res2*$^+$ may have an additional function during sexual development.

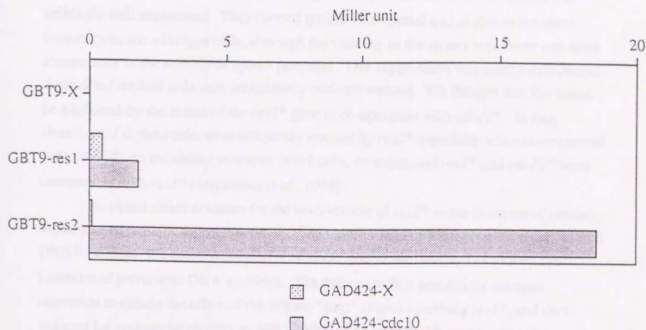


Fig.12. Interaction of Res1 and Res2 with Cdc10 in the two-hybrid system. The reporter strain SFY526 was transfected with indicated plasmids. An interaction between the fusion proteins expressed by the specified plasmids is demonstrated by the β-galactosidase activity of transformant. The activity is shown in Miller units. GBT9-X and GAD424-X are vector plasmids without insert.

As described before, when meiosis is initiated from diploid cells, the *res1*$^+$ gene is required for the "start" of premeiotic DNA synthesis. We, however, found that when the h^{90} *Δres1* mutant was induced to conjugate and perform meiosis, this defect was strikingly well suppressed. They formed typical four-spored asci at almost the same frequency as the wild type cells, although the viability of the spores was lower and some abnormality in the number of spores persisted. This suppression was totally unexpected since *Δres1* diploid cells very inefficiently initiated meiosis. We thought that this could be explained by the action of the *res2*$^+$ gene in co-operation with *cdc10*$^+$. In fact, *Δres1*/*Δres1* diploid cells were efficiently rescued by *res2*$^+$ especially when co-expressed with *cdc10*$^+$. In the ability to rescue *Δres1* cells, co-expressed *res2*$^+$ and *cdc10*$^+$ were comparable with *res1*$^+$ (Miyamoto *et al.*, 1994).

To obtain direct evidence for the involvement of *res2*$^+$ in the initiation of meiosis, we employed haploid meiosis inducible by the inactivation of a temperature-sensitive Pat1 (Pat1^{ts}) kinase. We constructed *pat1-114 Δres2* double mutants and examined their initiation of premeiotic DNA synthesis. The cells were first arrested by nitrogen starvation to reduce the effect of the mitotic "start" system involving *res1*$^+$, and then induced for meiosis by shifting to 34°C. Within 6hr after Pat1^{ts} inactivation, the majority of *pat1-114* single mutant cells completed premeiotic DNA synthesis. However, when the same experiment was done with *pat1-114 Δres2* double mutant cells, most of the cells remained as 1n DNA without entering premeiotic S phase (Miyamoto *et al.*, 1994). However, the inability of *Δres2* cells to initiate meiosis was partially suppressed when rapidly growing *pat1-114 Δres2* double mutant cells were shifted to 34°C without prior nitrogen starvation, and many cells underwent haploid meiosis. Consequently, these results indicate that *res2*$^+$ indeed acts for the "start" of premeiotic DNA synthesis, and that this function is likely to be substituted at least partly by the mitotic "start" system involving *res1*$^+$.

res2$^+$ plays an additional role in meiotic division

In addition to a role in the initiation of premeiotic DNA synthesis, *res2*$^+$ seems to have another function in meiotic division. When logarithmically growing h^{90} *Δres2* cells were starved for nitrogen, they conjugated and efficiently initiated premeiotic DNA synthesis but had a severe abnormality in meiotic division. The mutant was unable to proceed through the normal meiotic process and arrested mostly at the stage of binucleate cells (Miyamoto *et al.*, 1994). Perhaps as a consequence of this, most of the mutant asci had only two or one, or even no spores. Moreover, DNA was unevenly partitioned into the spores. However, this defect was not suppressed by *res1*$^+$ gene. Since the majority of rapidly growing *Δres2* cells can initiate premeiotic DNA synthesis upon nitrogen starvation, these results indicate that under such conditions, the main defect in *Δres2* cells was either at the final stage of DNA synthesis or at meiotic division.

All the results presented here lead us to conclude that the fission yeast contains two functionally overlapping parallel cell cycle "start" systems, Res1-Cdc10 and Res2-Cdc10, the former of which plays a major role in mitotic "start" whereas the letter in meiotic "start" and meiotic division.

Table I. Strain List

strain	genotype
L968	h^{90}
L972	h^{-S}
K137-10	h^{-S} *ade6-M216 leu1-32*
SO5	h^{-S} *pat1-114 ura4-294*
AS1	h^{+N} *cdc10-129 leu1-32*
AS2	h^{-S} *cdc10-129 ura4-294*
AS5	h^{-S} *cdc10-129 leu1-32*
DP2	h^{-S}/h^{+N} *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32*
D1787	h^{-S}/h^{+N} *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 res1*$^{+}$*/res1::ura4*$^{+}$
DC107	h^{-S}/h^{+N} *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 cdc10*$^{+}$*/cdc10::ura4*$^{+}$
D1787-5A	h^{-S} *ade6-M210 ura4-D18 leu1-32*
D1787-5B	h^{-S} *ade6-M216 ura4-D18 leu1-32 res1::ura4*$^{+}$
D1787-5C	h^{+N} *ade6-M216 ura4-D18 leu1-32 res1::ura4*$^{+}$
D1787-5D	h^{+N} *ade6-M210 ura4-D18 leu1-32*
K123-14A	h^{+N} *ade6-M210 ura4-D18 leu1-32 res1::ura4*$^{+}$
K123-14B	h^{-S} *ade6-M216 ura4-D18 leu1-32*
K123-14C	h^{+N} *ade6-M210 ura4-D18 leu1-32*
K123-14D	h^{-S} *ade6-M216 ura4-D18 leu1-32 res1::ura4*$^{+}$
K130-1A	h^{90} *ade6-M210 ura4-D18 leu1-32*
K130-1B	h^{-S} *ade6-M216 ura4-D18 leu1-32 res1::ura4*$^{+}$
K130-1C	h^{-S} *ade6-M210 ura4-D18 leu1-32*
K130-1D	h^{90} *ade6-M216 ura4-D18 leu1-32 res1::ura4*$^{+}$
K160-A5	h^{90} *ura4-D18 res1::ura4*$^{+}$
M106	h^{90} *ura4-D18 leu1-32 res1::ura4*$^{+}$

strain	genotype
K134	h^{-S}/h^{+N} *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 res1^{+}/res1::ura4^{+}*
K135	h^{-S}/h^{+N} *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 res1::ura4^{+}/res1::ura4^{+}*
M101	h^{-S}/h^{+N} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 res2^{+}/res2::ura4^{+}*
M102-1B	h^{+N} *ade6-M210 leu1-32 ura4-D18 res2::ura4^{+}*
M105	h^{-S}/h^{+N} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 res1^{+}/res1::ura4^{+} res2^{+}/res2::ura4^{+}*
M107-1A	h^{-S} *leu1-32 ura4-D18 res2::ura4^{+} pat1-114*
SO6	h^{-S} *leu1-32 pat1-114*
SO7	h^{90} *ade6-M210 ura5-294 leu2-120 mei1-B102*
SFY526	*Mata ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 can^{r} gal4-542 gal80-538 URA3::GAL1-lacZ*

Table I. Continued

Section 2

A zinc finger protein controls the onset of premeiotic DNA synthesis and meiotic division of fission yeast via interacting with Res2-Cdc10 start-specific transcriptional regulatory complex

Isolation of *rep1*$^+$ gene

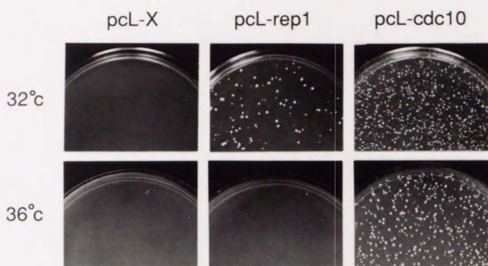
To isolate new regulators that play a crucial role in the initiation of mitotic as well as meiotic DNA synthesis, we screened an *S. pombe* expression cDNA library for multicopy suppressors of the temperature-sensitive *cdc10-129* mutant (AS1) as described previously (Okazaki *et al.*, 1990), and obtained 3 independent cDNA clones that had such activity. Among them, two were *res1*$^+$, and the remaining one was a new gene. It rescued the *cdc10-129* mutant but only at modest non-permissive temperatures up to 33°C (Figure 12A) and was unable to rescue a *Δcdc10* mutant (data not shown). However, this cDNA clone effectively suppressed the heat (36°C)- and cold (23°C)-sensitivities of a *Δres1* mutant (K123-14D) (Figure 12B). This gene was named *rep1*$^+$ since it is required for entry into premeiotic DNA synthesis as described below.

The nucleotide and predicted amino acid sequences of the *rep1*$^+$ gene were determined from the cDNA and the corresponding genomic DNA (Figure 13). The *rep1*$^+$ gene contains an open reading frame encoding a 472 amino acid protein with a calculated molecular weight of 52,617. An amino acid homology search using the Swiss Prot Data Base found that the carboxyl-terminus contains one noticeable zinc finger motif, C-C-X1-C-X12-H-X3-C-X1-H (see underline in Figure 13). This sequence contains two consensus structures, C-X2-C-X12-H-X3-C and C-X1-C-X12-H-X5-H. The latter type is found in one of the three zinc finger motifs of the Swi5 and Ace2 transcription factors of budding yeast (Stillman *et al.*, 1988; Butler and Thiele, 1991; Dohrmann *et al.*, 1992). The importance of the zinc finger motif for the *rep1*$^+$ function was revealed by deletion analysis. The pcL vector containing *rep1*$^+$ with a deletion in this region (pcL-rep1 Δ*Nru*I-*Pvu*II) displayed greatly reduced activity to rescue the temperature-sensitive *cdc10-129* mutant (Figure 14).

The first nucleotide of cDNA corresponds to adenine at -215 from the putative initiation codon, and two sets of the Ste11-responsive element called "TR box" TTCTTTGTTY are present at -581 to -572 and -568 to -559. Ste11 transcriptionally regulates many genes inducible by nitrogen starvation (Sugimoto, *et al.*, 1991). Perhaps due to the presence of the TR boxes, the *rep1*$^+$ gene was induced by nitrogen starvation (see below).

A

host : cdc10^{ts}



B

host : Δres1

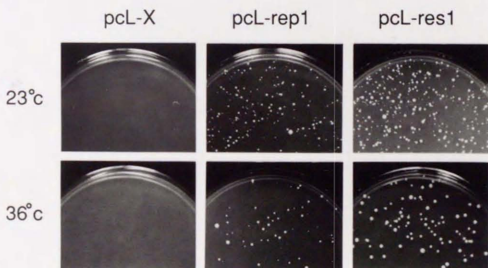


Fig.12. Suppression of *cdc10*^{ts} and *Δres1* mutants. The temperature-sensitive *cdc10-129* mutant (AS1) (**A**) and *Δres1* mutant (K123-14D) (**B**) were transformed with the indicated plasmids and incubated on MMA plates at the specified temperatures. pcL-X is a pcL vector plasmid with no insert and was used as a negative control.

```
-1499                                 AAGCTTATAACCAGAAGACTTGATACTGGTGAAGAATACGGTAAAACCAAACTTTCCAA
-1440 TATAGTTATTGGCGAATGGAGTTGTACTTTAAGCCAAGAATCATGGAGCCAAACAAAGCTCCACGATAAAAGAAGACGTGACTTTGGTGA
-1350 AGCTCAACTGAACCAGTATTTGAATTATTGCGGAGGATGCTTCTTTTGGACTTATAAATTCCTTCACGGAAAAGGTGGAGATTGGGATTT
-1260 TAGATCTGTTGTAGAGGACAAGGTAATCAATTACCCTCCTCCTCCCCCTACCGAAAACAAAGCCATGCCGGCCTTACTTGAACAAAGTCG
-1170 GGATCAAAACTTTGGTGGACATTGCTATTATTGGGATCAGAAACAACATGATCATCCATATGAACATGATTTATATGTTAAAGGATGGAA
-1080 TCAAGCCTGGGAGGACTACATTGAGTTTCTTCAGCATGGGGCCATGATCGGATTTCCTCGTGCATGGACTCAAAAAAGGATGACAAGTAT
 -990 TTCTTCTGCTTCTGCTTGGGAATATCGTGACGGTATGAATGCGGCCTGGTTGCATCTCGAGCGTATGGGCTTTCTCAATCCTTTTCGTCA
 -900 CCCATAATTCCATAATGTCTGTCTTAATGACCTTTATACTCTATGTTTATGCTTTTGTTTATCCGCACAAAAATTTAATGTGTTTGGTAA
 -810 CCCTCCTGCTAATCTCTATTCCCTTACCCACTTTTTGTCGTTAAATTTTGCTTGTTGTATTCACATCCTTGTTACATATGTTTAATTTTG
 -720 GTAGATTAAAAATTCTGTTGATTTTCTTTTATAAAATTCTCTAAAAGATAATTAAAGGATTAATATTAACGACTTACTATTGAATTTTTT
 -630 AATAGAGAAATGAAATAATAGAACGTTTAAATAAAGAATCCAAGCATTTTTCTTTGTTTACATCCTTTGTTTACAATACGCAATCTTAGC
 -540 ATAGTAAAACAAAGAAAGGATACGTAGTAGTGCGTACGTATTTTACCCACACTCGCTTTTTACCGCACTCTTGAACTATATATACATGTA
 -450 CTACCTCGAGCTGGAAGTAGTACAGTAAAATTCGTATATTGCTATTAATCCTTTTTTATCTTATTTAAACATCAAGGAGTAGTATTTATT
 -360 GACGTTATCAAAGCAAAACTTTACTTTGACACTGTATTAATACACCAGGCATCTCTCTATTACATAGTGTCGGCTACAATTGACTTAAAA
 -270 GAATTGTTTTAAATCTAATTGCTAAAGCTGAAACTGTTTAAAATTTAGAATATTTAAACTTATACATTATATTAAACTTTTATGCAATTT
 -180 AGCTTGACGGCAATACGATAATATCTTTTTAATTCCTAGGTCTCATATATTATTCTTGCTGTTGAGCTTAAAACCATAAAACAGCCTTAC
  -90 GTTTGTTTCTGAACGACAAACTATTACCATATATTGAATTGACTTTCCTGAATTTATACCCTTATTTTCTTTATTAAAGCTTTGAACGTC
    1 ATGGATTCTGATCGTTGTTTAACAGACGAAATTTCATTAAATACGCTTTCATCTACTTTTGCTAGTGATAATAACTTAAGAAGGAAAGAG
    1 M  D  S  D  R  C  L  T  D  E  I  S  L  N  T  L  S  S  T  F  A  S  D  N  N  L  R  R  K  E
   91 AATTTTTGAAAAGTAGATACCCTTCAAAATTTGATTTAAATGTTTCAATGTTAACGAAGAGTGATGACGTTGGAAAGACCCCGTTTTCA
   31 N  F  L  K  S  R  Y  P  S  K  F  D  L  N  V  S  M  L  T  K  S  D  D  V  G  K  T  P  F  S
  181 ATTTTCGATTCACCAAGCAATCCTGGCTTTTCTCGGTCGCACCAAATGTGTTCTGACAAAAACAAGAGTCCCTTCCTCTTTGATAAGATT
   61 I  F  D  S  P  S  N  P  G  F  S  R  S  H  Q  M  C  S  D  K  N  K  S  P  F  L  F  D  K  I
  271 AGGGATGAACCCGTTTCTGTCGATCCCGTTAAACTTATCATTAATAATCGGAACAAACGGAAAATTAATAGACAGAAATCAAGGCTTCAA
   91 R  D  E  P  V  S  V  D  P  V  K  L  I  I  N  N  R  N  K  R  K  I  N  R  Q  K  S  R  L  Q
  361 GGCCTTTATCGGTCCGATGCTAATGGTTTGCAACCATTGAATTCTGAAAATGTCAAAATGAAAAAATCAACTGCCTTGTCGTTGACAAGT
  121 G  L  Y  R  S  D  A  N  G  L  Q  P  L  N  S  E  N  V  K  M  K  K  S  T  A  L  S  L  T  S
  451 TCACCCTTAAACTCTTGGAAAACTGATTTCAAAACACCGCCGAAAGCTAATGTTGTTTGTATCTCTTTAGTTATTGAAGGAGACGGCTGC
  151 S  P  L  N  S  W  K  T  D  F  K  T  P  P  K  A  N  V  V  C  I  S  L  V  I  E  G  D  G  C
  541 GCATCATTGCTGTACGAAGATTTGAATCAGATTTCCAATAGTTGCCCTGAAGTAGCACCGAACCGTCAGAACGCTCTCTTTTCAGACGAA
  181 A  S  L  L  Y  E  D  L  N  Q  I  S  N  S  C  P  E  V  A  P  N  R  Q  N  A  L  F  S  D  E
  631 ACGCTGACAACTATGTTCTATAGTGGTGTCACGGAGGATGAGGGCTCATGTAACAATTTACTACAATCTAGCTTCGGTGATGATCTGGAC
  211 T  L  T  T  M  F  Y  S  G  V  T  E  D  E  G  S  C  N  N  L  L  Q  S  S  F  G  D  D  L  D
  721 TTGGGAATGCAACGGTCTGCAACTTGGGCACCGGGCTATAATTATCCAAGCAAATTTGATAGCATACCTTTTGCTTCAGCATCTCCAAAA
  241 L  G  M  Q  R  S  A  T  W  A  P  G  Y  N  Y  P  S  K  F  D  S  I  P  F  A  S  A  S  P  K
  811 ATAAAAGCAGCTTTCCCTTTCGATCCCAACGTTTATGCATTGAATACTAACGATGGTCCAGTCACGAGTACTGATAATAATTGTGATCAA
  271 I  K  A  A  F  P  F  D  P  N  V  Y  A  L  N  T  N  D  G  P  V  T  S  T  D  N  N  C  D  Q
  901 TTAAACACTAGGATGCAGGCATGGAATAATGGCTTTAATTACTCAAACCTAAATGGTGATATCCAATATCCTGCTGTTACACCAAAATTT
  301 L  N  T  R  M  Q  A  W  N  N  G  F  N  Y  S  N  L  N  G  D  I  Q  Y  P  A  V  T  P  K  F
  991 TTTCAACAAGAAGGCCGGGCCCTTAATGTTTCCGACTGTAATTTTGGAAATGAAGAAATTGCTTACGGTGGAATTCCTATGCGTGTTTGT
  331 F  Q  Q  E  G  R  A  L  N  V  S  D  C  N  F  G  N  E  E  I  A  Y  G  G  I  P  M  R  V  C
 1081 CATAGTGATAGCACATTATGTGATGCCAGAATTGCAGCTAAGCAAGCTTTAAAAGGTAAAAGGCAATATGACACATCAACATCAAAAGCA
  361 H  S  D  S  T  L  C  D  A  R  I  A  A  K  Q  A  L  K  G  K  R  Q  Y  D  T  S  T  S  K  A
 1171 GAGCTCTCTACTCCCCCATCAAAACGAAGGCATATTGATGATGCCGATTTAGTTTTTCACAGTAGTCCCTTACTGTCACGCTCGCGATTC
  391 E  L  S  T  P  P  S  K  R  R  H  I  D  D  A  D  L  V  F  H  S  S  P  L  L  S  R  S  R  F
 1261 ATTTGTTGCTACTGCACGAAGCCTTTCCTTTCAATTAGTAAACTTCAGGAACATGAGTCCTCGTGTTCACATGTTGAGCGACTCTTTGGG
  421 I  C  C  Y  C  T  K  P  F  L  S  I  S  K  L  Q  E  H  E  S  S  C  S  H  V  E  R  L  F  G
 1351 TTTGCCCCAAATCGTCTTTATGATGATGGTGATGGATTTTTGGGTTCGAGTTTTTGCAGTGATTGGTAAGATTATAGCATGTTTCGCTGC
  451 F  A  P  N  R  L  Y  D  D  G  D  G  F  L  G  S  S  F  C  S  D  W  *
 1441 ATCTATTTGATTGTCCAGCAGCTGAGATTGCTACGATTTATTCTTTATTTAAAACACATTTATTTCATGCTTTATTACTTATCATTAATC
 1531 TTGTACTTTAATTTAATTTTTCATAGTTATTAATCTTTAATTAGTGTTTATGAGGCGATCTTGAA
```

Fig.13. Nucleotide and deduced amino acid sequences of the *rep1*$^+$ gene (EMBL sequence database accession number X77518). The amino acid sequence is shown below the DNA sequence in a single-letter code. Nucleotide numbering starts at the initiation codon of the predicted *rep1*$^+$ translation product. The first nucleotide of the cDNA at position -215 is indicated by arrowhead. The zinc finger motif is underlined. Two TR boxes, a Ste11-responsive *cis*-element, are boxed.

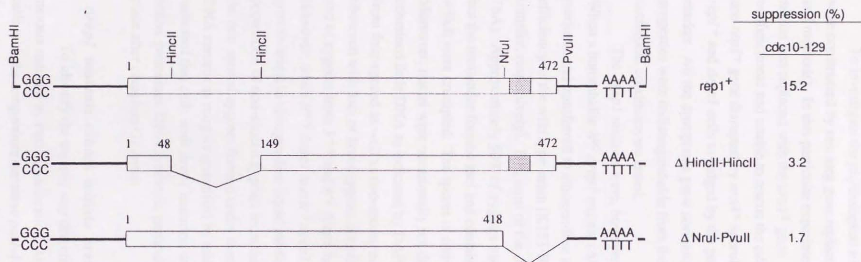


Fig.14. Deletion analysis of the Rep1. The intact *rep1*$^{+}$ cDNA is shown at the top. The protein coding region is boxed and the zinc finger motif is shaded. The amino acid numbers are shown at the each protein coding region. The *cdc10*^{ts} mutant suppression activities of deletion mutants are indicated in the right column as percentages of suppressed cells per stably transfected cells. The *Hinc*II-*Hinc*II deletion rises in-frame connection.

rep1$^+$ is required for meiosis but not for mitosis

To investigate the physiological role of *rep1*$^+$, several independent null mutants were constructed by one-step gene replacement as illustrated in Figure 15 (see Materials and methods). In this particular experiment, approximately 80 % of the *rep1*$^+$ coding region was replaced with the *ura4*$^+$ gene. The resulting disrupted *rep1*$^+$ gene was nonfunctional and unable to rescue the *cdc10*^{ts} and *Δres1* mutants. Diploid cells with one *rep1*$^+$ gene disrupted by *ura4*$^+$ sporulated and germinated with a 1:1 segregation of *rep1*$^+$ and *Δrep1* cells as judged by the presence and absence of the uracil auxotrophic marker. All the disruptants grew normally, and their cell morphology and growth properties were indistinguishable from those of the wild type strain under all the nutritional conditions we tested.

The *Δrep1* mutants were, however, found to have a defect in a meiotic process. When a homothallic h^{90} *Δrep1* mutant (AH1) was grown to a mid-log phase in minimal medium, and transferred to nitrogen-free minimal medium, they conjugated at the same efficiency as the wild type strain (K153-B25) (66 % and 72 % at the time of 25 hr after transfer, respectively). But, most of the *Δrep1* mutants sporulated abnormally (Figure 16A). Approximately 50% of zygotes had no spore, showing a typical *mei* phenotype, but the remainder formed asci and contained various numbers (1-4) of spores, some of which were crumpled. The spores in one- or two-spored asci were apparently larger. Moreover, nuclei were occasionally not divided evenly, and crumpled spores often contained little DNA as indicated by DAPI staining. Only 10% of the spores recovered from four-spored as well as two-spored asci was viable. Similar abnormality was observed with asci of homozygous *Δrep1/Δrep1* diploid cells (A181) (data not shown) and of zygotes from h^{+N} and h^{-S} *Δrep1* haploid cells (AH2 and AH3) (Figure 16B). However, when h^{+N} *Δrep1* and h^{-S} *Δrep1* were separately cultured to a complete growth-arrest in nitrogen-free liquid medium and then allowed to conjugate, the population of non-spored zygotes increased to more than 90% (Figure 16C). Almost all the non-spored zygotes formed under these conditions had one nuclei with a only 2n DNA content as roughly quantified by microscopic photometry. All these results indicated that cells with *Δrep1* mutation were unable to undergo meiosis and arrested before premeiotic DNA synthesis, particularly when conjugation was allowed to take place after complete G1 arrest.

Δrep1 mutants cannot initiate premeiotic DNA synthesis

To identify the meiotic step defective in *Δrep1* mutants, we employed haploid meiosis induced by Pat1^{ts} inactivation. Upon shifting to a non-permissive temperature, cells with the temperature-sensitive *pat1-114* mutation initiate and successfully complete the meiotic process in a synchronized fashion, regardless of its ploidy, nutritional conditions and availability of a mating partner (Iino and Yamamoto, 1985). But spores

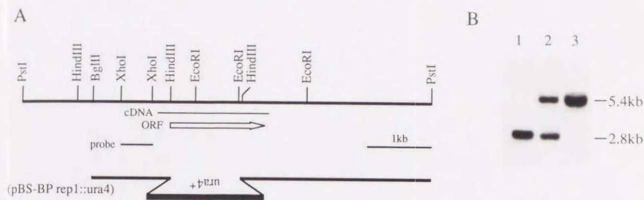


Fig.15. Disruption of the *rep1*$^+$ gene.

A. Restriction map of the *rep1*$^+$ gene. The *rep1*$^+$ cDNA, the Rep1 open reading frame, the 0.5 kb *Xho*I fragment used as a probe for Southern hybridization, and the fragment used for disruption are shown below the map. The upside-down ura4$^+$ denotes the *ura4*$^+$ gene inserted in the opposite transcriptional orientation.

B. Southern blot detection of the disrupted chromosomal *rep1*$^+$ gene. The *rep1*$^+$ gene was disrupted as described in Materials and methods. Genomic DNA was prepared from each strains, digested with *Pst*I and *Eco*RI, separated by agarose gel electrophoresis and blotted to a nitrocellulose filter. Lane 1, wild type (L972); Lane 2, diploid strain carrying the *rep1::ura4*$^+$ on one chromosome (D18); Lane 3, haploid strain carrying the *rep1::ura4*$^+$ (A18-3). The *rep1*$^+$ specific hybridization probe used is shown in the panel **A.** The 2.8 kb and 5.4 kb bands correspond to the intact and disrupted *rep1*$^+$ alleles, respectively.

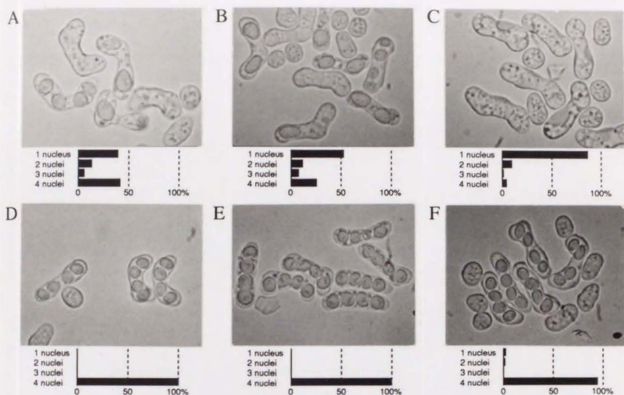


Fig.16. The morphology of the *Δrep1* mutant.

A. Homothallic h^{90} *Δrep1* (AH1) were cultured in MM+N to 1 x 10^7 cells/ml and shifted to MM-N.

B, C. Heterothallic h^+ *Δrep1* (AH2) and h^- *Δrep1* (AH3) were cultured separately in MM+N to 1 x 10^7 cells/ml, shifted to MM-N, and immediately mixed (**B**) or mixed after an 8 hr.-culture in MM-N (**C**).

D, E, F. Homothallic h^{90} *rep1*$^+$ (K153-B25) or heterothallic h^+ *rep1*$^+$ (SA21) and h^- *rep1*$^+$ (L972) were used as positive controls for **A**, **B** and **C**. Filled boxes under the each photograph indicate the percentages of asci containing the indicated number of nucleus counted after DAPI staining.

formed from haploid cells in this way contain fewer numbers of chromosomes and, therefore, retain little viability. Nevertheless, this allowed us to investigate the meiotic step defective in the *Δrep1* mutant. We, therefore, constructed a h^{-S} *pat1-114 Δrep1* double mutant (AP1) and investigated its meiotic process. When the *pat1-114 Δrep1* cells were induced to initiate meiosis by a temperature shift, approximately 80% of the cells failed to form spores and remained as 1 nucleus cells, the same phenotype as that of the conjugation-induced *Δrep1* mutant (Figure 17B). The failure to proceed meiosis was not due to its acquisition of the ability to grow vegetatively, such as the *pat1-114 mei2* double mutation (Iino and Yamamoto, 1985), and the *pat1-114 Δrep1* double mutant was, in fact, unable to grow at non-permissive temperatures (Figure 17A). To determine if the double mutant was indeed unable to initiate premeiotic DNA synthesis, the double mutant that was grown to a mid-log phase was incubated in nitrogen-free minimal medium for 14 hr to bring to complete G1 arrest. A small fraction of the cells was arrested in G2 under these conditions and was, therefore, eliminated by elutriation prior to use. The elutriation-purified G1-arrested cells were then induced to initiate meiosis by exposure to a non-permissive temperature (34°C) with sampling cells every hour. The progression of meiosis was monitored by flow cytometry and DAPI staining (Figure 18A and 18B). The same experiments were performed with the *pat1-114* single mutant cells (K149-1) as a positive control. In *pat1-114* single mutant cells, premeiotic DNA synthesis was initiated within 2 hr after temperature shift, and completed within 4 hr followed by the first meiotic division yielding 2 nuclei cells, which peaked at 5 hr, and then by the second division. The cell viability, as determined by their ability to form colonies after plating on YEA plates at 23°C, dropped abruptly after 2 hr, presumably reflecting that the commitment to start meiotic division was made near the end of premeiotic DNA synthesis. On the contrary, *pat1-114 Δrep1* double mutant cells were unable to start premeiotic DNA synthesis and continued to remain as one nucleus cells with no apparent production of 2 or more nuclei cells (Figure 18A, 18B and 18C). Remarkably, the double mutant retained a high cell viability even 12 hr after induction (Figure 18B). These results led us to conclude that the inactivation of *rep1*$^{+}$ results in an arrest before the onset of premeiotic DNA synthesis. Consistent with this conclusion, in the *pat1-114 Δrep1* strain, *mei2*$^{+}$ mRNA remained elevated, and *cdc22*$^{+}$, a gene required for premeiotic DNA synthesis, underwent virtually no induction (Figure 18D).

As already shown (Figure 12), overexpression of *rep1*$^{+}$ rescues a *Δres1* but not *Δcdc10* mutant, and the *res2*$^{+}$ gene, which acts for the "start" of premeiotic DNA synthesis as well as for meiotic division, absolutely requires Cdc10 protein for its activity. This led us to speculate that *rep1*$^{+}$ might execute its function through interaction with the Cdc10-Res2 transcriptional regulator complex and that if this was indeed so, *rep1*$^{+}$ might influence *res2*$^{+}$ expression since *res2*$^{+}$ transcript was highly induced during conjugation (Miyamoto *et al.*, 1994). We, therefore, examined the level of *res2*$^{+}$

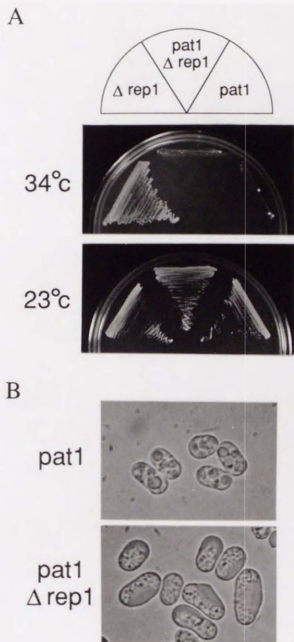
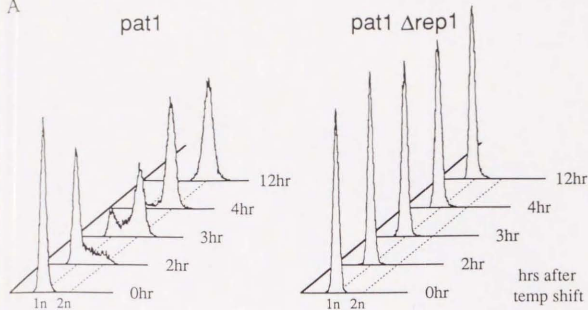


Fig.17. Phenotype of *pat1-114 Δrep1* double mutant.

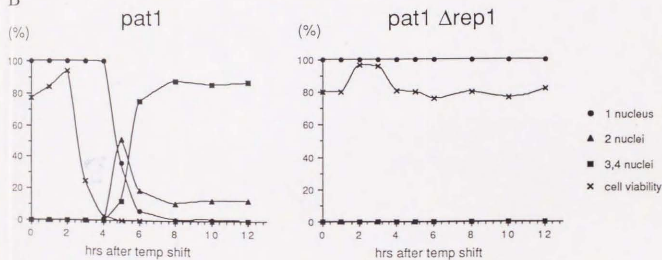
A. Temperature-sensitive phenotype of *pat1-114 Δrep1* double mutant. *Δrep1* single mutant (AH3), *pat1-114 Δrep1* double mutant (AP1) and *pat1-114* single mutant (K149-1) were inoculated on MMA and incubated at indicated temperatures.

B. The morphology of the *pat1-114* single mutant (K149-1) and *pat1-114 Δrep1* double mutant (AP1). Cells were cultured in MM+N to 7 x 10^6 cells/ml at 24°C, shifted to pre-warmed MM-N and incubated for 24 hr. at 34°C.

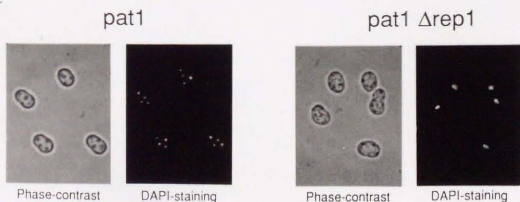
A



B



C



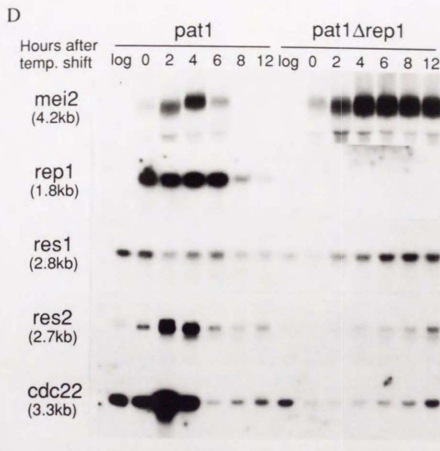


Fig.18. The *rep1*$^+$ gene product is required for entry into premeiotic DNA synthesis. The elutriation-purified G1-arrested cells (*h*$^{-S}$ *pat1-114 Δrep1* (AP1) and *h*$^{-S}$ *pat1-114* (K149-1)) were shifted up to 34°C to start synchronous meiosis, and aliquots were taken at indicated times to monitor the progression of meiosis (see Materials and methods).

A. Flow cytometry patterns of cells. The panels show frequency histograms of the number of cells at the indicated time.

B. Time course of cell viability and proportion of cells containing one to four nuclei.

C. The morphology of cells after for 12 hr. incubation. Left panels: Phase-contrast; Right panels: DAPI-staining

D. Northern blot analysis of *mei2*$^+$, *rep1*$^+$, *res1*$^+$, *res2*$^+$ and *cdc22*$^+$ transcripts. RNA was prepared from each aliquot at the indicated time and Northern blot analysis was done with 10 μg of total RNA.

transcript during meiosis in both the *pat1-114* single and *pat1-114 Δrep1* double mutant strains. We performed several independent experiments which gave representative results (Figure 18D). In *pat1-114* single mutant cells, *rep1*$^+$ mRNA in this particular experiment, which was detected already before induction of meiosis perhaps due to slight inactivation of Pat1^{ts} even at 24°C, underwent significant induction at 2 hr, continued to be induced until 6 hr when the second division completed, and quickly disappeared thereafter. The *res2*$^+$ mRNA was induced in a similar time course but disappeared slightly ahead of *rep1*$^+$ mRNA, a time when the first division completed. In *pat1-114 Δrep1* double mutant cells, however, *res2*$^+$ mRNA underwent no induction. On the other hand, *res1*$^+$ mRNA, which appears to be significantly decreased in *Δrep1* cells in Figure 18D, was actually expressed throughout with only a slight change in the presence or absence of *rep1*$^+$. These results led us to conclude that the induction of *res2*$^+$ during meiosis depends on *rep1*$^+$ function when meiosis is induced after complete G1 arrest.

Inability of *Δrep1* mutant to initiate premeiotic DNA synthesis seems to be partially suppressed when rapidly growing cells were induced to meiosis. We, therefore, investigate whether *res2*$^+$ transcript is expressed under this condition. A homothallic h^{90} *Δrep1* mutant (K158-A1) was grown to a mid-log phase in minimal medium and transferred to nitrogen-free minimal medium to induce conjugation and meiosis. Under these conditions, a majority of the *Δrep1* cells underwent premeiotic DNA synthesis with induction of the *res2*$^+$ transcript (Figure 19) and formed spores although abnormalities in meiotic division still persisted. Thus, rapidly growing cells contain a Rep1-like factor which can induce *res2*$^+$ expression but can not execute meiotic division in meiotic cycle. However, induction of *res2*$^+$ transcript is not the sole function of Rep1. In mitotic cycle, *rep1*$^+$ effectively rescued the *cdc10-129* and *Δres1* cells without induction of *res2*$^+$ transcript (Figure 20), suggesting that Rep1 may have a function as an activator of the Res2-Cdc10 complex.

rep1$^+$ mRNA is induced by nitrogen starvation and mating pheromones in a Mei2-independent pathway

Many genes acting in meiosis are induced during nitrogen starvation and conjugation. Since *rep1*$^+$ is required for and induced during meiosis, we investigated its inducers and the genes responsible for its induction. RNA was prepared from log-phase and nitrogen-starved cultures of cells with various genetic backgrounds, and the level of *rep1*$^+$ mRNA was determined by Northern blot analysis (Figure 21). Nitrogen starvation induced *rep1*$^+$ mRNA very strongly in homothallic h^{90} haploid cells (L968) but relatively weakly in heterothallic h^- haploid cells (L972), indicating that mating pheromone(s) is a major inducer (Figure 21A). This was confirmed by no or greatly diminished induction of *rep1*$^+$ transcript in *ste11* (C756-1A) and *Δgpa1* (JZ453) mutants (Figure 21B), which

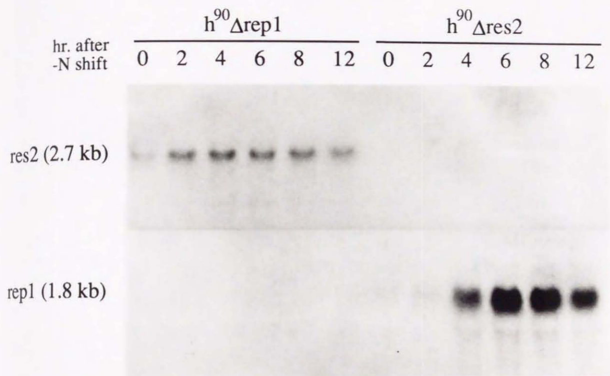


Fig.19. *res2*$^+$ transcript is expressed when rapidly growing *Δrep1* cells are induced to undergo conjugation and meiosis. Time course of *res2*$^+$ and *rep1*$^+$ induction in h^{90} homothallic *Δrep1* (K158-A1) and *Δres2* (K159-A1) cells after nitrogen starvation.

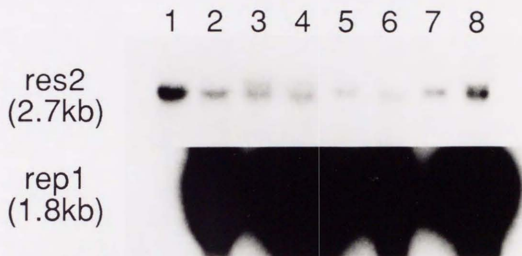
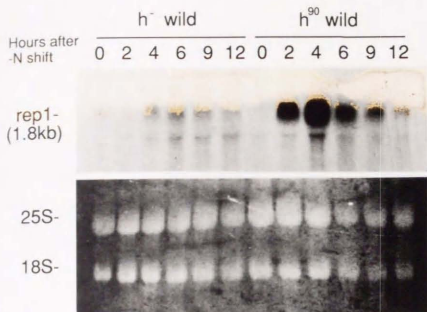


Fig.20. Overexpression of *rep1*$^+$ in heterothallic haploid cell in nitrogen-rich medium does not induce *res2*$^+$ transcript.

Wild type (h^{-S} *leu1-32* (K150-A6); lane 1-4) or *cdc10*^{ts} mutant (h^{-S} *cdc10-129 leu1-32* (AS1); lane 5-8) cells were transfected with plasmids and grown in MM+N at 30°C (wild type) or 32°C (*cdc10*^{ts}) to mid-log phase. RNA was prepared from each aliquot and Northern blot analysis was done with 30 μg of total RNA. Lane 1, wild type + pcL-X; Lane 2, wild type + pcL-rep1; Lane 3, wild type + pREP1-X; Lane 4, wild type + pREP1-rep1; Lane 5, *cdc10*^{ts} + pcL-cdc10; Lane 6, *cdc10*^{ts} + pcL-rep1; lane 7, *cdc10*^{ts} + pREP1-cdc10; Lane 8, *cdc10*^{ts} + pREP1-rep1

A



B

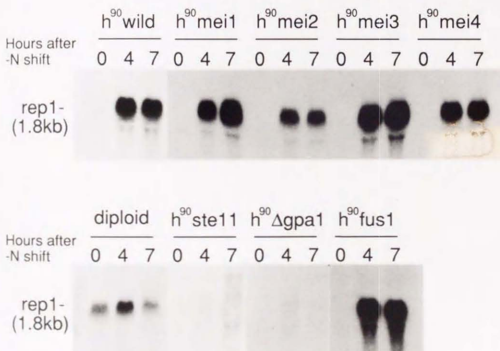


Fig.21. Northern blot analysis of the *rep1*$^+$ transcript.

A. Time course of *rep1*$^+$ induction in heterothallic h^- (L972) and homothallic h^{90} (L968) wild type cells after nitrogen starvation. Below the autoradiograph is the ethidium bromide-stained gel before transfer.

B. Expression of the *rep1*$^+$ transcript in indicated strains after nitrogen starvation. Total RNA was prepared from h^{90} wild type (L968), h^{90} *mei1* (AS2), h^{90} *mei2* (K100-8B), h^{90} *mei3* (AS3), h^{90} *mei4* (AS4), h^+/h^- diploid (DP2), h^{90} *ste11* (C756-1A), h^{90} *Δgpa1* (JZ453), h^{90} *fus1* (C508-2A). All lanes have 20 μg of total RNA.

are unable to produce and respond to mating pheromone(s) (Leupold, *et al.*, 1991), and to transduce signals from the mating factor receptors (Obara, *et al.*, 1991), respectively.

As described above (Figure 13), the *rep1*$^+$ promoter contains two copies of the Ste11-responsive element called TR box, which is present in many genes induced by nitrogen starvation, such as *mei2*$^+$, *ste4*$^+$, *matP*$^+$, *matM*$^+$ and *ste11*$^+$ itself (Sugimoto, *et al.*, 1991; Okazaki, *et al.*, 1991). The moderate *rep1*$^+$ induction in h^- haploid cells by nitrogen starvation is likely to be the consequence of direct activation via the Ste11-responsive element whereas the strong induction by mating pheromones may be mediated by some unidentified transcriptional regulatory element(s) and/or factor(s). Consistent with this conclusion, in diploid zygotes, *rep1*$^+$ was already turned on in nitrogen-rich medium and underwent only slight induction by nitrogen starvation (Figure 21B). Moreover, *rep1*$^+$ was induced in a *fus1* mutant (C508-2A)which normally responses to mating pheromones and undergoes sexual agglutination but not subsequent cell fusion (Egel and Egel-Mitani, 1974). The induction of *rep1*$^+$ is, however, independent of the *mei1*$^+$-*mei3*$^+$-*pat1*$^+$-*mei2*$^+$ cascade. *rep1*$^+$ was induced in the mutants of those genes with extents and time courses comparable to those of wild type cells (Figure 21B).

The transcript of *rep1*$^+$ was difficult to detect in the logarithmically growing cells. Consistent with this, *cdc10*^{ts} mutant could be rescued by only *rep1*$^+$ which was expressed from a foreign promoter like the SV40 promoter (see Figure 12), and the genomic *rep1*$^+$ gene expressed by its own promoter had no suppression activity .

Although the *rep1*$^+$ gene has a positive role in the initiation of meiosis, constitutive expression of *rep1*$^+$ from the SV40 promoter is not sufficient to induce conjugation or meiosis in the absence of nitrogen starvation, pheromone signals and diploidy that are required to proceed with normal meiotic program.

Table II. Strain List

strain	genotype
L968	h^{90}
L972	h^{-S}
SA21	h^{+S}
K156-A6	h^{-S} *leu1-32*
K153-B25	h^{90} *leu1-32*
AS1	h^{+N} *cdc10-129 leu1-32*
K123-14D	h^{-S} *ade6-M216 leu1-32 ura4-D18 res1::ura4*$^{+}$
DP2	h^{-S}/h^{+N} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18*
D18	h^{-S}/h^{+N} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 rep1*$^{+}$*/rep1::ura4*$^{+}$
A18-3	h^{-S} *ade6-M216 leu1-32 ura4-D18 rep1::ura4*$^{+}$
A181	h^{-S}/h^{+N} *ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 rep1::ura4*$^{+}$*/rep1::ura4*$^{+}$
AH1	h^{90} *leu1-32 ura4-D18 rep1::ura4*$^{+}$
AH2	h^{+N} *ura4-D18 rep1::ura4*$^{+}$
AH3	h^{-S} *leu1-32 ura4-D18 rep1::ura4*$^{+}$
K149-1	h^{-S} *pat1-114*
AP1	h^{-S} *pat1-114 ura4-D18 rep1::ura4*$^{+}$
AS2	h^{90} *mei1-B102*
K100-8B	h^{90} *mei2-2*
AS3	h^{90} *mei3-B71*
AS4	h^{90} *mei4-P572*
C756-1A	h^{90} *ste11 leu1-32*
JZ453	h^{90} *ade6-M21 leu1-320 ura4-D18 gpa1::ura4*$^{+}$
C508-2A	h^{90} *fus1-B20*
K158-A1	h^{90} *ura4-D18 rep1::ura4*$^{+}$
K159-A1	h^{90} *ura4-D18 res2::ura4*$^{+}$

III. Discussion

As aforementioned, two types of *cis*-acting transcriptional elements, SCB and MCB, are involved in the regulation of genes expressed in late G1 or early S phase in *S. cerevisiae*. Remarkably, some of the genes are essential for progression from G1 into S phase, indicating that these elements play a crucial role in the regulation of "start" in the cell cycle. SCB is present in the gene specifying the HO endonuclease, an enzyme involved in mating type switching, and those encoding *S. cerevisiae* G1 cyclins, *CLN1*, *CLN2*. *CLN*s are required for the activation of Cdc28 (homologue of Cdc2) kinase, and activated Cdc28 is in turn required for the "start" of S phase. On the other hand, MCB is present in the gene encoding a subunit of ribonucleotide reductase, a key enzyme for the biosynthesis of deoxyribonucleotide substrates for DNA synthesis, of both *S. cerevisiae* and *S. pombe*. This gene is specifically expressed in late G1 and S phases.

The Swi4-Swi6 complex activates SCB. DSC1, which activates MCB, is similarly composed of the Swi6 protein in *S. cerevisiae* (Lowndes *et al.*, 1992b; Dirick *et al.*, 1992) and the Cdc10 protein in *S. pombe* (Lowndes *et al.*, 1992a). Cdc10 is homologous with Swi4 and Swi6, all containing two copies of the cdc10/SWI6/ankyrin motif. This motif has been found in various mammalian proteins involved not only in transcriptional regulations such as the rat GABPβ subunit (LaMarco *et al.*, 1991), human NFκ-B/KBF1 precursor (Bours *et al.*, 1990; Kieran *et al.*, 1990), bcl-3 (Ohno *et al.*, 1990) and MAD3 (Haskill *et al.*, 1991) but also in cytoskeletone such as ankyrin (Lux *et al.*, 1990), signal transductions and other unknown functions (reviewed by Blank *et al.*, 1992). Although the function of this motif in these proteins is unclear, the structural similarity indicates that Cdc10, Swi4 and Swi6 are closely related to one another, and perhaps have evolved from a common ancestor. The fact that Swi4 and Swi6 form a complex and activate SCB in *S. cerevisiae* indicates that Cdc10 may have a Swi4-like partner.

Res1 protein is an association partner of Cdc10 and requires for mitotic and meiotic "start" in *S. pombe*

The Swi4-like protein encoded by *res1*$^{+}$, a new *cdc* gene essential for the "start" of S phase which we isolated as a dual suppressor of *pat1*^{ts} and *cdc10*^{ts} mutants, is one of this putative partner. The structural resemblance of Res1 to Swi4 and Cdc10 to Swi6 is striking. Besides the two copies of the cdc10/SWI6/ankyrin motif, Res1 have a homologous sequence with Swi4 in their amino-terminal region whereas Cdc10 and Swi6 have such a sequence in their carboxy-terminal region (Figure 5). The region of amino acids 36-155 of Swi4 protein, which is necessary and sufficient for its binding to SCB (Primig *et al.*, 1992), corresponds exactly to the domain homologous with Res1

protein. As shown in Figure 6, this region is also required for the Res1 function. Similarly, the functional domain of Cdc10 resides in its carboxy-terminal half (Aves *et al.*, 1985).

Functional resemblance is also evident between Res1 and Swi4. The *SWI4* gene can rescue the *swi6* mutation, but the *SWI6* gene can not rescue the *swi4* mutation (Andrews and Herskowitz, 1989b; Breeden and Nasmyth, 1987b). Similarly, the $res1^+$ gene can rescue the $\Delta cdc10$ mutation, but the $cdc10^+$ gene can not rescue the $\Delta res1$ mutation (Figure 10).

Finally, if Res1 is a partner of Cdc10, inactivation of either gene is expected to produce similar if not identical defective phenotypes, and this holds true. Mutational inactivation of $res1^+$ or $cdc10^+$ indeed induces the same G1 as well as meiotic arrests with very similar cell morphologies (Figure 8, 9). Furthermore, Res1 actually associates with Cdc10 *in vivo* (Figure 11) and both proteins are contained in a protein complex bound to the MCB *cis*-element (Caligiuri and Beach, 1993).

S. pombe contains two functionally redundant, structurally related parallel transcriptional regulatory systems, Res1-Cdc10 and Res2-Cdc10

Since the $res1^+$ gene can complement the $\Delta cdc10$ mutant but not vice versa, the function crucial for the "start" of the cell cycle resides in Res1 but not Cdc10. However, cells deleted for the $res1^+$ gene could grow at 30°C, though poorly. This paradox is resolved by identification of the $res2^+$ gene. The Res2 protein is highly homologous with Res1 throughout the entire region and contains the 'Res/Swi4 motif', the presumed DNA binding domain, in its amino-terminal region (Figure 5). Reflecting this structural similarity, Res2 is functionally redundant with Res1. Accordingly, the presence of either Res1 or Res2 is sufficient for cells to initiate premitotic and premeiotic DNA synthesis.

However, there are several distinctions between Res1 and Res2. Deletion of the $res2^+$ gene cripples meiosis but has little effect on the mitotic cell cycle, indicating that Res2-Cdc10 plays a dominant role in the initiation of premeiotic DNA synthesis whereas Res1-Cdc10 do so in the initiation of mitotic DNA synthesis. In addition, Res2 significantly differs from Res1 in their relationship to Cdc10. The binding affinity of Res1 to Cdc10 is relatively low, and overexpressed Res1 is able to act without Cdc10. On the contrary, Res2 has a very high binding affinity to Cdc10 (Figure 11) and almost absolutely requires Cdc10 for its function (Miyamoto *et al.*, 1994). This indicates that Res2 acts in closer relation with Cdc10, and that the putative Res2-Cdc10 and Res1-Cdc10 transcriptional complexes themselves may differently be regulated and may play different roles. In fact, expression of the $res1^+$ gene is constitutive with only a slight change under the conditions we tested whereas $res2^+$ transcript is highly induced during conjugation and meiosis, consistent with their biological roles (Figure 18D; Obara-Ishihara and Okayama, 1994; Miyamoto *et al.*, 1994). This induction requires the $rep1^+$

gene. In addition, Res1-Cdc10 and Res2-Cdc10 differ further in function. The latter has an additional role in meiotic division. Rapidly growing *Δres2* cells appear to enter premeiotic DNA synthesis normally but perform severely crippled meiotic division: they display a terminal phenotype of partial *mei* (meiosis deficient) with production of unsporulated cells and asci containing only one or two inviable spores (Miyamoto *et al.*, 1994). This defect can not be rescued by the *res1*$^+$ gene.

This functional distinction may in turn suggest that Res1-Cdc10 and Res2-Cdc10 recognize different *cis*-elements. However, this may be incorrect. Res1-Cdc10 has been reported to recognize MCB (Caligiuri and Beach, 1993). Zhu *et al.* (1994) isolated the *pct1*$^+$ gene, identical to *res2*$^+$, as a gene whose product associates with Cdc10 and binds to MCB in yeast. Thus, at least under certain conditions, both Res1-Cdc10 and Res2-Cdc10 complexes recognize MCB. How both complexes could differ in function while they recognize the same regulatory sequence? There may be several distinct systems regulating the Res1-Cdc10 and Res2-Cdc10 complexes to exert their functional distinction. The Rep family members may be included such systems (see below).

Members of the *cdc10*$^+$*/SWI4* family are also identified in the yeast *Kluyveromyces lactis* (Koch *et al.*, 1994). The high structural and functional conservations of these proteins among distantly related three yeasts suggest that their families may exist in all eukaryotes. The human transcription factor E2F/DRTF1 constitutes an entity analogous with *cdc10*$^+$*/SWI4* family. E2F binds an *cis*-element similar to the SCB and MCB and is involved in the regulation of G1 specific expression of genes required for S phase, such as DHFR encoding dihydrofolate reductase (Means et al., 1992; reviewed by Nevins, 1992a, b). However, it is unlikely that E2F is a mammalian counterpart of Res/Cdc10. E2F displays little similarity to the *cdc10*$^+$*/SWI4* family member (LaThangue and Taylor, 1993). In mammals there may be functional and structural homologues of Res/Cdc10.

rep1$^+$ gene controls the initiation of premeiotic DNA synthesis via induction and activation of Res2

The *rep1*$^+$ gene was isolated as a multicopy suppressor of inability of a *cdc10*^{ts} mutant to grow in the mitotic cycle. However, our data indicate that *rep1*$^+$ plays a crucial role in the initiation of meiosis. Inactivation of *rep1*$^+$ had no apparent effect on the mitotic cell cycle, but blocked the onset of premeiotic DNA synthesis (Figure 18A). However, we found that the inability of *Δrep1* mutants to initiate premeiotic DNA synthesis is greatly influenced by growth conditions before conjugation. When meiosis was induced after complete growth arrest, cells almost absolutely required *rep1*$^+$ for the induction of *res2*$^+$ and initiation of premeiotic DNA synthesis (Figure 18). But, when rapidly growing *Δrep1* cells were induced, a considerable number of cells initiated premeiotic DNA synthesis and successfully formed spores (Figure 16A and 16B), even

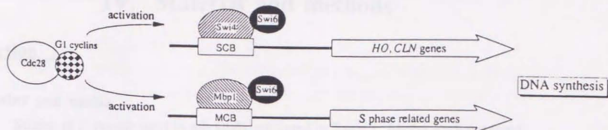
though abnormalities in the number and DNA content of spores persisted. This phenotype is strikingly similar to *Δres2* mutants. Arrested *Δres2* cells are unable to initiate premeiotic DNA synthesis, but this inability is efficiently suppressed when rapidly growing *Δres2* cells are induced for conjugation and meiosis although meiotic abnormality can not be rescued. Despite its role in meiosis, the *rep1*$^{+}$ gene can effectively suppress not only *cdc10*^{ts} but also *Δres1* mutants in the mitotic cycle without induction of *res2*$^{+}$ (Figure 12 and 20). All these results suggest that Rep1 not only induces but also activates Res2. Consistent with this, we recently isolated as another dual multicopy suppressor of *cdc10*^{ts} and *Δres1* mutant the *rep2*$^{+}$ gene which encodes one zinc finger protein with certain homology to Rep1 (Nakashima *et al*, unpublished results). Characterization of this gene led to the conclusion that Rep2 protein is an essential component of the active Res2-Cdc10 complex in mitotic cycle, and without it Res2-Cdc10 binds to but fails to activate MCB. Thus, the one zinc finger proteins Rep1 and Rep2 play a key role in the activity of the Res2-Cdc10 complex in meiotic and mitotic cycles, respectively. This in turn suggests that there may be another Rep-like factor which activates Res1-Cdc10.

How are Res1-Cdc10 and Res2-Cdc10 regulated?

MCB-containing promoters are periodically activated with peak in late G1 or early S phase. However, little is known about how this periodicity is generated. In meiotic cycle, the transcriptional regulation of *res2*$^{+}$ is an important factor for this control. The expression of the *res2*$^{+}$ gene is highly induced during conjugation by the action of Rep1. In addition, as already discussed, the activity of Res2-Cdc10 is regulated by Rep1, which is also induced by nitrogen starvation (Figure 22).

In mitotic cycle, however, the expression of *res1*$^{+}$, *res2*$^{+}$, *cdc10*$^{+}$ and *rep2*$^{+}$ does not change significantly (Aves *et al.*, 1985; Obara-Ishihara and Okayama, 1994; Nakashima *et al*, unpublished results), indicating that Res1-Cdc10 and Res2-Cdc10 are regulated by means other than transcription. The interaction of Res to Cdc10 or the binding of the complexes to MCB could be regulated to induce periodicity. Alternatively, Rep2 and putative Rep2-like factor for Res1-Cdc10 could be the target for the regulation. With this view, the possibility of regulation by phosphorylation has been explored. One obvious candidate for regulation kinase is Cdc2 kinase though it is not required for "start" of premeiotic DNA synthesis (Beach *et al.*, 1985). The levels of DSC1 activity change during the cell cycle, depending on the activity of Cdc2 kinase (Reymond *et al.*, 1993) (Figure 22). However, despite extensive studies by us and others, there is no indication that Cdc2 kinase directly regulate Res1 or Res2.

S. cerevisiae



S. pombe

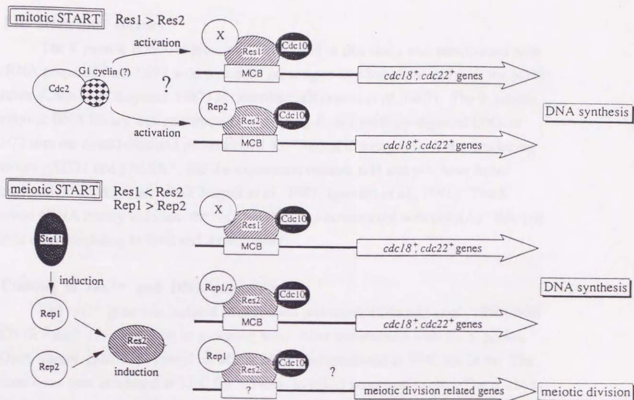


Fig.22. A model illustrating regulatory systems controlling the "start" specific transcription in budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*.

IV. Material and methods

Section 1

Strains and media

Strains of *S. pombe* used in this study are listed in Table I. Media were prepared as described previously (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974; Nurse, 1975; Okazaki *et al.*, 1990; Moreno *et al.*, 1990).

Libraries and vectors

The *S. pombe* cDNA expression library used in this study was constructed with mRNA prepared from L972 wild type cells growing to late-log phase and with the pcD2 vector (Chen and Okayama, 1987) as described (Okayama *et al*, 1987). The *S. pombe* genomic DNA library was constructed by inserting *Eco*RI-partially-digested DNA of L972 into the *Eco*RI-digested pBluescriptII KS$^+$ vector (Stratagene). The transducing vectors pAU21 and pALSK$^+$, and the expression vectors pcU and pcL have been described (Okazaki *et al.*, 1990; Nagata *et al.*, 1991; Igarashi *et al.*, 1991). The *S. pombe* cDNA library to isolate the *res2*$^+$ cDNA was constructed with poly(A)$^+$ RNA of L972 cells according to Seed and Arufo (1987).

Cloning of *res1*$^+$ and DNA sequencing

The *res1*$^+$ gene was isolated as described previously (Okazaki *et al.*, 1990) with SO5 (*h*$^{-S}$ *pat1-114 ura4-294*) as a cloning host. After transfection with the *S. pombe* cDNA library, cells were spread on MMA plates and incubated at 23°C for 24 hr. The plates were then incubated at 32°C for 3-5 days to select suppressed cells. The colonies that formed were isolated and subjected to an instability test which distinguishes authentic transformants from phenotypic revertants. cDNA clones were recovered in *E. coli* from candidates that passed the instability test. The suppression activity of the recovered cDNAs was confirmed by subsequent transfection into the host strains. DNA sequence analysis was performed by the dideoxynucleotide method (Sanger *et al.*, 1977) after subcloning into M13-derived vectors. All sequences described in this paper were confirmed by sequencing both strands.

Deletion analysis

Full or variously truncated *res1*$^+$ cDNAs were excised with appropriate restriction enzymes from pcD2-res1 and inserted into the pcL vector. Δ*Sac*I-*Kpn*I and Δ*Sac*I-*Xba*I (see Figure 6) were made by digestion of the full length *res1*$^+$ cDNA with *Sac*I and *Kpn*I or *Sac*I and *Xba*I followed by blunt-ending with the Klenow fragment and ligation.

These deletion mutants were confirmed by DNA sequencing to contain in-frame deletions as described.

These intact cDNA and deletion mutants were transfected into the *cdc10-129* mutant (AS1) and the suppression activities were calculated as percentages of suppressed cells at 32°C per stably transfected cells at 23°C. The abilities to complement *Δres1* (K123-14D) mutant was determined as percentages of complemented cells at 36°C per stably transfected cells at 30°C.

Gene disruption

A null mutation allele of *cdc10*$^+$ was constructed as follows. A genomic DNA containing the *cdc10*$^+$ gene was isolated by complementation of a *cdc10*^{ts} strain. The 2.3 kb *Xho*I fragment in the coding region of the *cdc10*$^+$ gene was replaced by the 1.8 kb *Hin*dIII genomic DNA fragment of the *ura4*$^+$ gene. The 5.8 kb *Kpn*I-*Sma*I fragment encompassing the disrupted *cdc10*$^+$ gene was used to transform the diploid strain DP2 (h^{+N}/h^{-S} *ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18*) and stable ura$^+$ transformants were isolated. Southern blot hybridization confirmed that one copy of the *cdc10*$^+$ gene in 11 independently isolated diploid transformants was replaced by the *ura4*$^+$ gene. All spores formed from these disruptants germinated but ceased to grow in uracil-free media as described before (Marks *et al.*, 1992).

The null mutation of the *res1*$^+$ gene was constructed as follows. A genomic DNA fragment containing the *res1*$^+$ gene was isolated from an *S. pombe* genomic library by colony hybridization. The 4.5 kb *Bam*HI-*Eco*RI genomic DNA fragment containing the entire *res1*$^+$ gene was subcloned into pUC19 (pUC-BEres1). The *Sac*I-*Sca*I fragment containing more than 80% of the *res1*$^+$ coding region (511 of 637 amino acids) was replaced with the *ura4*$^+$ *Hin*dIII fragment (pUC-BEres1::ura4, Figure 7). The diploid strain DP2 was transformed with the *res1::ura4*$^+$ allele excised from pUC-BEres1::ura4 by *Bam*HI and *Eco*RI digestion, and stable ura$^+$ were isolated.

A null mutant of *res2*$^+$ was constructed as follows. The 1.8 kb *Eco*T22I-*Pst*I fragment containing about 77% of the *res2*$^+$ coding region was replaced with the 1.8 kb end-filled *Hin*dIII fragment of the *ura4*$^+$ gene. The *Bam*HI-*Bss*HII fragment of the disrupted *res2*$^+$ gene containing a short vector sequence at one end was transfected into a diploid strain DP2 and stable ura$^+$ transformants ware isolated. Disruptants were first identified by PCR using a *ura4*$^+$ specific primer (5'-TTGGTGTTGGAACAGAATAAA-TT-3' as a sense primer) and a 3' primer (5'-CACAAATGCATACATATAGCCA-3' as an antisense primer)which corresponded to a sequence downstream of the *Sau*3AI restriction site in *res2*$^+$. The disruptant (M101) was confirmed by Southern blot analysis using the 2.0 kb *Hin*dIII-*Eco*RI fragment as a probe.

Flow cytometry

Flow cytometry was performed as described previously (Costello *et al.*, 1986) using the FACScan system and the CellFIT Cell Cycle Analysis program (Becton Dickinson). The *cdc10*^{ts} mutant (AS2) was growth arrested by incubating at 37°C for 2 hr, and used as a standard marker for 1n and 2n DNA content.

Assay for conjugation and sporulation

Conjugation capabilities of *Δres1* or wild type cells were assayed according to the method of Nurse and Bisset (1981). The *Δres1* strain K123-14D (*h*$^{-S}$ *ade6-M216 leu1-32 ura4-D18 res1::ura4*$^{+}$) was cultured to a mid-log phase (4-5 x 10^6 cells/ml) at 30°C in minimal medium. Cells were re-inoculated into fresh minimal medium (18°C) at a concentration of 1-2 x 10^6 cells/ml and cultured for 8 hr at 18°C to arrest cell cycle progression. Cells were then washed, resuspended in nitrogen-free minimal medium preincubated at 18°C, mixed with a 10 times excess of *mei1* mutant cells (SO7, *h*^{90} *ade6-M210 ura5-294 leu2-120 mei1-B102*) and further incubated 18°C. After a 48 hr incubation the mating cell mixture was gently sonicated and plated on minimal medium agar to determine the number of diploid cells formed by conjugation. The strain K137-10 (*h*$^{-S}$ *ade6-M216 leu1-32*) was used as a positive control.

The ability of *Δres1* cells to undergo meiosis and sporulation was determined as described by Grallert and Sipiczki (1991). Diploid strains homozygous (K135) or heterozygous (K134) for *Δres1* were constructed as follows. The K123-14D (*h*$^{-S}$ *ade6-M216 leu1-32 ura4-D18 res1::ura4*$^{+}$) cells were mixed with K123-14C (*h*$^{+N}$ *ade6-M210 leu1-32 ura4-D18*) or K123-14A (*h*$^{+N}$ *ade6-M210 leu1-32 ura4-D18 res1::ura4*$^{+}$), spotted on SPA, and incubated at 30°C overnight. The cell mixture was spread on MMA containing leucine and phloxin B. The colonies which stained dark red were isolated and used for further study. The rapidly growing diploid cells were suspended in water and spotted on two SPA plates. One was incubated at a permissive temperature of 30°C, and the other was incubated at a non-permissive temperature of 23°C. Sporulation was examined under a microscope.

Isolation and structural analysis of a *res2*$^{+}$ clone

The *res2*$^{+}$ gene was isolated by phenotypic suppression of *Δres1* mutant (K123-14D). The *Δres1* cells were cultured at 30°C to a mid-log phase in EMM2 medium containing 0.5% glucose, adenine and leucine, and transfected with the *S. pombe* genomic library as described (Okazaki *et al.*, 1990). The cells were incubated at 30°C for 16 hr on MMA plates supplemented with adenine and then at 21°C for 5-6 days. Colonies formed were then examined by the instability test to distinguish authentic transformant from phenotypic revertants (Okazaki *et al.*, 1991). Plasmid DNAs were recovered from authentic transformants, cloned in *E. coli* and tested for their activity.

Active plasmid clones were analyzed by Southern blotting using the *Pvu*II fragment of *res1*$^{+}$ as a probe. One clone which did not hybridize with the probe was digested with *Bss*HII endonuclease, and the 3.4 kb insert was subcloned into the M13, pALSK^{+} or pcL vectors. *res2*$^{+}$ cDNA clones were isolated by colony hybridization. DNA sequence were determined by the dideoxy method (Sanger *et al.*, 1977) using Sequenase version 3 (USB).

Tetrad analysis

The *res1*$^{+}$/*Δres1 res2*$^{+}$/*Δres2* diploid cell (M105) was constructed by crossing M102-1B with K123-14D. M105 cells were streaked onto a MEA plate and incubated at 30°C 3 days. After asci were formed, spores were isolated from each ascus and plated onto YES medium followed by incubation at 30°C for 3-4 days. Germinated cells were analyzed for their genotype. *Δres2* (M102-1B) and *cdc10-129* (AS2) cells were crossed to generate *Δres2 cdc10-129* spores.

Two-hybrid assay

Two-hybrid assay was performed using the Matchmaker Two-Hybrid System (Clontech).

The *res1*$^{+}$ cDNA which was introduced a *Nde*I site into its initiation codon was digested with *Nde*I, blunt-ending with Klenow fragment and digested with *Bam*HI. The *Nde*I/blunt-*Bam*HI fragment containing the entire *res1*$^{+}$ coding region was subcloned into *Eco*RI/blunt-*Bam*HI digested pGBT9 vector (GBT9-res1). The amino-terminal region of *res2*$^{+}$ cDNA and *cdc10*$^{+}$ genomic DNA were amplified by PCR using oligonucleotides res2-MetRI and res2-Salrev (5'-GCGCGAATTCATGGCTCCACGTT-CTTCCGC-3' and 5'-ACCTTATACTTTGTGGCC-3') for *res2*$^{+}$ and cdc10-MetRI and cdc10-Nderev (5'-GCGCGAATTCATGGCTTCAGCCAATTTTATTCG-3' and 5'-AA-CCCCATATCGCTTTGC-3') for *cdc10*$^{+}$. The PCR products were digested with *Eco*RI-*Sal*I (*res2*$^{+}$) and *Eco*RI-*Nde*I (*cdc10*$^{+}$). The *Eco*RI-*Sal*I fragment of *res2*$^{+}$ was subcloned into *Eco*RI-*Bam*HI digested pGBT9 vector together with *Sal*I-*Sau*3AI fragment containing carboxy-terminal region of Res2 excised from pcL-res2 (GBT9-res2). The *Eco*RI-*Nde*I fragment of *cdc10*$^{+}$ was subcloned into *Eco*RI-*Bam*HI digested pGAD424 vector together with *Nde*I-*Bam*HI fragment containing carboxy-terminal region of Cdc10 excised from pAUSK^{+}-cdc10 (GAD424-cdc10). The integrity of the amplified fragments and the in-frame fusions with the Gal4 DNA binding domain or activator domain were confirmed by sequencing.

Northern blot analysis

S. pombe strains were grown at 30°C to 8 x 10^6 cells/ml in appropriately supplemented SSL+N, and total RNA was prepared according to Elder *et al.* (1983). Ten micrograms of RNA were electrophoresised in a 1% agarose gel containing 1.1M formaldehyde, 10mM sodium phosphate buffer (pH 7.2) and blotted onto a nylon membrane (Biodine). Hybridization was performed in 6 x SSPE containing 5 x Denhardt's solution, 0.1% SDS and 170μg/ml of salmon sperm DNA with the ^{32}P-labeled 1.4 kb *res2*$^+$ *Eco*RI-*Hin*dIII fragment as a probe. Membranes were washed in 0.2 x SSC, 0.1% SDS at 65°C.

Suppression of "start" gene mutants by *res2*$^+$

The ability to suppress growth defect of *cdc10-129* and *Δcdc10* mutants was assayed as follows. The *h^{-S} cdc10-129 leu1-32* (AS5) cells were transfected with the indicated plasmids. One-half of transfected cells was selected for leu$^+$ at the temperatures specified, and the other half at 25°C, a permissive temperature for *cdc10-129*. The ratios of colonies formed at the temperatures to those formed at 25°C are expressed as percentage suppression. A *cdc10*$^+$*/Δcdc10 Δura4/Δura4* diploid strain (DC107) was transfected with the indicated plasmids and selected for leu$^+$. The transformed cells were then sporulated. The percentage suppression of the *Δcdc10* mutation was calculated as the ratio of ura$^+$ colonies to the total germinated spores. Therefore, the percentage of full suppression is 50%.

The ability to suppress meiosis defects of *Δres1/Δres1* diploid cells was assayed as follows. *Δres1* haploid cells (K123-14C, K123-14D) transformed with the indicated plasmids were mated to form diploid *Δres1* cells and then induced for meiosis. A total 200 cells were examined and asci with four spores were counted.

Pat1^{ts}-induced haploid meiosis assay

The *h^{-S} pat1-114 Δres2* (M107-1A) and *h^{-S} pat1-114* (SO6) cells were grown at 23°C to 1 x 10^7 cells/ml in SSL+N. Cells were harvested, washed with SSL-N, suspended in the same medium at a density of 6 x 10^6 cells/ml and starved for nitrogen by incubation at 23°C for 14 hr. The temperature was then shifted to 34°C to initiate meiosis and cell aliquots were taken at indicated times to monitor the progression of meiosis. Flow cytometry was performed as described previously (Costello *et al.*, 1986) using the FACScan system and CellFIT cell cycle analysis program (Becton Dickinson).

Section 2

Yeast strains and media

The *S. pombe* strains used in this study are listed in Table II. A null mutation allele of *cdc10*$^{+}$ was described (Section 1). Media were prepared as described previously (Egel and Egel-Mitani, 1974; Gutz *et al.*, 1974; Okazaki *et al.*, 1990; Moreno *et al.*, 1990).

Libraries and vectors

The *S. pombe* cDNA expression library used in this study was described (Section 1). To prepare *S. pombe* genomic DNA libraries, high molecular weight DNA prepared from the wild type strain (L972) was partially digested with *Sau*3AI and inserted into the *Bam*HI-digested pBluescript II KS^{+} vector (Stratagene). The transducing vectors pAL19 and pALSK^{+}, and the expression vectors pcL and pREP1 were described (Okazaki *et al.*, 1990; Igarashi *et al.*, 1991; Nagata *et al.*, 1991; Maundrell, 1993).

Cloning of *rep1*$^{+}$ and DNA sequencing

The *rep1*$^{+}$ gene was isolated as described previously (Okazaki *et al.*, 1990; Tanaka *et al.*, 1992) with h^{+N} *cdc10-129 leu1-32* (AS1) as a cloning host. After transfection with the *S. pombe* cDNA library, cells were spread on MMA plates and incubated at 23°C for 24 hr. The plates were then incubated at 32°C for 3-5 days to select suppressed cells. cDNA clones were recovered in *E. coli* from candidate colonies and their suppression activity was assayed by transfection into the AS1.

DNA sequencing was performed by the dideoxynucleotide method (Sanger *et al.*, 1977) after subcloning into M13-derived vectors. The sequences were confirmed by sequencing both strands.

Gene disruption

The null mutant stain of the *rep1*$^{+}$ gene was constructed as follows. A genomic DNA fragment containing the *rep1*$^{+}$ gene was isolated from an *S. pombe* genomic library by colony hybridization. The 5.5 kb *Bgl*II-*Pst*I genomic DNA fragment containing the entire *rep1*$^{+}$ gene was subcloned into the *Bam*HI-*Pst*I sites of the modified pBluescriptII KS^{+} vector in which the *Hin*dIII site had been eliminated (pBS-BPrep1). The 1.1 kb *Hin*dIII fragment containing about 80% of the *rep1*$^{+}$ coding region was replaced by the 1.8 kb *Hin*dIII genomic DNA fragment of the *ura4*$^{+}$ gene (pBS-BPrep1::ura4, Figure 16A). Inactivation of *rep1*$^{+}$ in this construct was confirmed by its inability to rescue the *cdc10-129* mutant in cDNA form. A diploid strain (DP2) was transformed with the *rep1::ura4*$^{+}$ DNA excised from pBS-BPrep1::ura4 with *Mfl*I and *Pst*I endonucleases. Stable ura^{+} transformants were selected, and the genomic structures around the *rep1*$^{+}$ locus were analyzed by Southern blot hybridization using the *Xho*I fragment of *rep1*$^{+}$ as

a probe. One of the transformants (D18) containing the expected structure was sporulated. After ethanol treatment, random spores were tested for the ura$^+$ phenotype.

Pat1^{ts}-induced haploid meiosis assay

The h^{-S} *pat1-114 Δrep1* (AP1) and h^{-S} *pat1-114* (K149-1) cells were grown in MM+N at 24°C to 1 x 10^7 cells/ml. Cells were then harvested, resuspended in MM-N at density of 6 x 10^6 cells/ml and cultured for 14 hr at 24°C to arrest in G1. Under these conditions, approximately 20% of the cells were arrested in G2. The G1-arrested fraction was prepared using a Beckman JE-5.0 elutriation system with a J6-MC centrifuge. Two litters of the nitrogen-starved culture was pumped at 100 ml/min into the rotor which was spinning at 4000 r.p.m. and keeping at 24°C. After loading, rotor speed and pump rate were gradually decreased ~2500 r.p.m. and ~60 ml/min respectively. To harvest the small cells arrested in G1, rotor speed was decreased a little more, and about 4 x 10^9 cells were eluted off. Elutriation-purified G1-arrested cells were shifted up to 34°C to start meiosis, and aliquots were taken at indicated times to monitor the progression of meiosis.

Cell viability was determined as percentages of cells that form colonies on YEA plates at 23°C. To count one to four nuclei, cells were fixed with final 70% ethanol, stained with final 1 μg/ml of DAPI (4,6-diamidino-2-phenylindole) and observed using a fluorescent microscope. Flow cytometry was performed according to Costello *et al.* (1986) using the FACScan system and the CellFIT Cell Cycle Analysis program (Becton Dickinson). For Northern blot analysis, total RNA was prepared from each aliquot at the indicated time.

RNA preparation and Northern blot analysis

S. pombe strains were grown in SSL+N with appropriate supplement at 26°C to 8 x 10^6 cells/ml. After cells were shifted to SSL-N at a density of 1 x 10^7 cells/ml, aliquots were taken at indicated times. Total RNA was prepared, and Northern blot analysis was done as described previously (Elder *et al.*, 1983; Nagata *et al.*, 1991).

V. Acknowledgments

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VII. Summary

In the fission yeast *Schizosaccharomyces pombe*, the *cdc10*$^+$ gene plays a crucial role in the "start" of mitotic and meiotic cycles as a transcriptional regulator of some S phase specific genes. We have isolated two new cell division cycle genes, *res1*$^+$ and *res2*$^+$, which can suppress the *cdc10*^{ts} mutant. *res1*$^+$ and *res2*$^+$ specify 72 kd and 73 kd proteins, respectively, and share high sequence and structure similarity. They have significant homology with Cdc10 of *S. pombe* and Swi4, Swi6 and recently identified Mbp1 of *Saccharomyces cerevisiae*, all of which have two copies of the cdc10/SWI6/ankyrin motif. In addition to this motif, Res1, Res2, Swi4 and Mbp1 proteins share a remarkable homology in their amino-terminal region, whereas Cdc10 and Swi6 do so in their carboxy-terminal region. Moreover, the amino-terminal region is essential for the Res1 function as is for the Swi4 function. Thus, structurally, Res1 and Res2 resemble Swi4 and Mbp1 while Cdc10 resembles Swi6.

A disruptant of *res1*$^+$ grows poorly at 30°C with severe heat- and cold-sensitivities, and completely arrests in G1 at 36°C and 23°C. The arrested disruptant retains a full conjugation ability. In addition, *Δres1/Δres1* diploid cells hardly undergo meiosis. Therefore, we conclude that the function of *res1*$^+$ gene is required for "start" in both mitotic and meiotic cycle. Analogous to the relationship of *SWI4* to *SWI6*, the *res1*$^+$ gene effectively rescues *cdc10*^{ts} and *Δcdc10* mutants, but the *cdc10*$^+$ gene can not rescue the *Δres1* phenotype. Thus, striking similarities exist in both structural and functional relationships between Res1 and Swi4, and between Cdc10 and Swi6. In view of the fact that Swi4 and Swi6 form a transcription factor complex and activate promoters containing the Swi4/6 dependent cell-cycle box, we predicted that Res1 was an association partner of Cdc10 which activates *Mlu*I cell-cycle box-containing promoters. This prediction has recently been confirmed by us and others.

The *res2*$^+$ gene is largely redundant in function with *res1*$^+$ and is required for the initiation of mitotic and premeiotic DNA synthesis. However, unlike Res1, Res2 possesses extremely high affinity with Cdc10 and strongly depends on Cdc10 for its activity. *res2*$^+$ transcript is highly induced during conjugation and has an important role in "start" of premeiotic DNA synthesis. Moreover, *res2*$^+$ plays an additional role in meiotic division which is not substituted by *res1*$^+$. Thus, the fission yeast contains two functionally overlapping parallel "start" systems, Res1-Cdc10 and Res2-Cdc10, the former of which plays a major role in mitotic cycle whereas the latter in meiotic cycle.

In addition, we identified a novel gene, *rep1*$^+$, which regulates the Res2-Cdc10 transcriptional activator complex during meiotic cycle. The *rep1*$^+$ gene encodes a 53 kd protein with one zinc finger motif and effectively suppresses a null mutation of the *res1*$^+$ gene but only partially a temperature-sensitive mutant of the *cdc10*$^+$ gene, both of which

are required for the onset of mitotic as well as premeiotic DNA synthesis. Deletion of *rep1*$^{+}$ has no apparent effects on the mitotic cell cycle or conjugation, but blocks the initiation of premeiotic DNA synthesis and fails to induce the *res2*$^{+}$ gene. This defect is partially suppressed when rapidly growing cells are induced to conjugate. Thus, the *rep1*$^{+}$ function is at least partly substituted by those of the genes controlling the "start" of the mitotic cell cycle involving *res1*$^{+}$. The *rep1*$^{+}$ gene itself is highly induced during conjugation, and this induction is dependent on both nitrogen starvation and mating pheromones. Both *Δres2* and *Δrep1* mutants display a very similar defect in meiosis which can not reciprocally be suppressed by *rep1*$^{+}$ and *res2*$^{+}$. Thus, we conclude that inducible *rep1*$^{+}$ controls the initiation of premeiotic DNA synthesis via induction of *res2*$^{+}$ transcript and activation of the Res2-Cdc10 complex in meiotic cycle.

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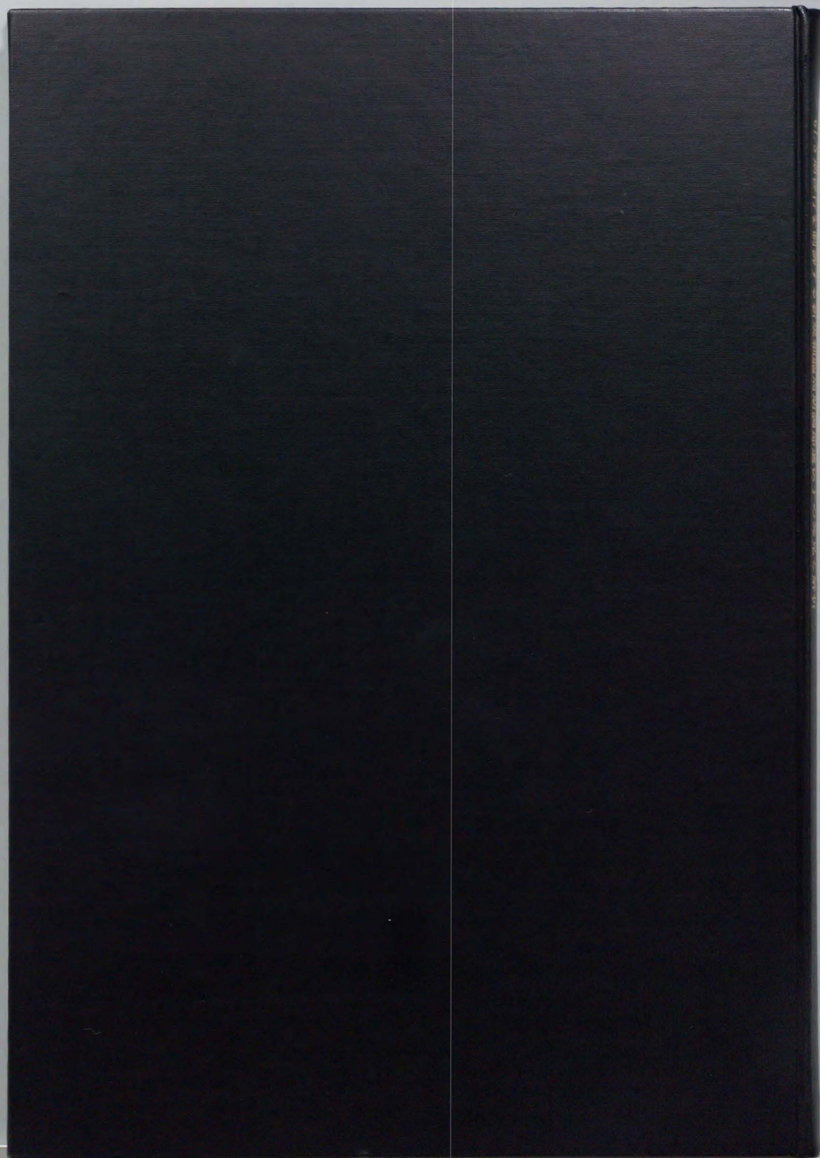

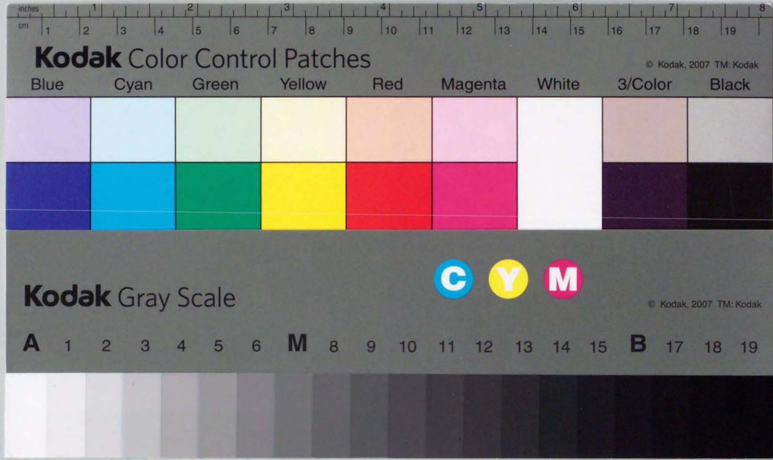
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