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Novel Factor Highly Conserved among Eukaryotes Controls Sexual Development in Fission Yeast

NORIKO OKAZAKI,[1] KOEI OKAZAKI,[1] YOSHINORI WATANABE,[2] MARIKO KATO-HAYASHI,[2] MASAYUKI YAMAMOTO,[2] AND HIROTO OKAYAMA[1,3]*

Okayama Cell Switching Project, ERATO, JRDC, Sakyo-ku, Kyoto 606,[1] and Department of Biochemistry, Graduate School for Science,[2] and Department of Biochemistry, Faculty of Medicine,[3] University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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In the fission yeast *Schizosaccharomyces pombe*, the onset of sexual development is controlled mainly by two external signals, nutrient starvation and mating pheromone availability. We have isolated a novel gene named *rcd1*$^+$ as a key factor required for nitrogen starvation-induced sexual development. *rcd1*$^+$ encodes a 283-amino-acid protein with no particular motifs. However, genes highly homologous to *rcd1*$^+$ (encoding amino acids with >70% identity) are present at least in budding yeasts, plants, nematodes, and humans. Cells with *rcd1*$^+$ deleted are sterile if sexual development is induced by nitrogen starvation but fertile if it is induced by glucose starvation. This results largely from a defect in nitrogen starvation-invoked induction of *ste11*$^+$, a key transcriptional factor gene required for the onset of sexual development. The striking conservation of the gene throughout eukaryotes may suggest the presence of an evolutionarily conserved differentiation controlling system.

When starved for nutrient, the fission yeast *Schizosaccharomyces pombe* arrests in G_1, but if mating partners are available, it resumes sexual development. The cells that have committed to sexual development subsequently perform conjugation, meiosis, and sporulation. The commitment to this alternative pathway requires the action of the Ste11 transcriptional factor. This factor is essential for the activation of many genes needed for the initiation and progression of conjugation and meiosis. Among them are mating-type genes, *ste* genes (including *ste11*$^+$), *mei2*$^+$, and *rep1*$^+$ (11, 19, 24, 33, 34, 38, 40). Therefore, it is conceivable that the *ste11*$^+$ gene and its product serve as key targets for the regulation of the onset of sexual development.

Nitrogen starvation and carbon starvation are two major nutrient exhaustion signals that trigger sexual development. The cyclic AMP (cAMP)-Pka1 pathway mediates mostly a carbon source signal and partly a nitrogen source signal and negatively regulates *ste11*$^+$ expression (2, 10, 15, 16, 33). The *pac2*$^+$ gene, whose physiological role is unknown, also represses *ste11*$^+$ expression (20). In addition, a stress signal transduced by the Wis1-Phh1/Sty1/Spc1 mitogen-activated protein kinase kinase–mitogen-activated protein kinase cascade is essential for *ste11*$^+$ induction in response to nutrient starvation (18), which is mediated by the Atf1/Gad7 transcriptional factor (17, 35). Despite extensive studies, little is understood about the specific factors and mechanisms responsible for nitrogen starvation-invoked *ste11*$^+$ induction.

In the regulation of sexual development, Pat1/Ran1 kinase plays a unique role (1, 13, 14). Its primary role is to block the onset of meiosis until conjugation takes place, by inactivating Mei2, a key factor triggering the onset and progression of meiosis (39). The function of Ste11 protein itself is modulated by direct phosphorylation by Pat1 kinase, although its function in starting conjugation seems to be unchanged (22). Consequently, inactivation of Pat1 kinase in haploid cells unconditionally induces lethal meiosis, which can be suppressed by inactivation of the *mei2*$^+$ gene. Since *ste11*$^+$ is required for the expression of *mei2*$^+$ (33), any factors that inhibit *ste11*$^+$ expression or its function would rescue the *pat1* lethality. Based on this assumption, we recently screened a gene library for multicopy suppressors of the *pat1* lethality and isolated a new gene, named *rcd1*$^+$, that is required for *ste11*$^+$ expression specifically induced by nitrogen starvation. Strikingly, genes highly homologous to *rcd1*$^+$ are present in many eukaryotes including humans. In this communication, we report the structure and function of this new gene and discuss the possibility of the presence of a highly conserved differentiation control mechanism throughout the eukaryotes.

MATERIALS AND METHODS

Yeast manipulations. The strains of *S. pombe* used in this study are listed in Table 1. Media were prepared as described previously (4, 8, 25, 28). EMM medium (25) contained 0.5% NH_4Cl and 2% glucose unless specified and was sterilized by filtration.

Conjugation capability was measured as follows. Cells were cultured to the mid-log phase (4×10^6 to 6×10^6 cells/ml) at 30°C in EMM medium and resuspended at 10^7 cells/ml in NH_4Cl-free EMM medium or in EMM medium with the indicated concentration of NH_4Cl and glucose. After incubation overnight or for the indicated times, aliquots of the cells were gently sonicated and the conjugated cells were counted by microscopy.

The ability to perform meiosis and sporulation was assayed as follows. Heterothallic *h*$^-$ *rcd1* and *h*$^+$ *rcd1* cells were grown on YES plates (25) overnight, diluted to 10^7 cells/ml with H_2O, and spotted on ME plates (25). Both conjugated and sporulated cells were counted, and the frequencies of conjugation and sporulation of conjugated cells were calculated.

Flow cytometry was performed as described previously (36) with the FACScan system and the CellFIT cell cycle analysis program (Becton Dickinson).

DNA manipulations. The *S. pombe* cDNA expression library used in this study has been described previously (29) and a *Sau*3AI genomic library was constructed by inserting partial *Sau*3AI-digested L972 genomic DNAs into the *Bam*HI-digested pBluescriptII KS$^+$ vector (34). The pALSK$^+$ and the simian virus 40 early promoter-driven pcL vectors have been described previously (12, 26). The *rcd1*$^+$ cDNA was isolated by suppression of SO5 (*h*$^-$ *pat1-114 ura4-294*) as described previously (36). The genomic DNA fragment containing *rcd1*$^+$ was isolated from an *S. pombe Sau*3A1 genomic library by colony hybridization. The human RCD1 cDNA was isolated from the human foreskin fibroblast cDNA

* Corresponding author. Mailing address: Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Phone: 03-5689-0876. Fax: 03-3815-1490. E-mail: okayama@m.u-tokyo.ac.jp.

TABLE 1. Fission yeast strains used in this study

Strain	Genotype
L972	*h*$^-$
L968	*h*^{90}
SO3	*h*$^-$ *leu1-32*
TI-120	*h*^{90} *leu1-32*
SO5	*h*$^-$ *pat1-114 ura4-294*
SO6	*h*$^-$ *pat1-114 leu1-32*
DP2	*h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 ura4-D18/ura4D18 leu1-32/leu1-32*
NN1	*h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 ura4-D18/ura4D18 leu1-32/leu1-32*
NS1	*h*$^-$/*h*$^+$ *ade6-M210/ade6-M216 ura4-D18/ura4D18 leu1-32/leu1-32 rcd1::ura4*$^+$*/rcd1::ura4*$^+$
NS2	*h*$^-$ *rcd1::ura4*$^+$ *ura4-D18*
NS3	*h*$^+$ *rcd1::ura4*$^+$ *ura4-D18*
NS4	*h*^{90} *rcd1::ura4*$^+$ *ura4-D18*
NS5	*h*^{90} *rcd1::ura4*$^+$ *ura4-D18 leu1-32*
NS6	*h*$^-$ *pat1-114*
NS7	*h*$^-$ *pat1-114 rcd1::ura4*$^+$ *ura4-D18*
NS8	*h*$^-$ *pat1-114 rcd1::ura4*$^+$ *ura4-D18 leu1-32*
NS9	*h*$^-$ *ste11-1*
NS10	*h*$^-$ *mei2-2*
NS11	*h*^{90} *pka1::ura4*$^+$ *ura4-D18*
NS12	*h*^{90} *pka1::ura4*$^+$ *rcd1::ura4*$^+$ *ura4-D18*
NS13	*h*^{90} *wis1::his1*$^+$ *his1-102*
NS14	*h*^{90} *wis1::his1*$^+$ *rcd1::ura4*$^+$ *his1-102 ura4-D18*
NS15	*h*^{90} *pac2::ura4*$^+$ *ura4-D18*
NS16	*h*^{90} *pac2::ura4*$^+$ *rcd1::ura4*$^+$ *ura4-D18*
NS17	*h*^{90} *cig2::ura4*$^+$ *ura4-D18*
NS18	*h*^{90} *cig2::ura4 rcd1::ura4*$^+$ *ura4-D18*

library pcD2-Basinger (3) by colony hybridization with a partial human RCD1 sequence amplified by PCR against the cDNA library with primers that were synthesized based on the sequence information from the Wash U-Merck EST project (R38452 yh89b11.r1 cDNA clone 136893 5′). The DNA sequence was determined by the dideoxynucleotide method (30).

Gene disruption. Two types of *rcd1* deletion mutants were constructed. In one type, the 0.75-kb *Nru*I-*Aat*II fragment containing 88% of the *rcd1*$^+$ coding sequence was replaced by the *ura4*$^+$ gene. In the other, the *Spe*I fragment corresponding to amino acids 77 to 129 was replaced by *ura4*$^+$ (7). Inactivation of *rcd1*$^+$ in each construct was confirmed by its inability to rescue the *pat1-114* mutant. The diploid strain DP2 was transformed with each disrupted *rcd1* fragment, stable ura$^+$ transformants were selected, and successful disruption was confirmed by Southern blot hybridization or by PCR detection. The ura$^+$ diploid cells were sporulated and germinated to obtain haploid *rcd1* disruptant cells.

Northern blot analysis. *S. pombe* cells were grown in EMM medium to 5×10^6 cells/ml. An aliquot of the cells was harvested, and the remainder were inoculated into EMM medium without NH_4Cl or EMM medium with 0.5% glucose at 10^7 cells/ml and incubated, with sampling of cell aliquots at the indicated times. Total RNA was prepared and Northern blot analysis was performed as described previously (27). The Northern blot membrane filter for human tissues used in this study was purchased from Clontech Laboratories, Inc. (human multiple-tissue Northern blots II). The hybridization probes used were the 0.85-kb fragment of *ste11*$^+$, the 0.87-kb fragment of *rcd1*$^+$, the 1.1-kb fragment of *fbp1*$^+$, the 0.95-kb fragment of *matPc*$^+$ and *matPi*$^+$, the 1.7-kb *Ban*II fragment of human RCD1, and the 3.3-kb *Pvu*II-*Hin*dIII fragment of *mei2*$^+$ (38). These probes were obtained by PCR amplification of cDNA libraries with appropriate primers.

Nucleotide sequence accession numbers. The DDBJ, EMBL, and GenBank accession number of the *rcd1*$^+$ gene is D87956, and that of human RCD1 is D87957.

RESULTS

Isolation of the *rcd1*$^+$ gene. To identify new elements controlling the onset of cell differentiation, we screened an *S. pombe* expression cDNA library for genes that suppress the lethality of the temperature-sensitive *pat1-114* mutation, as described previously (28), and isolated several distinct clones. One new cDNA clone, named *rcd1*$^+$ (required for cell differentiation [see below]), was characterized further. The *rcd1*$^+$ cDNA placed under the control of the simian virus 40, cytomegalovirus or *nmt1*$^+$ promoter effectively suppressed the temperature-sensitive lethality of the *pat1-114* mutant at 35°C or higher temperatures (data not shown).

***rcd1*$^+$ encodes a highly conserved protein.** The *rcd1*$^+$ cDNA was 1.8 kb long. Because it had an in-frame termination codon upstream of the assigned initiation codon and was similar in size to the transcript determined by Northern blot hybridization, it was judged to contain the entire coding region, which is capable of encoding a 283-amino-acid protein (Fig. 1). The predicted protein product is leucine rich but has no apparent motifs or significant homology to any proteins with known function. However, it has strikingly high level of homology to the products of putative genes in four eukaryotes, *Saccharomyces cerevisiae*, *Arabidopsis*, *Caenorhabditis elegans*, and *Homo sapiens*, all of which were identified by the genome project (Fig. 1). Amino acid identity in the homologous region exceeds 70% among these species. However, there are some distinctions. The putative *S. cerevisiae* homolog of Rcd1 has an N-terminal extension, whereas the *C. elegans* homolog has a C-terminal extension. The *Arabidopsis* and *H. sapiens* homologs deposited in the DNA database appeared to be truncated at the N or C terminal, perhaps because of isolation of incomplete genes. We cloned a full-size cDNA for the human RCD1 homolog. The sequence shown in Fig. 1 is the one determined from this cDNA.

Comparison of the *rcd1*$^+$ cDNA with the genomic sequence isolated by colony hybridization indicated that there is no intron in the protein coding sequence. The *rcd1*$^+$ gene is transcribed into a 1.8-kb mRNA with no obvious oscillation in the mRNA level during nutrient starvation (data not shown).

Cells disrupted for *rcd1*$^+$ are sterile. To investigate the physiological role of *rcd1*$^+$, we constructed two types of *rcd1* null mutants. Two nonfunctional *rcd1* fragments in which either 30% of the middle of the coding region (*Spe*I-*Spe*I) or 80% of the coding region (*Nru*I-*Aat*II) was replaced with the *ura4*$^+$ cassette were constructed and transfected into a diploid strain. The resulting *rcd1*$^+$/*rcd1* disruptants were identified by Southern blotting, sporulated, and germinated to obtain haploid disruptants. The two types of haploid disruptants were phenotypically indistinguishable. Therefore, the *Spe*I disruptant was chosen as representative and further characterized.

The disruptant germinated and proliferated with the same growth ability as did wild-type cells. The only noticeable difference was slight elongation of the cells. Because multicopy *rcd1*$^+$ blocked sexual development induced by *pat1*$^+$ inactivation, we anticipated that the *rcd1* disruptant would be highly proficient for sexual development. However, instead, the disruptant was highly sterile when sexual development was induced by nitrogen starvation (Fig. 2a). This sterility was the result of *rcd1*$^+$ disruption because it was suppressed by ectopic expression of *rcd1*$^+$ and always cosegregated with *rcd1*$^+$ disruption during repeated backcrossing with the standard *ura4* strain. Interestingly, the sterility was slightly alleviated at 25°C, a suboptimal temperature for growth (Fig. 2b). In contrast, when starved for glucose, the disruptant was grossly normal and actively conjugated, albeit slightly (approximately twofold) less efficiently than wild-type cells at both temperatures (Fig. 2c and d). Starvation for both nitrogen and glucose also effectively induced the onset of sexual development of the disruptant, indicating that the disruptant was responsive to glucose starvation irrespective of the presence or absence of nitrogen starvation (Fig. 2e and f). The efficiency and viability of the spores formed by glucose starvation were the same as those of wild-type cells. The severe sterility is not a consequence of loss of the ability of the cell to sense nitrogen availability and starvation or of arrest in G_1 in response to

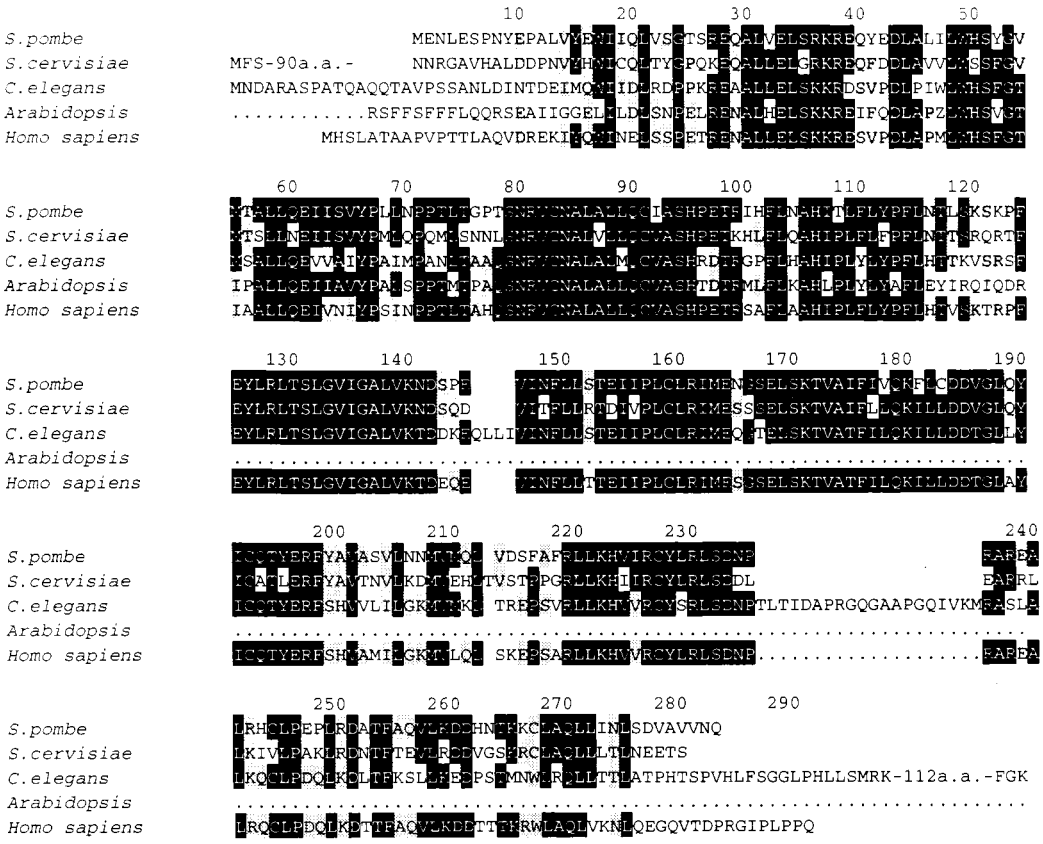

FIG. 1. Amino acid identity among Rcd1 and its homologs from *S. cerevisiae* (Z71564), *C. elegans* (U13875 C26E6.3) *Arabidopsis* (Z29188), and *H. sapiens*. Amino acids that match in any three proteins among these are shown against a black background, and those that match in any two proteins are against a shaded background. The DDBJ, EMBL, and GenBank accession numbers for the *rcd1*$^+$ and human RCD1 are D87956 and D87957, respectively.

nitrogen starvation, because the disruptant grew at the same rate and arrested in G_1 upon nitrogen starvation with the same time course and extent of G_1 arrest as those of wild-type cells (Fig. 3).

To gain a deeper insight into the function of *rcd1*$^+$, we examined the effect of *rcd1* disruption on the *pat1* phenotype. Contrary to expectation but consistent with the sterility, inactivation of *rcd1*$^+$ markedly suppressed the *pat1* phenotype, and the heterothallic *pat1-114 rcd1* double mutant grew at a nonpermissive temperature of 34°C without undergoing haploid meiosis (data not shown). The reason for the paradoxical *pat1* suppression by both overexpression and disruption of *rcd1*$^+$ is discussed below. When the *rcd1* disruptant was converted to diploid cells and the established *rcd1*/*rcd1* diploid cells were tested for their ability to perform meiosis and sporulation, they showed poor sporulation (Fig. 4a). However, this was caused not by a defect in meiosis or sporulation but by a defect in the initiation of sexual development. When the disruptant was induced to conjugate and the resulting poorly conjugated diploid cells were examined for their sporulation, they were indistinguishable from wild-type cells in sporulation efficiency (Fig. 4b) and also in both morphology and spore viability (data not shown); this is consistent with the lack of apparent defect in their conjugation, meiosis, and sporulation when induced by glucose starvation. The poor ability of the disruptant to perform diploid meiosis is likely to be a consequence of the absolute requirement of some factors controlling the onset of sexual development for meiosis and sporulation, such as *ste11*$^+$ for the expression of *mei2*$^+$ (33, 38).

Nitrogen starvation-induced *ste11*$^+$ expression is defective in Δ*rcd1* cells. Because *ste11*$^+$ plays a key role in the initiation of sexual development, we examined the effect of *rcd1*$^+$ inactivation on the induction of *ste11*$^+$. Glucose starvation and nitrogen starvation are two major nutrient exhaustion signals for *ste11*$^+$ induction. In heterothallic wild-type cells, *ste11*$^+$ mRNA was drastically induced during a 6-h starvation for nitrogen or glucose (Fig. 5a). However, in the *rcd1* disruptant, nitrogen starvation failed to induce *ste11*$^+$ but, consistent with the fertility, glucose starvation involved *ste11*$^+$ induction. To confirm these results, the time course of *ste11*$^+$ induction was examined not only for heterothallic cells but also for self-conjugative homothallic disruptant and wild-type cells. Again, irrespective of mating types, nitrogen starvation failed to strongly induce *ste11*$^+$ transcription in the disruptant whereas the response to glucose starvation was the same as that of wild-type cells (Fig. 5b and c). Nevertheless, it should be pointed out that nitrogen starvation-invoked *ste11*$^+$ induction was not totally lost in the disruptant, since a significant level of *ste11*$^+$ mRNA was detected in the nitrogen-starved disruptant. Moreover, in the homothallic disruptant, *ste11*$^+$ mRNA seemed to be induced slightly more strongly.

These results were obtained at 30°C. However, as mentioned above, the sterility of the disruptant was slightly alleviated at 25°C (Fig. 2). To examine any direct correlation between the degree of sterility and the extent of *ste11*$^+$ induction, the levels of nitrogen starvation-invoked *ste11*$^+$ induction in the homothallic disruptant and wild-type cells were compared at 25 and 30°C. As expected, nitrogen starvation induced *ste11*$^+$ to a

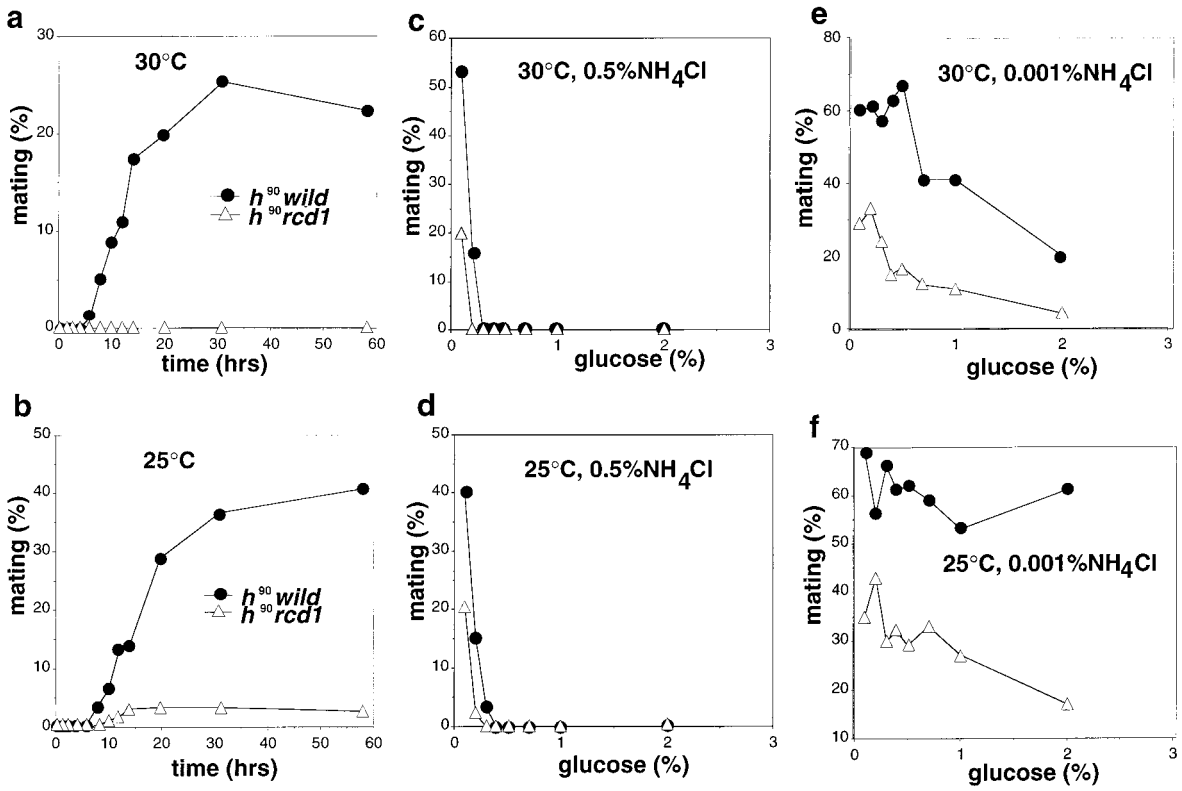

FIG. 2. Mating frequencies of the *rcd1* disruptant under various conditions. (a and b) Mating efficiencies of *rcd1* cells induced by nitrogen starvation. The h^{90} wild-type cells (L968) and h^{90} *rcd1* cells (NS4) were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended at 10^7 cells/ml in NH_4Cl-free EMM medium, and incubated at 30°C (a) or 25°C (b). (c and d) Mating efficiencies of *rcd1* cells induced by glucose starvation. The h^{90} wild-type cells (L968) and h^{90} *rcd1* cells (NS4) were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended at 10^7 cells/ml in EMM medium containing 0.5% NH_4Cl and with the indicated concentration of glucose and incubated at 30°C (c) or 25°C (d). (e and f) Mating efficiencies of *rcd1* cells induced by dual starvation for glucose and nitrogen. The h^{90} wild-type cells (L968) and h^{90} *rcd1* cells (NS4) were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended at 10^7 cells/ml in EMM medium containing 0.001% NH_4Cl and with the indicated concentrations of glucose and incubated at 30°C (e) or 25°C (f). Conjugated cells were counted at the indicated times, and their populations were calculated as percent mating.

higher level at 25°C than at 30°C, although the level was still slightly lower than in wild-type cells (Fig. 6). The induction of *mei2*$^+$ closely paralleled the *ste11*$^+$ induction. By contrast, in wild-type cells, the level of nitrogen starvation-invoked *ste11*$^+$ induction was the same at both temperatures. Perhaps reflecting the significant induction of *ste11*$^+$ at 25°C, *matPc*$^+$ and *matPi*$^+$ were partially induced in the disruptant. However, at this temperature, the level of *matP*$^+$ induction and the mating frequency (Fig. 2b) relative to *ste11*$^+$ induction were still noticeably low. These results indicate that *rcd1*$^+$ is required for nitrogen starvation-invoked *ste11*$^+$ mRNA induction at 30°C but not at 25°C. Furthermore, these results suggest that *rcd1*$^+$ is likely to play additional roles, such as induction of *matPc*$^+$ and *matPi*$^+$, independent of transcriptional regulation of *ste11*$^+$ expression.

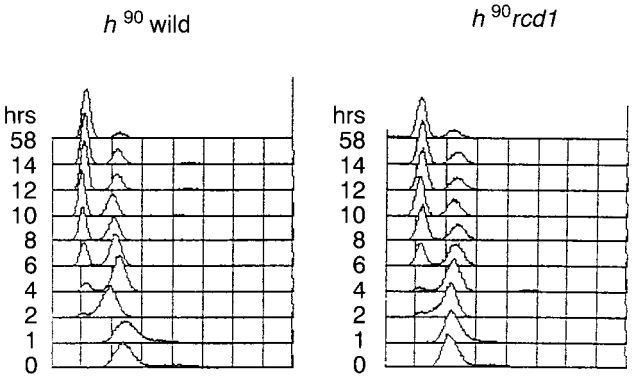

FIG. 3. *rcd1* disruptant responds to nitrogen starvation and arrest in G_1 in the same time course and to the same extent as wild-type cells. The same samples as for Fig. 2a were analyzed by flow cytometry. Fission yeast cells in logarithmic growth show only a 2C DNA content because of prolonged G_2 phase and late cell separation.

Pat1^{+s} inactivation-triggered *ste11*$^+$ induction is also defective in Δ*rcd1* cells. As mentioned above, deletion of *rcd1*$^+$ suppressed the lethality of *pat1*. To investigate the possibility of mechanistic similarity, we examined the level of *ste11*$^+$ induction in both *pat1-114* and *pat1-114 rcd1* cells after the shift to 34°C. *ste11*$^+$ was induced in the *pat1-114* mutant upon the shift to the nonpermissive temperature (Fig. 7). In the double mutant, however, *ste11*$^+$ induction was markedly diminished. Diminished *ste11*$^+$ induction was indeed the cause of *pat1* suppression because ectopic expression of *ste11*$^+$ or *mei2*$^+$ in the double mutant effectively led to reversion of the suppressed *pat1* lethality (Table 2). Thus, the *rcd1* disruptant responded to glucose starvation but not to nitrogen starvation or inactivation, indicating that *rcd1*$^+$ is required for *ste11*$^+$ induction invoked at 30°C by nitrogen starvation and by Pat1^{+s} inactivation.

Ectopic expression of *ste11*$^+$ restores fertility to *rcd1* cells. To resolve whether the sterility of the disruptant was attribut-

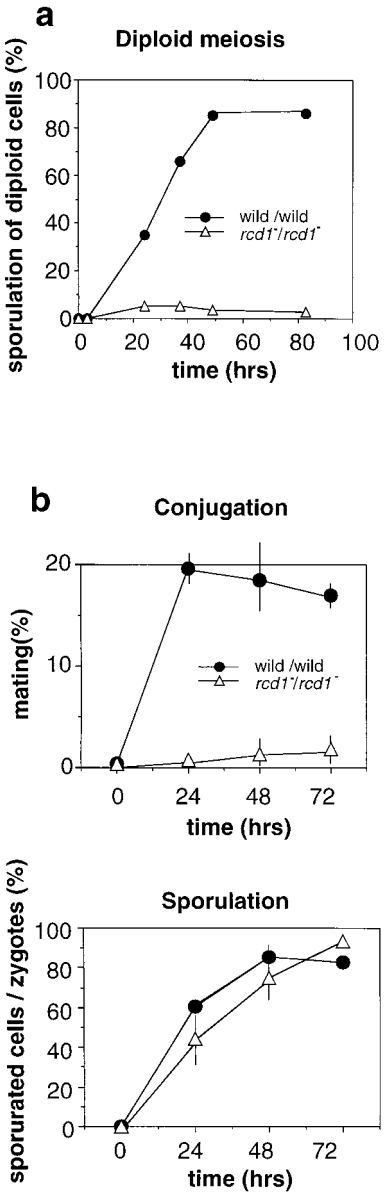

FIG. 4. *rcd1*$^+$ is not required for meiosis and sporulation after onset of sexual differentiation. (a) The homozygous *rcd1* diploid (NS1) and wild-type diploid cells (NN1) were grown in EMM medium to 2×10^6 cells/ml, washed, resuspended in NH_4Cl-free EMM medium at 5×10^6 cells/ml, and incubated at 30°C. (b) The heterothallic h^- cells (L972), h^- *rcd1* cells (NS2), h^+ cells (L975), and h^+ *rcd1* cells (NS3) were separately grown on YES plates overnight and mixed with opposite-mating-type but otherwise genotypically identical cells at a concentration of 10^7 cells/ml in H_2O and spotted on ME plates. Conjugated cells and sporulated cells were counted by microscopy. Conjugation was carried out on agar plates but not in liquid medium to increase the mating frequencies of the *rcd1* disruptant. The percentage of sporulated cells was calculated by counting the number of spore asci per conjugated cell. The data shown are means ± standard deviations in two independent experiments.

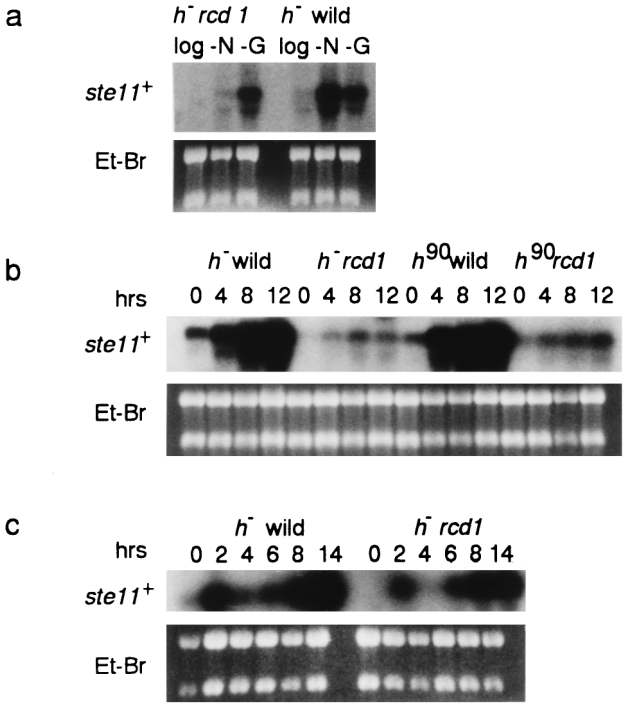

FIG. 5. The *rcd1* disruptant is defective in *ste11*$^+$ induction in response to nitrogen starvation but not to glucose starvation. (a) The h^- wild-type (L972) and h^- *rcd1* (NS2) cells were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended in NH_4Cl-free EMM medium or in 0.5% glucose containing EMM medium at 10^7 cells/ml, and incubated at 30°C for 6 h. Total RNA was then extracted from the cells. (b) The h^- wild-type (L972), h^- *rcd1* (NS2), h^{90} wild-type (L968), and h^{90} *rcd1* cells (NS4) were grown and incubated in NH_4Cl-free EMM medium under the same conditions as in panel a. The cells were harvested at the indicated times, and total RNA was extracted. (c) The h^- wild-type (L972) and h^- *rcd1* (NS2) cells were grown and incubated in EMM medium containing 0.5% glucose under the same conditions as in panel a. The cells were harvested at the indicated times, and total RNA was extracted. The level of the *ste11*$^+$ transcript was determined by Northern blot hybridization.

able largely to poor *ste11*$^+$ induction, we tested if ectopic expression of *ste11*$^+$ could restore fertility to the disruptant. As shown in Fig. 8, expression of *ste11*$^+$ indeed restored fertility to the disruptant to the same extent as in wild-type cells or cells obtained by expression of *rcd1*$^+$. This led us to conclude that the sterility of the *rcd1* disruptant was caused largely by poor induction of *ste11*$^+$ in response to nitrogen starvation.

***rcd1*$^+$ is required for *ste11*$^+$ induction independently of the cAMP-Pka1 and Wis1-Phh1 pathways and other known factors.** The cAMP-Pka1 and Wis1-Phh1 pathways mediate mainly glucose and stress signals, respectively, and critically control the onset of sexual development (18, 42). We therefore investigated the possible link of *rcd1*$^+$ to these pathways. For this purpose, a homothallic *rcd1 pka1* double mutant was constructed and compared to each single mutant for the ability to perform nitrogen starvation-induced conjugation. The *pka1* cells are highly proficient for conjugation even if not starved for nitrogen (23), whereas the *rcd1* cells were sterile, as described above. The *rcd1 pka1* cells were, however, fertile and showed an intermediate level of mating frequency, indicating that *rcd1*$^+$ is independent of the cAMP-Pka1 pathway (Fig. 9a).

Similarly, a homothallic *rcd1 wis1* double mutant was constructed and examined for its ability to perform conjugation. Both *rcd1* and *wis1* single mutants are highly but not completely sterile, each conjugating at low frequencies of 1 to 5% under the conditions used (18, 32) (Fig. 2). The *rcd1 wis1* double mutant was, however, completely sterile, and we failed to detect any conjugated cells throughout the entire experiment, indicating that the action of *rcd1*$^+$ is also independent of the Wis1-Phh1 stress signal pathway (Fig. 9b). The independence of *rcd1*$^+$ from both pathways was further confirmed by the lack of effect of *rcd1*$^+$ deletion on the glucose starvation-

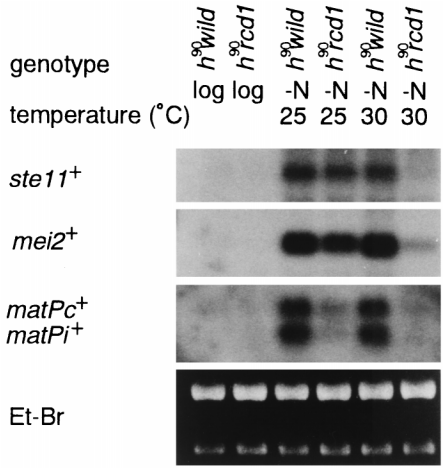

FIG. 6. The defect of the *rcd1* disruptant in nitrogen starvation-responsive *ste11*$^+$ induction is partially suppressed at 25°C. The h^{90} wild-type (L968) and h^{90} *rcd1* cells (NS4) were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended in NH_4Cl-free EMM medium at 10^7 cells/ml, and incubated for 6 h at 30 or 25°C. Total RNA was then extracted from the cells, and the levels of the *ste11*$^+$, *mei2*$^+$, *matPc*$^+$, and *matPi*$^+$ mRNAs were determined by Northern blot hybridization.

invoked *fbp1*$^+$ induction, which is regulated by both the cAMP-Pka1 and Wis1-Phh1 pathways via Atf1 (10, 32) (Fig. 10).

Overexpression of the *pac2*$^+$ gene blocks the onset of sexual development by repressing *ste11*$^+$ expression, and cells defective in *pac2*$^+$ could express *ste11*$^+$ and enter sexual development under incomplete starvation conditions, although the biological significance of *pac2*$^+$ is not understood (20). The possible relationship between *pac2*$^+$ and *rcd1*$^+$ was therefore examined by the same analysis. Again, *rcd1*$^+$ was suggested to be independent of *pac2*$^+$ (Fig. 9c).

The Cig2/Cyc17 cyclin inhibits sexual development and promotes the start of the cell cycle (5, 27). This cyclin inhibits sexual development by a mechanism independent of transcriptional regulation of *ste11*$^+$ (41). A similar analysis was carried out for the relation between *cig2*$^+$ and *rcd1*$^+$. As shown in Fig. 9d, the double mutant became fertile but not to the level of wild-type cells, indicating that *rcd1*$^+$ is independent of *cig2*$^+$, as expected.

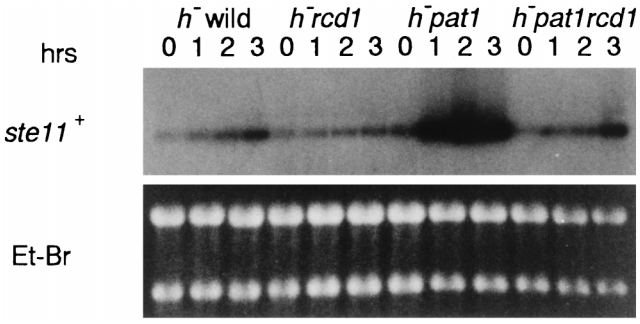

FIG. 7. The *rcd1* disruptant is defective in *ste11*$^+$ mRNA induction invoked by Pat1^{+s} inactivation. The h^- wild-type (L972), h^- *pat1-114* (NS6), and h^- *pat1-114 rcd1* (NS7) cells were grown in EMM medium at 23°C. When the cell density reached 10^7 cells/ml, each culture was shifted to 34°C and incubated for the indicated times. Total RNA was extracted from the cells, and the level of the *ste11*$^+$ mRNA was determined by Northern blot hybridization.

TABLE 2. Ectopic expression of *ste11*$^+$ reduces the growth ability of the *pat1* and *pat1 rcd1* mutants at the restrictive temperatures[a]

Strains	Plasmid	% Suppression[b]
h^- *pat1-114*	pcLX	0.1
	pcL*rcd1*$^+$	53.1
	pcL*ste11*$^+$	<0.1
	pcL*mei2*$^+$	<0.1
h^- *pat1-114 rcd1*	pcLX	37.1
	pcL*rcd1*$^+$	42.2
	pcL*ste11*$^+$	2.1
	pcL*mei2*$^+$	2.7

[a] The h^- *pat1-114 leu1* and h^- *pat1-114 rcd1 leu1* cells were transfected with the indicated plasmids. The transfected cells were spread on MMA medium plates and incubated at 23°C overnight and then at the restrictive temperature of 34 or 23°C. The numbers of leu$^+$ colonies were counted after incubation for 3 days.

[b] The percent suppression of *pat1-114* mutation was calculated by dividing the number of colonies that grew at 34°C by the number that grew at 23°C.

The human RCD1 homolog is expressed in various tissues. Given the biological role of *rcd1*$^+$ in fission yeast, we examined tissue-specific expression of the human RCD1$^+$ homolog by Northern blot analysis. As shown in Fig. 11, it was expressed in a variety of human tissues, but its expression was particularly high in the testes, ovaries, and thymus, the tissues in which cell growth and differentiation are actively taking place.

DISCUSSION

As is apparent from the data presented above, *rcd1*$^+$ is a newly identified differentiation-controlling factor that is crucial for nitrogen starvation-invoked onset of sexual development in fission yeast. Although the molecular mechanism by which *rcd1*$^+$ controls sexual development is not fully understood, *rcd1*$^+$ is at least essential for *ste11*$^+$ induction in response to nitrogen starvation at the normal growth temperature. To date, three different factors or pathways have been identified that

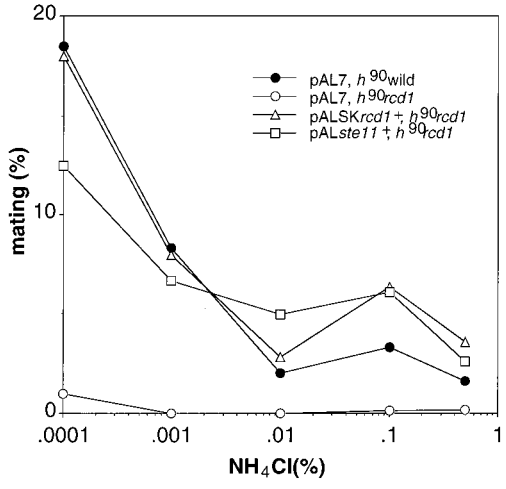

FIG. 8. Ectopic expression of *ste11*$^+$ restores fertility to the *rcd1* disruptant. The h^{90} *rcd1 leu*$^-$ cells harboring empty pAL7, pAL7 carrying *ste11*$^+$, or pALSK carrying *rcd1*$^+$ were grown in EMM medium to 5×10^6 cells/ml. The cells were washed, resuspended in EMM medium with the indicated concentration of NH_4Cl at 10^7 cells/ml, and incubated at 25°C for 22 h. The numbers of zygotes and nonzygotes were counted. The pAL7 vector was described previously (28).

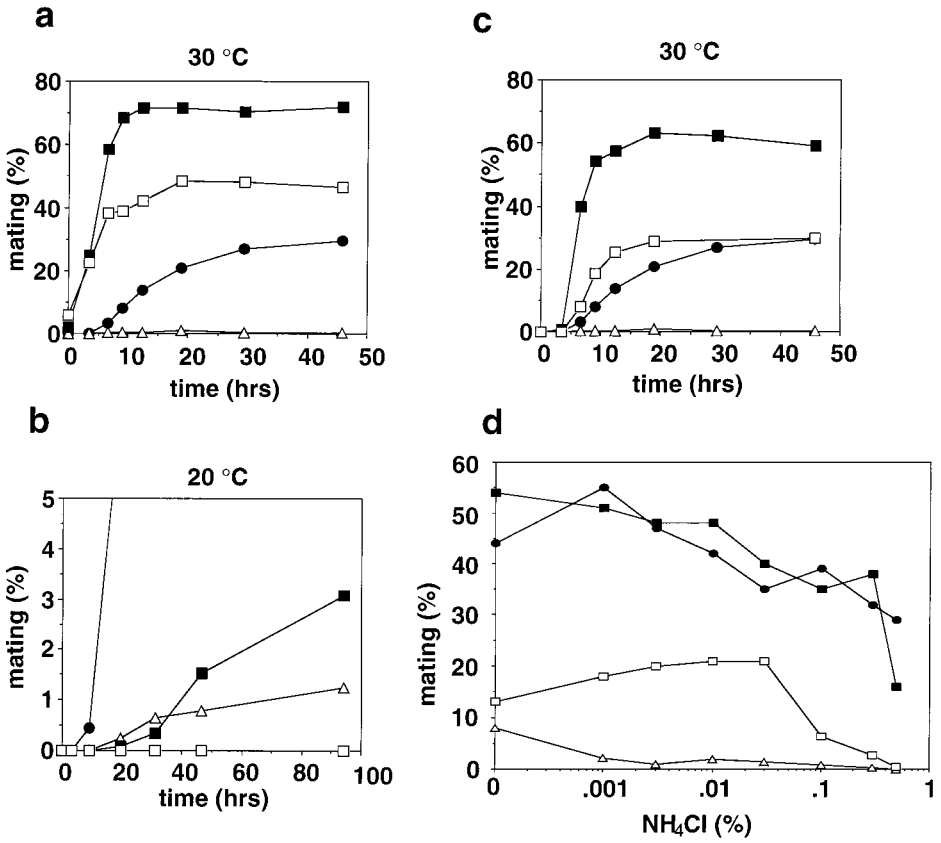

FIG. 9. *rcd1*$^+$ controls differentiation independently of the cAMP-Pka1 (a) and Wis1-Phh1 (b) pathways, Pac2 (c), and Cig2 (d). (a to c) The h^{90} wild-type (solid circles), h^{90} *rcd1* (open triangles), h^{90} *pka1* (solid squares), and h^{90} *pka1 rcd1* (open squares) cells (a), the h^{90} wild-type (solid circles), h^{90} *rcd1* (open triangles), h^{90} *wis1* (solid squares), and h^{90} *rcd1 wis1* (open squares) cells (b), and the h^{90} wild-type (solid circles), h^{90} *rcd1* (open triangles), h^{90} *pac2* (solid squares), and h^{90} *rcd1 pac2* (open squares) cells (c) were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended in NH_4Cl-free EMM medium at 10^7 cells/ml, and incubated at 30°C for the indicated times. Conjugated cells were counted, and their percent populations were calculated by dividing twice the number of conjugated cells by the total cell number. (d) The h^{90} wild-type (solid circles), h^{90} *rcd1* (open triangles), h^{90} *cig2* (solid squares), and h^{90} *rcd1 cig2* (open squares) cells were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended in EMM medium with the indicated concentrations of NH_4Cl at 10^7 cells/ml, and incubated at 30°C for 24 h. Conjugated cells were counted at the indicated times, and their percent populations were calculated as above.

regulate the expression of *ste11*$^+$: (i) the cAMP-Pka1 cascade, which mainly mediates a signal for carbon source; (ii) the Wis1-Phh1 pathway, which mediates a stress signal; and (iii) Pac2, whose physiological role is unknown. As is known, starvation for nitrogen source is the most effective signal for the induction of the onset of sexual development. However, none of these *ste11*$^+$ regulatory systems seems to specifically mediate the nitrogen starvation signal. Rcd1 is the fourth factor that controls *ste11*$^+$ expression. All the data indicate that the sys-

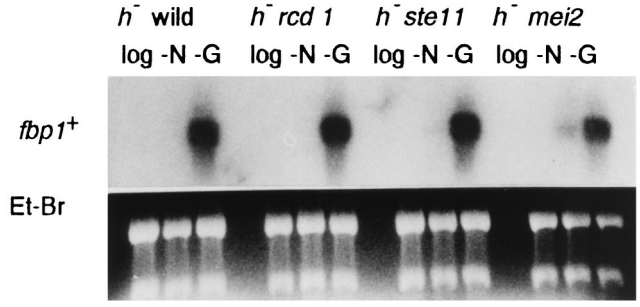

FIG. 10. *fbp1*$^+$ mRNA is fully inducible in *rcd1* disruptants in response to glucose starvation. The h^- wild-type (L972), h^- *rcd1* (NS2), *ste11* (NS9), and h^- *mei2* (NS10) cells were grown in EMM medium to 5×10^6 cells/ml, washed, resuspended in NH_4Cl-free EMM medium (−N) or EMM medium containing 0.5% glucose (−G) at 10^7 cells/ml, and incubated at 30°C for 6 h. Total RNA was extracted from the cells, and the level of the *fbp1*$^+$ transcript was determined by Northern blot hybridization. Fbp1, fructose-1,6-bisphosphatase.

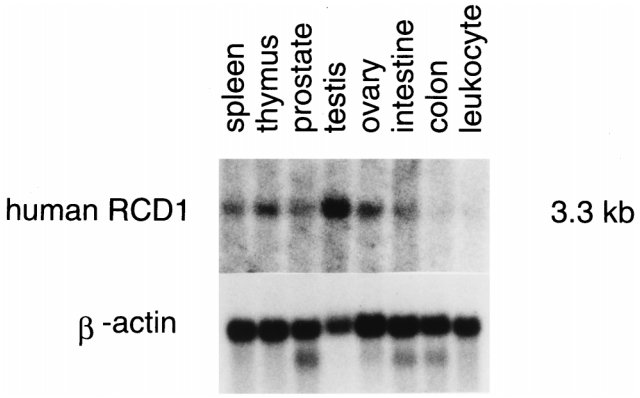

FIG. 11. Tissue-specific expression of human RCD1. Each lane contains approximately 2 μg of poly(A)$^+$ RNA prepared from human spleen, thymus, prostate, testes, ovaries, small intestine, colon (mucosal lining), or peripheral blood leukocytes probed with human RCD1 (top) or with the β-actin gene (bottom).

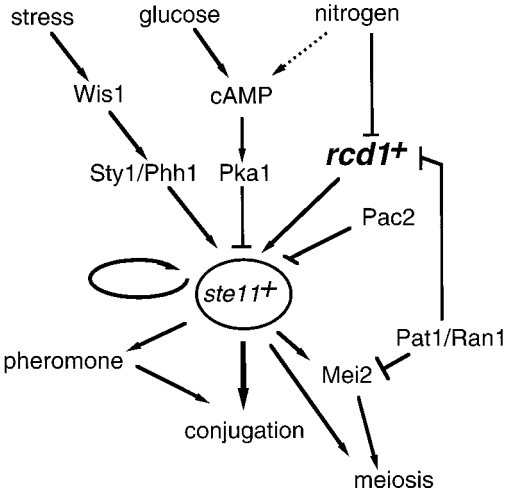

FIG. 12. Proposed model for the regulation of *ste11*$^+$ expression by Rcd1 and other known factors. Rcd1 is required for *ste11*$^+$ induction in response to nitrogen starvation and Pat1^{+s} inactivation. In addition to *ste11*$^+$ induction, Rcd1 controls differentiation via a mechanism independent of *ste11*$^+$ regulation, which is not shown here because of ignorance of its molecular target. The Wis1-Sty1/Phh1 stress signal pathway (18) positively regulates *ste11*$^+$ expression, and the cAMP-Pka1 pathway (15) and Pac2 (20) negatively regulate *ste11*$^+$ expression. In addition, the Ste11 molecule positively autoregulates its own expression (33).

tem involving Rcd1 is independent of these three *ste11*$^+$-regulatory systems and is uniquely responsible for controlling *ste11*$^+$ expression by nitrogen starvation and Pat1^{+s} inactivation signals (Fig. 12). Rcd1 is essential for nitrogen starvation-invoked *ste11*$^+$ induction but is not a component of the general nitrogen signal cascade, because cells lacking *rcd1*$^+$ still respond to nitrogen starvation and can arrest in G_1 in a time course indistinguishable from that of wild-type cells (Fig. 3). Interestingly, sterility and lack of *ste11*$^+$ induction in the *rcd1* disruptant are partially suppressed at 25°C or lower temperatures. The reason for this phenomenon is unknown. Rcd1 might be involved in stabilizing the possibly heat-labile nitrogen starvation-responsive *ste11*$^+$ regulatory system, or there might be two redundant factors, one of which is Rcd1 and functions exclusively at relatively high temperatures. *rcd1*$^+$ seems to play another role besides the transcriptional control of *ste11*$^+$. At 25°C, *ste11*$^+$ was significantly induced in the *rcd1* disruptant by nitrogen starvation, yet both induction of *matPi*$^+$ and *matPc*$^+$ and conjugation were poor (Fig. 6). These results suggest that *rcd1*$^+$ is likely to control differentiation by a mechanism independent of transcriptional regulation of *ste11*$^+$ expression. This mechanism may involve posttranscriptional control of *ste11*$^+$, because the sterility of the *rcd1* disruptant was effectively rescued by ectopic overexpression of *ste11*$^+$ (Fig. 8).

Deletion of *rcd1*$^+$ suppresses the *pat1-114* mutant, because *rcd1*$^+$ is required for *ste11*$^+$ induction by Pat1^{+s} inactivation (Fig. 7). Paradoxically, *rcd1*$^+$ was initially isolated as a multicopy suppressor of the *pat1-114* lethality. An entirely different mechanism seems to be involved in this suppression. The *rcd1*$^+$ gene was also independently isolated and found to inhibit the activity of a dominant active mutant of the Mei2 protein and to strongly bind to Mei2 in the budding yeast two-hybrid system (39a). The inhibition of Mei2 seems to be evident only in an overproduced situation, since deletion of *rcd1*$^+$ apparently did not influence the efficiency of meiosis and sporulation following conjugation (Fig. 4b).

One striking finding in the present work is the evolutionary conservation of the *rcd1*$^+$ gene throughout eukaryotes. Budding yeast, plants, worms, and humans all contain its structural homologs. Amino acid homology exceeds 70% among the products of these homologs. Despite such a high level of similarity, *rcd1*$^+$ could not functionally be substituted, at least by the human homolog (29a). Although the physiological role of those homologs is not known because they were identified by the genome-sequencing project, the level of tissue-specific expression of the human RCD1 homolog, which is particularly high in the testes, ovaries, and thymus, is certainly consistent with its possible involvement in differentiation control. Conservation of regulatory factors is not restricted to Rcd1. Ste11 contains a typical high-mobility group (HMG) motif (33). The recently identified TCF1 is a HMG protein and is essential for the terminal differentiation of lymphocytes (37), whereas p38, a homolog of Phh1, mediates stress signals and controls the growth and differentiation of lymphocytes (6, 9, 21). Moreover, cAMP is a well-identified regulator of the growth and differentiation of mammalian cells (31). Such similarity in several distinct factors may suggest that the system controlling the onset of differentiation found in fission yeast might well be conserved throughout the eukaryotes.

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