Outer Plaque Assembly and Spore Encapsulation Are Defective during Sporulation of Adenylate Cyclase-deficient Mutants of *Saccharomyces cerevisiae*

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ABSTRACT Sporulation in diploid cells homozygous for the *cyr1-2* mutation of the yeast *Saccharomyces cerevisiae* was examined. This mutation causes a defect in adenylate cyclase and temperature-sensitive arrest in the G1 phase of the mitotic cell cycle. The *cyr1-2/cyr1-2* diploid cells were able to initiate meiotic divisions, but produced predominantly two-spored asci at the restrictive temperature. Temperature-sensitive period for production of two-spored asci was ~12 h after the transfer of cells to the sporulation medium. The levels of cAMP increased during this period in the wild type and *cyr1-2/cyr1-2* diploid cells incubated at the permissive temperature, but remained at an extremely low level in the *cyr1-2/cyr1-2* diploid cells incubated at the restrictive temperature. Dyad analysis of the *cyr1-2* strain indicated that meiotic products were randomly included into ascospores. Fluorescent microscopy of the *cyr1-2/cyr1-2* diploid cells incubated at the restrictive temperature revealed that individual haploid nuclei were enclosed in each of the two spores after meiosis. About half of the *cyr1-2/cyr1-2* diploid cells entered normal meiosis I producing two normal spindle pole bodies with inner and outer plaques, and the other half entered abnormal meiosis I producing one normal spindle pole body and one defective spindle pole body without outer plaque. At meiosis II, some cells contained a pair of normal spindle pole bodies and other cells contained pairs of normal and abnormal spindle pole bodies.

Sporulation in diploid cells of *Saccharomyces cerevisiae* involves the production of haploid nuclei by meiotic division and the completion of ascospore formation. Increase and fluctuation in the levels of intracellular cAMP during sporulation of yeast has been reported (1, 2). We isolated adenylate cyclase-deficient mutants, *cyr1*, which were arrested at the G1 phase of the mitotic cell cycle in the absence of cAMP (3–5). Diploids of yeast homozygous for temperature-sensitive *cyr1* mutations permitted the initiation of meiosis but resulted in the frequent production of two-spored asci at the restrictive temperature (6). Therefore, it has been suggested that cAMP works as a positive effector at the start of mitosis, but as a negative effector on the initiation of meiosis (4, 6).

Two-spored asci are formed under certain physiological and genetic controls. It has been reported that two-spored asci produced by physiological control contain nonsister meiotic products (7), diploid nuclei (8), or abnormal meiotic products (9) depending on the sporulation condition, and those produced by genetic control contain nonsister meiotic products (10), random meiotic products (11), or diploid nuclei (12) depending on the mutation. Our purpose was to study the role of cAMP in the control of sporulation in *S. cerevisiae*. The production of cAMP was essential to complete the random enclosure of haploid meiotic products in prospore walls.

MATERIALS AND METHODS

Yeast Strains: Two diploid strains of *S. cerevisae* were used in this study. Strain G435 is a wild-type diploid (6). Strain AM77 is a homozygous diploid for *cyr1-2*, a temperature-sensitive allele of the *cyr1* locus (6). All strains used in this study were derived from the same wild type strain, P-28-24C, and interpreted to be isogenic (6).

Media: Minimal medium contained 2% of glucose and 0.67% of yeast nitrogen base without amino acids (Difco Laboratories, Inc., Detroit, MI). Rich medium (YPGlu) was prepared by dissolving 20 g glucose, 20 g peptone, and 10 g yeast extract in 1 liter of distilled water. Presporulation medium (YPA) was prepared by adding 1% of potassium acetate to YPGlu medium instead of glucose. Liquid sporulation medium (acetate) contained 10 g potassium acetate

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per liter of distilled water. Solid sporulation medium contained 2.5 g yeast extract, 20 g potassium acetate, 0.5 g glucose, and 20 g agar in 1 liter of distilled water.

Sporulation Procedure: Vegetative cultures were grown in liquid YPA medium at 25°C. Cells were harvested when the cultures reached a titer of ~10^7 cell/ml, washed with water, and resuspended in sporulation medium at the same titer for sporulation. When meiosis was followed at the permissive condition, cultures of temperature-sensitive mutants were incubated at 25°C. When the restrictive condition was required, the cultures were transferred to 33.5°C. Sporulation was examined by light microscopy for samples mounted in a hematocytometer, and 200–500 cells or asci were counted per sample. Sporulation efficiency is expressed as the number of asci divided by the sum of asci and nonsporulating cells. The frequency of each ascus type is expressed as the number of asci containing the particular number of spores divided by the total number of asci or by the sum of asci and nonsporulating cells.

Fluorescent Microscopy: Samples were placed on a glass slide, and a drop of staining solution which contained 10 μg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) and 500 μg/ml ribonuclease A (Sigma Chemical Co.) (13) was immediately added to them. The materials were then squashed gently under a coverslip. When nuclei in spores were stained, the materials were squashed strongly. Thus propidium iodide staining and ribonuclease treatment were simultaneously performed at 20°C for 30 min. Observations of fluorescence were made with a UVFL 100x objective using an ultraviolet excitation filter (545 nm) in combination with a 610-nm suppression filter. Photographs were taken on 35-mm Fuji 400 film. To determine the relative DNA content per nucleus, we measured the intensity of fluorescence emitted from a propidium iodide–stained nucleus by use of a Zeiss MPM 03-Fluorometer (Carl Zeiss, Inc., Ober Kochen, West Germany) equipped with a 100x objective. The area of the individual nucleus was optically isolated by the 4-μm microscopical photometer diaphragm.

Electron Microscopy: Cells subjected to 33.5°C in sporulation medium for 0–24 h were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h. Fixation of culture was preceded by treatment for 10 min at 25°C with 0.1 M β-mercaptoethanol in 0.02 M EDTA and 0.2 M Tris-HCl buffer (pH 8.0) to facilitate later removal of walls. After glutaraldehyde fixation, walls were removed by incubation of the washed cells with Zymolyase 5000 (Kirin Brewery Co. Ltd., Takasaki, Japan) (0.1 mg/ml) in 0.2 M phosphate buffer. Cells were subsequently postfixed for 60 min at 20°C in 2% osmium tetraoxide in 0.1 M phosphate buffer (pH 7.0), washed well with distilled water, treated for 60 min at 20°C with 2% uranyl acetate, dehydrated, and embedded in Spurr resin. Blocks were serially sectioned on a Sorvall MT-2 ultramicrotome (DuPont Co., Wilmington, DE) into 80-nm thick sections; the ribbons were picked up on the formvar films on 1 × 2-mm oval single hole grids, stained successively with uranyl acetate and lead citrate, and viewed in a JEOL 200CX electron microscope (JEOL, Akishima-shi, Japan) at 100 kV.

cAMP Assay: The cAMP content was measured by the protein binding assay using the cAMP assay kits (Amersham International, Buckinghamshire, England) as described by Brown et al. (14).

Protein Measurement: Protein was measured by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RESULTS

Time Course of Two-spored Ascus Formation in cyr1 Homozygous Diploids

Sporulation was examined in diploids of yeast homozygous for the temperature-sensitive mutation, *cyr1-2*, which causes a temperature-sensitive requirement for cAMP. The *cyr1-2/cyr1-2* diploid cells (AM77) initiated sporulation ~5 h after the wild-type diploid cells at 33.5°C and frequently produced two-spored asci at the restrictive temperature (Fig. 1). The time course of differential appearance of two-spored and four-spored asci under the normal sporulation condition was examined by shaking wild-type and *cyr1-2* homozygous diploid cells in liquid sporulation medium at 33.5°C for various lengths of time. The wild-type diploid cells produced predominantly three- or four-spored asci 14–20 h after suspending in sporulation medium and few one- or two-spored asci after 20 h (Fig. 1 *a*). The *cyr1-2* diploid cells produced predominantly one- or two-spored asci after 18 h (Fig. 1 *b*). The kinetics of formation of two-spored asci in the mutant cells is

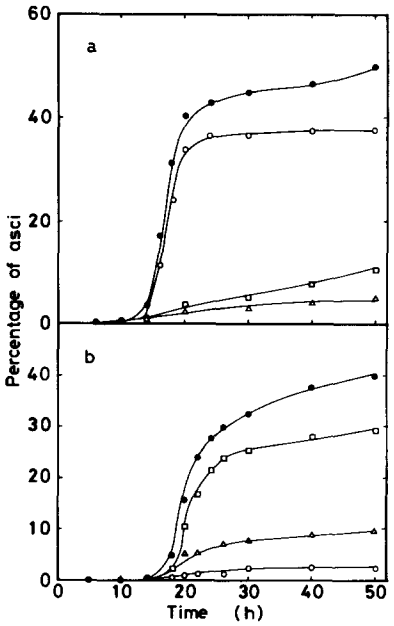

FIGURE 1 Time course of sporulation of wild-type and *cyr1-2* homozygous diploid strains at 33.5°C. Strain G435 (*a*) and AM77 (*b*) cells were cultivated at 33.5°C in the liquid sporulation medium for various periods, and asci were observed. The number of total asci (●), three- or four-spored asci (○), two-spored asci (□), and one-spored asci (△) were counted.

similar to that of three- or four-spored asci in the wild-type cells, but different from that of two-spored asci in the wild-type cells incubated at 33.5°C. The total percent sporulation in *cyr1-2* homozygous diploid cells was significantly less than that in the wild-type diploid cells at 33.5°C.

The *CYR1/cyr1-2* heterozygous diploid (AM70) was sporulated, and four-spored asci were dissected. All 11 asci tested showed a 4+:0− segregation for growth at 25°C, but they showed a 2+:2− segregation at 35°C. Each segregant was crossed with a haploid *cyr1-2* strain, and the resultant diploids were tested for the frequency of ascus types at 33.5°C. All the CYR1/*cyr1-2* diploids obtained yielded four-spored asci, but all the *cyr1-2/cyr1-2* diploids yielded primarily two-spored asci at 33.5°C. The result indicates that the *cyr1-2* mutation causes the production of two-spored asci.

When *cyr1-2* diploid cells grown in presporulation medium at 25°C were first shaken in liquid sporulation medium at 33.5°C and then shifted to 25°C, about half of cells formed three- or four-spored asci if the cells were shifted to 25°C within 12 h, but the number of two-spored asci increased in cultures shaken at 33.5°C for 12 h or more (Fig. 2). Conversely, when the cells were first incubated at 25°C and then shifted to 33.5°C, a few three- or four-spored asci were formed if the cells were shifted to 33.5°C within ~17 h, and the number of three- or four-spored asci increased in cultures shaken at 25°C for 12 h or more (Fig. 2). Wild-type diploid cells produced primarily three- or four-spored asci (~70%) at either 25 or 33.5°C, and no significant variation in the distribution of ascus types was observed after shifting the sporulation temperature (data not shown). These results indicate that the commitment to production of two-spored asci becomes irreversible ~12 h after the transfer to the sporulation medium, and the temperature-sensitive period is completed ~17 h after the transfer.

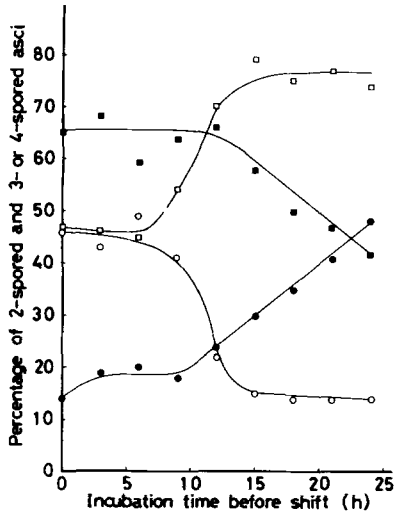

FIGURE 2 Temperature shift of *cyr1-2* homozygous diploid cells under the sporulation condition. AM77 cells cultivated in YPA medium at 25°C were divided into two subcultures. The first culture (○, □) was cultivated at 33.5°C after the transfer to the liquid sporulation medium, and at the indicated period an aliquot of the culture was shifted to 25°C. Conversely, the second culture (●, ■) was cultivated at 25°C, and at the indicated period an aliquot of the cultures was shifted to 33.5°C. Percentages of two-spored (□, ■) and three- or four-spored asci (○, ●) were obtained after 48 h in sporulation medium.

cAMP Level Produced during Sporulation

Wild-type diploid cells produced a constant level of cAMP during growth at 25°C and 33.5°C whereas *cyr1-2* diploid cells grown at 25°C produced a lower level of cAMP than that of wild-type cells, and cAMP levels in the cells grown first at 25°C for 2 h and then at 33.5°C decreased significantly (Fig. 3). The cAMP levels of wild-type and *cyr1-2* diploid cells decreased to minimum values after the transfer to the presporulation (YPA) medium (Fig. 4). The cAMP levels of wild-type cells transferred to the sporulation (acetate) medium increased and the peak of cAMP level was observed after incubation at 33.5°C. The *cyr1-2* cells accumulated cAMP during incubation at 25°C in the sporulation medium, although the maximum level of cAMP produced was about half of that produced by wild-type cells. No such increase of cAMP level was observed in the *cyr1-2* cells incubated at 33.5°C in the sporulation medium (Fig. 4). It is pointed out from this result that the increase of cAMP level is observed when three- or four-spored asci were formed, and that the maximum levels of cAMP were attained at about the same time when the execution point of two- or four-spored asci formation was observed (Fig. 2 and 4).

Dyad Analysis of Two-spored Asci

The *cyr1-2* diploid strain contains two auxotrophic markers (*leu1* and *gal7*) and the different mating type loci (*MAT*) in heterozygous condition. The segregation of these markers was examined in spores obtained from two-spored asci. The segregation patterns observed in these asci are summarized in Table I. If chromosome segregation is normal, wild-type (+), and mutant (m) ascospores, or *MATα* (+) and MATa (m) ascospores, with respect to markers present in heterozygous condition, are expected to occur in a 1(+):1(m) ratio. The three loci examined, distributed over three chromosomes, segregated roughly in this ratio. The segregation of a marker closely linked to its centromere may be used to determine whether spores present in two-spored asci of the *cyr1-2* diploid are a random or nonrandom sample of the four haploid nuclei resulting from meiosis. Two markers, *leu1* and *gal7* are ~3 and 6 cM from the respective centromeres of chromosome VII and II (16). If the two spores formed are always sister spores (i.e., products of the same second division of meiosis), the spores formed would be expected to be identical with respect to their genotypes at each locus (i.e., m,m or +,+) in 94 and 88% of cases. If the two spores formed always represent nonsister spores (i.e., products from each of the second divisions of meiosis), then the two spores would be expected to differ in their genotypes at each locus in 97 and 94% of cases. If the two spores formed are a random sample of the four haploid products of meiosis, then one-third of the two-spored asci formed would be expected to be identical with respect to their genotypes at each locus, and two-thirds would be expected to be different.

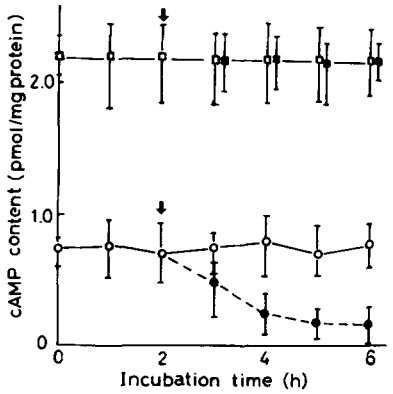

FIGURE 3 The effects of temperature on cAMP levels in wild-type and *cyr1-2* homozygous diploid cells. Strain G435 (□, ■) and AM77 (○, ●) cells cultivated at 25°C in YPGlu medium were harvested at the time indicated (□, ○), and cAMP content in these cells was measured. After 2 h each aliquot of these cultures was shifted to 33.5°C (■, ●) in the same medium, and cAMP content was measured. The arrow indicates the time of temperature shift. Error bars indicate SEM.

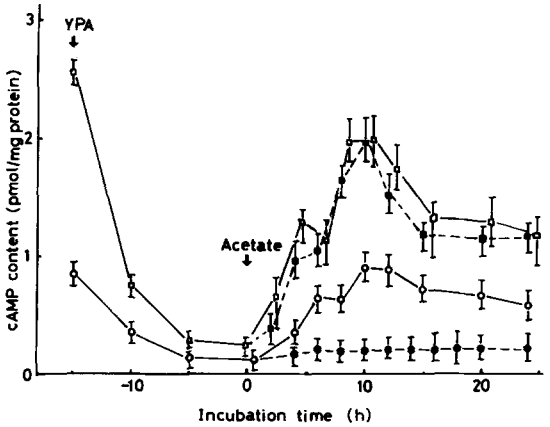

FIGURE 4 Cyclic AMP levels during sporulation of wild-type and *cyr1-2* homozygous diploid cells. G435 (□, ■) and AM77 (○, ●) cells were cultivated first in YPA medium at 25°C for 15 h and then in acetate medium at 25°C (□, ○) or 33.5°C (■, ●). The cells were harvested at various times, and cAMP levels of these cells were measured. Error bars indicate SEM.

In the samples of two-spored asci formed in the *cyr1-2* diploid cells, approximately one-third consisted of m,m or +,+ spore pairs with respect to the three markers examined (Table I). This distribution indicates meiotic products incorporated into ascospores in the *cyr1-2* diploids are random sample of the four haploid products from meiosis.

Observation of Meiotic Nuclear Phenotypes by Propidium Iodide Staining

To observe the sporulation process, we grew the *cyr1-2* homozygous diploid cells in sporulation medium and stained them with propidium iodide. Fluorescent and phase-contrast/fluorescent photomicrographs that show nuclear behavior are presented in Fig. 5. Distribution of each nuclear pattern during the sporulation of *cyr1-2* homozygous diploid cells is presented in Table II. The nucleus of the *cyr1-2* diploid cells (Fig. 5*a*) enlarged soon after the transfer to sporulation medium at 33.5°C (Fig. 5*b*), and divided into two parts of nuclear material stained after ~4 h or later (Fig. 5*c* and Table II). Stained cells that contained four parts of nuclear material were observed mainly after 8 h or later (Fig. 5*d* and Table II); two prospores were formed after 14 h or later (Fig. 5*e* and Table II). Nuclear material excluded from prospores were well stained under the present condition, and became dispersed (Fig. 5*f*). Nuclei enclosed in the prospore wall were not stained unless strongly pressed.

To determine the relative DNA content per nucleus in spores, we recorded the relative intensity of fluorescence emitted from a propidium iodide–stained nucleus. The histograms representing the distribution of DNA content per nucleus in dividing haploid cells of *cyr1-2* strain, diploid cells of the wild-type strain, and two-spored asci from *cyr1-2* diploid cells were prepared. The results shown in Fig. 6 indicate that two nuclei in spores formed in *cyr1-2* homozygous diploid cells have the haploid level of DNA. The presence of two haploid spores in an ascus implies that the second meiotic division occurred, and two haploid nuclei were enclosed in two prospores.

TABLE I

Segregation of Heterozygous Markers in Two-spored Asci Formed in cyr1-2 Homozygous Diploid Cells

	Type of asci*			
	I	II	III	I + II
Marker	+ : +	m : m	+ : m	I + II + III
LEU1	28	13	67	0.38
GAL7	12	28	68	0.37
MAT	19	21	68	0.37

* Two-spored asci of diploid (AM70-1D [α *cryl-2 leu1*] [4] × AM137-2D [**a** *cry1-2 gal7-2*]) were analyzed for segregation of heterozygous markers. + : +, m : m, and + : m indicate *LEU1* : *LEU1*, *leu1* : *leu1*, and *LEU1* : *leu1* at the *LEU1* locus, and *GAL7* : *GAL7*, *gal7* : *gal7*, and *GAL7* : *gal7* at the *GAL7* locus, or *MAT*α : *MAT*α, *MAT***a** : *MAT***a**, and *MAT*α : *MAT***a** at the *MAT* locus. Strain AM137-2D was constructed by crossing AM26-2A (**a** *cryl-2*) (4) × G211-6A (α *gal7-2 his7*) (4).

Ultrastructural Analysis of Sporulation of cyr1-2 Homozygous Diploid Cells

Cells of the *cyr1-2* homozygous diploid cells subjected to sporulation at 33.5°C were fixed and embedded for serial section. More than 50 serial sections of each stage of sporulation were observed. About 40% of cells observed entered normal meiosis I with duplicated spindle pole bodies which contained inner and outer plaques, and were associated with microtubules as shown by Byers and Goetsch (17) and Moens and Rapport (18). However, the remaining cells entered abnormal meiosis I with duplicated spindle pole bodies, one of which lacked its outer plaque (Fig. 7, *a* and *b*). Even at the later stage of meiosis I, one spindle pole body had no clear outer plaque (Fig. 7, *c* and *d*). Subsequently, at meiosis II, two types of spindle were observed. The first type had one normal spindle pole body with inner and outer plaques and the abnormal one without outer plaque (Fig. 8, *a* and *b*), and the second type had the normal duplicated spindle pole bodies of both plaques (Fig. 8, *c* and *d*). Each normal spindle pole body was incorporated into prospore wall but the abnormal one was not (Fig. 9). At the final stage of sporulation, two matured spores were observed in an ascus (Fig. 10).

DISCUSSION

It has been reported that the *cyr1* locus of yeast is the structural gene for adenylate cyclase, and that the temperature-sensitive *cyr1-2* mutant strain produced an altered adenylate cyclase

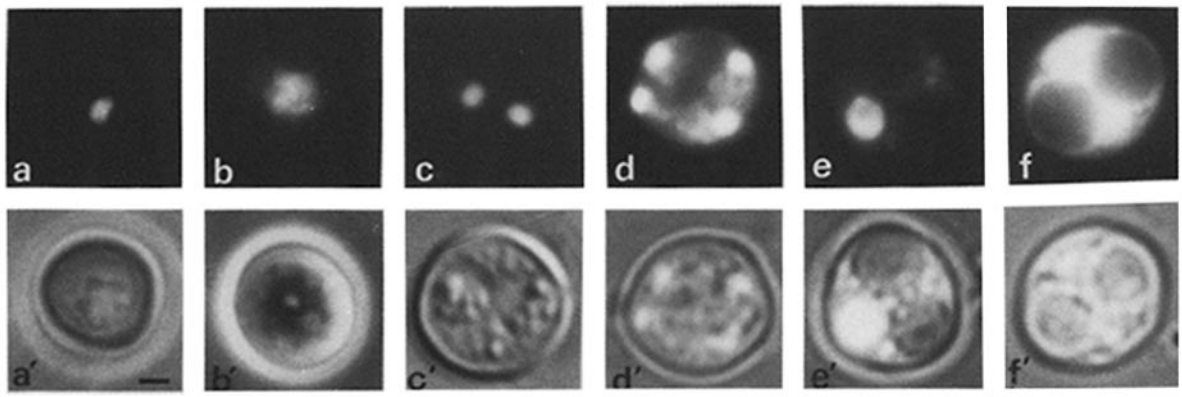

FIGURE 5 Distribution of nuclear material at various times during sporulation of *cyr1-2* homozygous diploid cells at 33.5°C. AM77 cells were incubated in sporulation medium at 33.5°C for 0 (*a* and *a'*), 6 (*b* and *b'*), 8 (*c* and *c'*), 10 (*d* and *d'*), 12 (e and e'), and 16 (*f* and *f'*) h. Sporulating cells stained with propidium iodide were photographed under phase-contrast/fluorescent (*a'–f'*) and fluorescent (*a–f*) microscope. *a* and *a'*, a diploid cell; *b* and *b'*, a cell containing a large part of nuclear material (an early stage of meiosis I); *c* and *c'*, a cell containing two parts of nuclear material (a late stage of meiosis I); *d* and *d'*, a cell containing four parts of nuclear material (a late stage of meiosis II); *e* and *e'*, a cell containing two parts of nuclear material and two prospores; *f* and *f'*, a two-spored ascus. Bar, 1.0 μm. × 4,000.

TABLE II

Time Course of Meiosis and Two-spored Ascus Formation in cyr1-2 Homozygous Diploid Strain

Incubation time*	Nuclear phenotypes and types of ascus‡					
	I	II	III	IV	V	VI
2	100.0	0	0	0	0	0
4	96.5	3.5	0	0	0	0
6	88.5	11.0	0.5	0	0	0
8	76.0	18.0	6.0	0	0	0
10	69.5	20.0	10.5	0	0	0
12	69.5	16.5	14.0	0	0	0
14	70.0	10.0	14.0	0	0.5	0.5
16	71.9	7.6	10.0	2.0	8.0	0.5
18	66.8	4.2	8.5	4.0	16.0	0.5
20	62.5	2.0	7.0	5.0	23.0	0.5
24	61.5	2.5	4.5	5.5	24.5	0.5
30	65.5	2.0	0.5	6.0	25.0	1.0
34	63.0	3.0	0	7.0	26.0	1.0
38	64.8	2.0	0	6.0	26.2	1.0

* The *cyr1-2* homozygous diploid cells were incubated at 33.5°C in sporulation medium. Incubation time is indicated as hours after the transfer to acetate medium.

‡ The cells were stained with propidium iodide and observed by fluorescent microscopy. Nuclear phenotypes and types of ascus were determined as shown in Fig. 5. *I*, one-nucleus cells (Fig. 5,*a* and *b*); *II*, first-division asci and asci with 2 nuclei (Fig. 5*c*); *III*, second-division asci and asci with 4 nuclei (Fig. 5*d*); *IV*, one-spored asci; *V*, two-spored asci (Fig. 5,*e* and *f*); *VI*, three- or four-spored asci. All values are indicated as percentage of total cells.

(5). The present data indicate that the *cyr1-2* homozygous diploid cells accumulated significantly low levels of cAMP at the restrictive temperature, and produced predominantly two-spored asci at the restrictive temperature but normal asci at the permissive temperature. These results suggest that production of a high level of cAMP is required for the normal sporulation in yeast. The dyad analysis of two-spored asci formed in *cyr1-2* diploid strain indicated the random segregation of meiotic products in two spores. The time course of sporulation of *cyr1-2* homozygous diploid cells incubated at the restrictive temperature showed that four parts of nuclear material were identified after the transfer of the cells to sporulation medium. This observation clearly indicates that meiosis I and meiosis II proceed normally in the mutant cells. The nuclei included in two-spored asci after meiotic nuclear division were haploid. Furthermore, the electron microscopy revealed that the defective spindle pole bodies which lacked its outer plaque were associated with microtubules and separated to the opposite poles. All these data suggest that the *cyr1-2* mutation has no deleterious effect on the function of chromosome separation, but interferes with normal spore wall formation. Several investigators observed the formation of two-spored asci in yeast. Esposito et al. (11) found that the *spo3* homozygous diploids produced two-spored asci that contained a random sample of haploid genomes. Cytological observation of *spo3* diploid cells revealed that these cells incubated at the restrictive temperature were defective in spore wall formation (19). In other studies, Okamoto and Iino (10, 20) reported the *hfd1-1* mutant which produced nonsister spores in two-spored asci, and Davidow et al. (7) found that the thermal arrest of meiosis yielded two-spored asci containing nonsister products of meiosis. Srivastava et al. (9) described that the two-spored asci arose due to interruption of the formation of the spindle apparatus at the second meiotic division and random chromosome loss.

About half of sporulating *cyr1-2* diploid cells showed normal meiosis I producing two normal spindle pole bodies, but the other half exhibited abnormal meiosis I producing one normal spindle pole body with inner and outer plaques, and one defective spindle pole body without outer plaque. At meiosis II, a part of cells contained a pair of normal spindle pole bodies with both plaques and prospore wall. Two spores formed in this type of cells should contain sister nuclei. The other part of the cells contained pairs of spindle pole bodies: normal ones with both plaques and prospore wall, and abnormal ones without outer plaque and prospore wall. Two spores formed in this type of cells should contain nonsister nuclei. Although the exact ratio of these two types of spindles was not obtained by the electron microscopic observation, it is expected that the half of sporulating cells that shows normal meiosis I produces two nonsister spores, and the half that shows abnormal meiosis I produces two sister spores in an ascus. Thus, the enclosure of the products of meiosis in two spores of *cyr1-2* diploid strain appears to be random with respect to the distribution of haploid genomes.

The cAMP levels of wild-type and *cyr1-2* diploid cells incubated in presporulation medium decreased rapidly to the minimum. After the transfer to the sporulation medium, the cAMP level of wild-type cells and *cyr1-2* cells incubated at the permissive temperature increased and reached to the maximum levels after 12 h, but that of *cyr1-2* cells incubated at the restrictive temperature was kept at the low level. It is pointed out that the time required to reach to the maximum level of cAMP after the incubation in sporulation medium (Fig. 4) coincided exactly the time required for the commitment to production of two-spored asci in sporulation medium (Fig. 2). These evidences clearly indicate the connection between the availability of cAMP and the ability to assemble an outer plaque during sporulation. Watson and Berry (1) measured the cAMP level of the wild-type yeast strain during sporulation, and indicated that the level of intracellular cAMP fluctuated but increased throughout the first 12 h of the sporulation phase. It is suggested that a low level of cAMP of

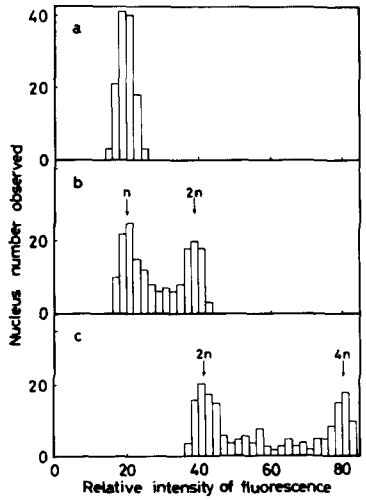

FIGURE 6 Frequency distribution of the relative intensity of fluorescence per nucleus stained with propidium iodide. Histograms were prepared for nuclei of spores of two-spored asci of *cyr1-2* homozygous diploid (*a*), nuclei of haploid *cry1-2* cells at the log phase (*b*), and wild-type diploid cells at the log phase (*c*). The peaks in the histogram indicated by *n*, *2n*, and *4n* correspond to the haploid, diploid, and tetraploid levels of DNA, respectively.

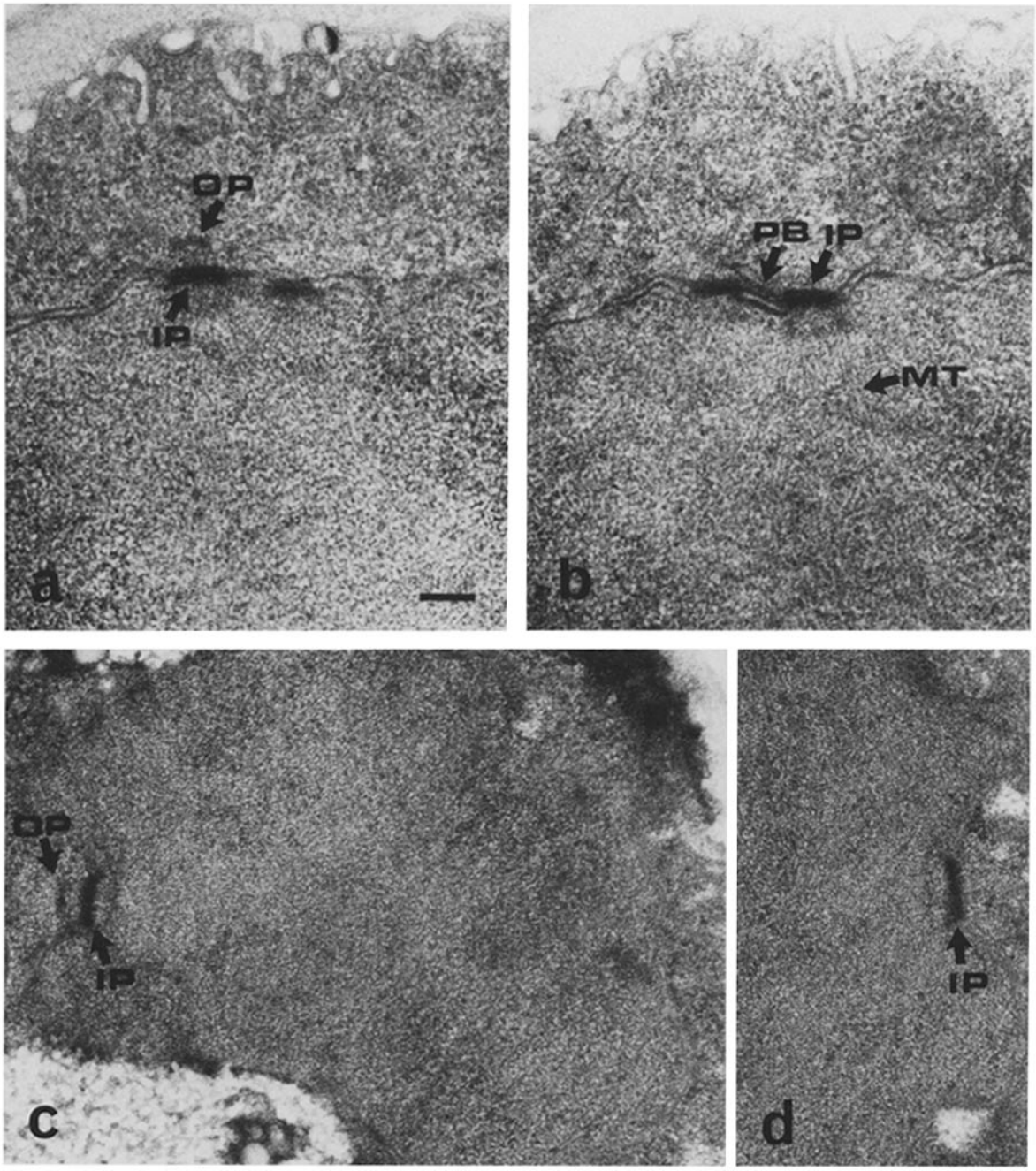

FIGURE 7 Electron micrographs of meiosis I spindles of *cyr1-2* homozygous diploid strain. (*a* and *b*) Serial sections of the same cell at an early stage of meiosis I. Replicated spindle pole bodies were arranged side by side. The two plaques are connected by a plaque bridge (*PB*) and associated with microtubules (*MT*). One of these plaques has only inner plaque (*IP*) but not outer plaque (*OP*). (*c* and *d*) Serial sections of the same cell at a later stage of meiosis I. One normal spindle pole body with inner and outer plaques and an abnormal one without outer plaque are observed. Bar, 0.1 μm. × 80,000.

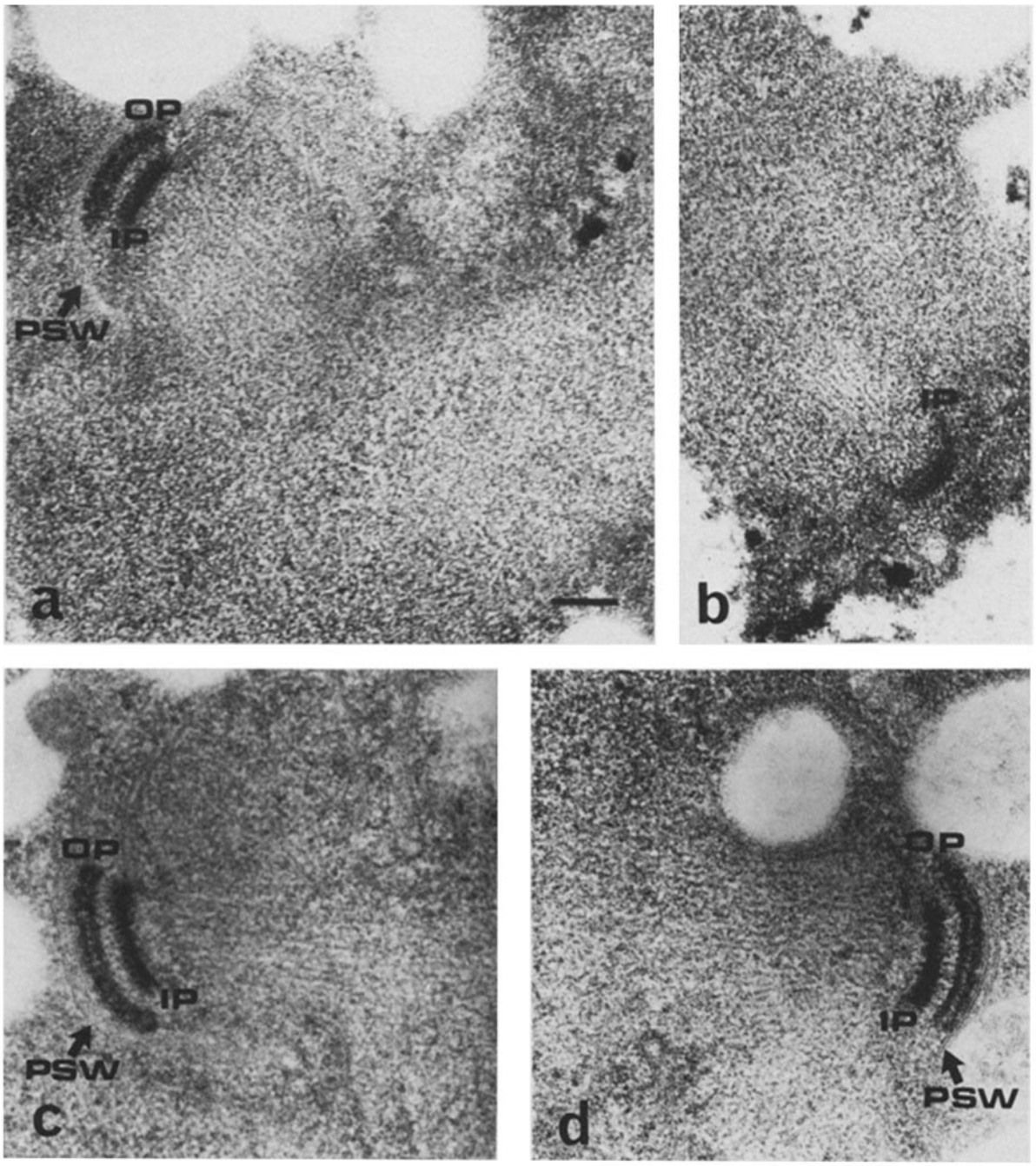

FIGURE 8 Electron micrographs of meiosis II spindles of *cyr1-2* homozygous diploid strain. (*a* and *b*) Serial sections of the same cell at meiosis II. One normal spindle pole body with inner (*IP*) and outer (*OP*) plaques associated with prospore wall (*PSW*), and an abnormal one without outer plaque and prospore wall were observed. (*c* and *d*) Serial sections of the same cell at meiosis II. Two normal spindle pole bodies associated with prospore wall were observed. Bar, 0.1 μm. × 100,000.

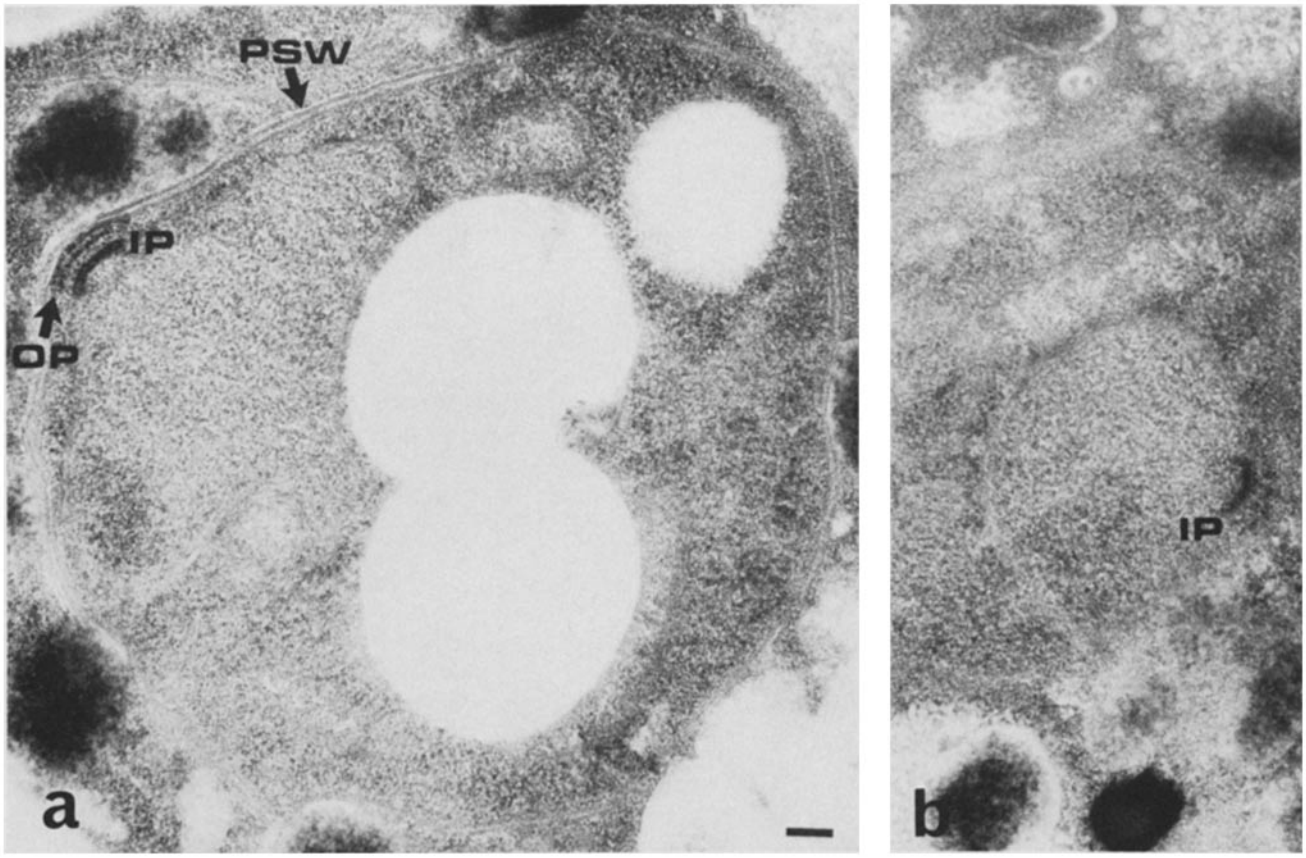

FIGURE 9 Electron micrographs of sporulating cells of *cyr1-2* homozygous diploid strain. Serial sections of the same cell at a final stage of meiosis II are shown. (*a*) One normal spindle pole body with inner (*IP*) and outer (*OP*) plaques is enclosed by prospore wall (*PSW*), and (*b*) an abnormal one without outer plaque associated no prospore wall. Bar, 0.1 μm. × 60,000.

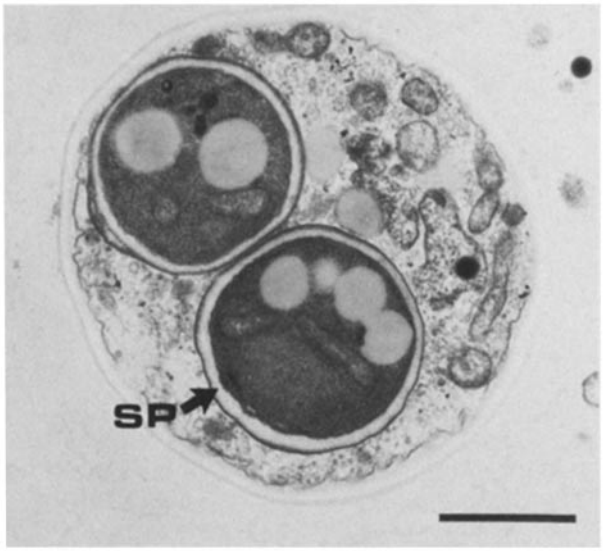

FIGURE 10 Electron micrograph of two mature ascospores (*SP*) in an ascus. Bar, 0.1 μm. × 18,000.

cyr1-2 cells incubated at the restrictive temperature is not enough to form all four ascospores in an ascus. Our previous studies indicated that cAMP works as a positive effector at the start of mitotic cell cycle but as a negative effector on the initiation of meiosis via the activation of cAMP-dependent protein kinase (6). The present results suggest that further production of cAMP is required for the enclosure of all meiotic products in prospore walls.

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