Cell Size Dependency of the Sporulation Process in the Yeast *Saccharomyces cerevisiae*

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(Received 25 April 1983; revised 11 August 1983)

Physiological changes during the sporulation process were compared between large and small cells prepared from stationary phase cells of *Saccharomyces cerevisiae*. There were marked differences in the sporulation capacity between cells of different size. NH_4^+ and methylamine did not block sporulation in large cells, but did in small cells. Large cells could sporulate in water without exposure to acetate sporulation medium, but small cells could not. During sporulation, small cells became insensitive to NH_4^+ and methylamine and acquired the ability to sporulate in water at an early stage. After acquiring the ability to sporulate in water, sporulation in small cells proceeded through a series of physiological changes common to those occurring in large cells. These results suggested that the initiation point of sporulation varied with cell size.

INTRODUCTION

In general, sporulation (meiosis and ascospore formation) in the yeast *Saccharomyces cerevisiae* can be easily induced when cells are transferred to acetate sporulation medium deprived of nitrogen sources. It provides a convenient system for studying control mechanisms of cell differentiation in eukaryotic cells.

The sporulation capacity of *S. cerevisiae* is known to vary during the vegetative cell cycle (Haber & Halvorson, 1972; Sando *et al.*, 1973). Cells can switch from the vegetative cell cycle to sporulation at the beginning of the cell cycle in G1 when exposed to sporulation conditions (Hartwell, 1974; Hirschberg & Simchen, 1977), and cells arrested in G1, such as stationary phase cells, can initiate either the mitotic cell cycle or meiosis according to the nutritional circumstances. The initiation point of the mitotic cell cycle is referred to as the 'Start' (Hartwell, 1974). Shilo *et al.* (1978) showed that meiosis was also initiated at 'Start', and many authors (Hartwell, 1974; Hirschberg & Simchen, 1977; Shilo *et al.*, 1978) suggested that meiosis had the 'Start' events common to those of the mitotic cell cycle (DNA-division cycle; Mitchison, 1971).

Cells prior to 'Start' cannot necessarily be induced to sporulate upon exposure to sporulation conditions. Small cells, even those in stationary phase, generally cannot sporulate without some period of vegetative growth (Haber & Halvorson, 1972; Sando *et al.*, 1973). This suggests that some physiological changes occurring with the growth of cells are also directly related to the initiation of sporulation.

The yeast strain FE-1 used in the present study is a natural variant having precocious sporulation ability (Sando, 1977). In stationary phase cells of this strain, even small cells can sporulate in acetate sporulation medium with high frequency. The prominent sporulation ability of small cells allowed us to investigate the relation between sporulation and growth by comparing sporulation of large and small cells. In the present study, timings of physiological

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changes during sporulation in cultures of large and small cells prepared from stationary phase cells were estimated and compared to deduce the relation between sporulation and growth.

METHODS

Organism and culture conditions. *Saccharomyces cerevisiae* laboratory strain FE-1 was cultured at 30 °C on a rotary shaker in a modified Burkholder's medium supplemented with 3.6% (v/v) tomato extract as reported previously (Ueki *et al.*, 1981).

Separation of large and small cells. Cells in stationary phase were harvested and washed with water by centrifuging at 800 *g* for 5 min. The cells were suspended in 2–3 ml of water at a density of 10^8 – 10^9 cells ml⁻¹, layered on 30 ml 35% or 30% (w/v) sorbitol solution, and centrifuged at 80–90 *g* for 40 min. Cells pelleted by the 35% sorbitol centrifugation were collected and used as a large-cell preparation. Cells remaining in the supernatant after the 30% sorbitol centrifugation were collected as a small-cell preparation. If necessary, the centrifugation in 35% or 30% sorbitol was repeated and the cell preparations were further purified.

Sporulation culture. Cells were cultured at 30 °C on a rotary shaker in 10 or 20 ml sporulation medium, composed of 60 mM-potassium acetate and 25 mM-potassium phosphate buffer (pH 6.89), at a density of 3×10^7 cells ml⁻¹. Mature asci were counted during culture, and percentages of sporulated cells were expressed as asci (%). To examine effects of inhibitors or temperature on sporulation, inhibitors were added or the temperature was shifted at intervals during sporulation culture, and vegetative cells and asci were counted to determine final percentages of sporulated cells at 24 h in the large-cell preparation and at 48 h in the small-cell preparation unless otherwise stated. The inhibitors were added at the concentration, or the temperature was shifted to the value at which sporulation was maximally inhibited when the inhibitor addition or the temperature shift was carried out at the start of culture.

Budding cells. Large cells were inoculated at a density of 3×10^7 cells ml⁻¹ into 20 ml of the vegetative growth medium with or without inhibitor and cultured at 30 °C on a rotary shaker, and budding cells were counted. A budding cell was defined as a cell bearing a bud smaller than 2 µm in the long axis, and was counted as one cell. The cells consisting of a mother cell and a bud larger than 2 µm in the long axis were counted as two cells.

Giemsa staining. Cells sampled during sporulation were fixed with ethanol/acetic acid (3:1, v/v) hydrolysed by 1 M-HCl, and stained with a buffered Giemsa solution (Sando & Miyake, 1971).

Analytical method. DNA was extracted by a modification of Schneider's method (Schneider, 1945) and measured by the method of Burton (1956) with calf thymus DNA as a standard.

RESULTS

Sporulation in large and small cells

Cell size distributions in large- and small-cell preparations are shown in Fig. 1. Volumes of stationary phase cells were usually in the range of 10–150 µm³. In the large-cell preparation,

Fig. 1. Cell size distributions in large- and small-cell preparations. Microphotographs were taken, and the long and short axes of 200–300 cells were measured in each cell preparation. Cell volumes were calculated assuming the cell shape to be ellipsoid. Mean values $\pm$ S.D. (μm^3) of cell volumes were (a) 40.2 ± 29.9 in stationary phase cells, (b) 74.2 ± 44.5 in large-cell preparation, and (c) 14.7 ± 9.2 in small-cell preparation. Mean values $\pm$ S.D. (μm) of the long axis were 4.72 ± 1.40 in stationary phase cells, 6.10 ± 1.40 in large-cell preparation, and 3.32 ± 0.66 in small-cell preparation.

Fig. 2. Sporulation and changes in DNA content of large and small cells. (a) Large-cell preparation, (b) small-cell preparation. ●, Percentage of asci; ○, DNA content expressed as relative values, taking the initial content as 1.

Fig. 3. Effects of inhibitors and treatment at 40 °C on sporulation of large cells. The large cells were treated with (a) 50 mM-hydroxyurea, (b) 5% ethanol, (c) high temperature (40 °C), (d) 10 μM-cycloheximide, and (e) 0.5 mM-isopropyl *N*-(3-chlorophenyl)-carbamate during sporulation culture, as described in Methods. ●, Control culture; ○, final percentage of asci in the treated culture plotted against the time of the beginning of the treatment.

more than 80% of cells were larger than 40 μm³, and more than 90% of cells in the small-cell preparation were smaller than 30 μm³.

The results in Fig. 2 show changes in DNA content and kinetics of sporulation in large and small cells. In the large cells, 60–80% of cells generally completed sporulation over a 2 h period beginning 8 h after transfer to sporulation medium. More than 90% of cells had formed asci by 12 h. DNA content in the large cells increased about twofold during the first 4 h. In the small cells, asci appeared after at least 10 h, 80–90% of cells finally formed asci. DNA content increased up to 12 h.

Effects of inhibitors and high temperature on sporulation in large cells

Final percentages of sporulated cells in the inhibitor- or 40 °C-treated cultures of large cells were plotted against the times of the addition of inhibitor or shift of temperature to investigate changes in the percentage of cells insensitive to inhibitor or high temperature during sporulation (Fig. 3).

Fig. 4. Effects of inhibitors and high temperature on vegetative growth. The large cells were cultured in the vegetative growth medium either at 30 °C in the presence or absence of (a) 50 mM-hydroxyurea and (b) 5% ethanol, or at (c) various temperatures. ○, Control culture; ●, inhibitor- or 40 °C-treated culture; ▲, 50 °C-treated culture.

The effects on sporulation of treatment with 50 mM-hydroxyurea are shown in Fig. 3(a). Upon the addition of hydroxyurea at 2 h of culture, only about 5% of cells could complete sporulation. During the next 2 h of culture, however, more than 80% of cells became insensitive to hydroxyurea, and about 90% of cells could sporulate when treated after 4 h. The percentage of insensitive cells increased almost in parallel with the appearance of asci in the control culture, with a time interval of about 6 h. This indicated that cells became insensitive to hydroxyurea about 6 h before the completion of sporulation.

The effects of treatment with 5% ethanol, high temperature (40 °C), 10 µM-cycloheximide and 0.5 mM-isopropyl *N*-(3-chlorophenyl)-carbamate on sporulation are shown in Fig. 3(b-f). In all the treatments, percentages of insensitive cells increased almost in parallel with the appearance of asci in the control culture. These results indicated that cells became insensitive to ethanol, 40 °C treatment, cycloheximide and isopropyl *N*-(3-chlorophenyl)-carbamate at about 4, 3.5, 2.5 and 0.5 h before the completion of ascus formation, respectively.

Ammonium sulphate and methylamine at 30 mM did not block sporulation in large cells.

Effects of inhibitors of sporulation and high temperature on vegetative growth

Effects of the inhibitors and high temperature on vegetative growth of large cells were examined (Fig. 4). In the control culture, budding cells appeared after 60 min and all the cells performed first budding during the next 60 min. The next bud formation on mother and daughter cells occurred after 140 min. In the presence of 50 mM-hydroxyurea (Fig. 4a), the first bud formation occurred normally, but formation of the next bud was delayed. Subsequently, cell proliferation proceeded slowly, and the cell density finally reached the level of that in the stationary phase of the control culture. In the ethanol-treated culture (Fig. 4b), bud formation occurred about 20 min later than in the control culture. Cells did not form buds when cultured at 50 °C, but at 40 °C bud formation proceeded with a delay of about 40 min (Fig. 4c). Cycloheximide (10 µM) completely blocked bud formation, and no cells formed buds for at least 10 h in the presence of 0.5 mM-isopropyl *N*-(3-chlorophenyl)-carbamate.

Readiness, commitment and meiotic events in large cells

Cells were taken at intervals from the sporulation culture, and transferred into water or vegetative growth medium to estimate percentages of cells in the readiness or commitment stage during sporulation culture (Simchen *et al.*, 1972). More than 80% of the large cells formed spores when cultured in water even without any exposure to sporulation medium. This indicated that most of the large cells had already entered the readiness stage during the pre-sporulation culture.

Fig. 5. Cell proliferation and sporulation after transfer of sporulating large cells into vegetative growth medium. Large cells were taken at 0 (●), 2 (△), 4 (▲), 6 (□), or 8 h (■) of sporulation, washed with water and transferred into vegetative medium at a density of 1.2×10^7 cells ml^{-1} . (a) Cell proliferation after the transfer; (b) sporulation after the transfer plotted against the time of the transfer (○, dashed line); ○ (solid line), sporulation without the transfer. Proportions of sporulated cells are expressed as percentages of the initial cell number.

Fig. 6. Meiotic development in large cells. Percentages of mature asci (○) and cells that passed metaphase I (●), anaphase I (▲) and anaphase II (■) are shown. The various stages of meiosis are represented diagrammatically.

Cell proliferation and sporulation after transfer into vegetative medium are shown in Fig. 5. A rapid increase in cell number occurred when cells were transferred into vegetative medium within the first 4 h of sporulation, but the lag-time was markedly prolonged and the growth rate reduced upon transfer after 6 h of sporulation (Fig. 5a). In contrast to cell proliferation, the number of cells committed to sporulate in the vegetative medium conspicuously increased between the fourth and sixth hour of sporulation, nearly in parallel with the appearance of asci in the control culture (Fig. 5b). The timing of transition to the commitment stage was estimated to be about 5 h before ascus formation.

Development of meiotic processes during sporulation of the large cells is shown in Fig. 6.

Fig. 7. Effects of inhibitors, treatment at 40 °C and the transfer into water on sporulation of small cells. The small cells were treated with inhibitors and at 40 °C by the procedure described in Methods, or transferred into water during sporulation culture as described in the text. (a) Treatment with 30 mM-methylamine, (b) 30 mM-ammonium sulphate, (c) 50 mM-hydroxyurea (dashed line) and 5% ethanol (solid line), (d) 40 °C (dashed line) and 10 μM-cycloheximide (solid line); (e) the transfer into water. ●, Control culture; O, final percentage of asci in the treated culture plotted against the time of the beginning of the treatment.

Most of the cells entered the stage after metaphase I by 6 h, and the subsequent process from anaphase I to telophase II proceeded rapidly. At 8 h, more than 70% of cells had nuclei at telophase II and about 20% of cells had already formed spore walls. The timings of development of meiotic anaphase I, anaphase II and telophase II were estimated to be about 6, 4.5 and 3.5 h before ascus formation, respectively.

Effects of inhibitors and high temperature on sporulation in small cells

The effects of treatment with inhibitors and high temperature on sporulation were also examined in small cells (Fig. 7). Sporulation in small cells was blocked by adding methylamine (Fig. 7a) or ammonium sulphate (Fig. 7b). Cells insensitive to these compounds appeared after a short period in sporulation medium, and increased nearly in parallel with the appearance of asci in the control culture. Timings of changes in sensitivity to methylamine and ammonia were estimated to be 9–10 h before ascus formation. Changes in sensitivity to hydroxyurea and ethanol during sporulation culture are shown in Fig. 7(c), and those to 40 °C and cycloheximide in Fig. 7(d). Results indicated that small cells became insensitive to hydroxyurea, ethanol, 40 °C treatment and cycloheximide at about 6, 5, 4 and 3 h before ascus formation, respectively. The small cells in the sporulation culture were transferred into water, and the timing of the transition to the readiness stage was estimated (Fig. 7e). The result indicated that small cells entered the readiness stage about 8 h before ascus formation.

DISCUSSION

As shown by Shilo *et al.* (1978), cells can initiate sporulation only at 'Start' in the G1 phase (Hartwell, 1974). Johnston *et al.* (1977) examined the relationship between growth and cell division using *cdc* mutants, and showed that growth, rather than progress through the DNA-division cycle, was rate-limiting to vegetative cell division. They concluded that growth to a critical size was a prerequisite for initiation of the DNA-division cycle. The same explanation could also apply in the case of sporulation: some growth being required before small cells acquired the ability to sporulate (Haber & Halvorson, 1972; Sando *et al.*, 1973).

Fig. 8. Timetable of physiological changes and meiotic events during sporulation. Timings of the changes in sensitivities to methylamine (MA), ammonia (Am), hydroxyurea (HU), ethanol (E), 40 °C (T), cycloheximide (CH), and isopropyl *N*-(3-chlorophenyl)-carbamate (IP) are shown. R and Cm represent timings of the transition to the readiness and commitment stages, respectively. M-I and A-I represent times when cells pass through metaphase I, anaphase I and anaphase II, respectively. Sm, Small cells; La, large cells.

In the present study, however, small cells of strain FE-1 could sporulate without any further period of vegetative culture. Although small cells formed buds during vegetative culture when the cell size had become equal to that of large cells, small cells sporulated without significant increase in size in the sporulation medium (data not shown). Thus, in the present strain, FE-1, it seemed that cell size was not a critical factor in the initiation of sporulation in small cells.

For vegetative cell division in various organisms, the time when sensitivity to an inhibitor is lost is called the 'transition point' (Mitchison, 1971). In the present study, the transition points during sporulation were determined to compare the sporulation processes in large and small cells. The timings of physiological transitions and meiotic events in large and small cells are shown in Fig. 8. In small cells, the change in sensitivity to NH_4^+ or methylamine and the transition to readiness stage occurred early in sporulation, followed by a series of physiological transitions common to those occurring in large cells. Although the timing could not be determined in small cells as precisely as in large cells because of a lesser degree of synchrony in the former, physiological transitions in small cells coincided approximately with those in large cells. These results suggested that large and small cells initiated sporulation at different points but proceeded through a common physiological process at least after the change in hydroxyurea sensitivity, occurring at the time when large cells entered anaphase I.

There are many reports about the effects of the inhibitors of sporulation used in the present study. The effect of NH_4^+ or methylamine, i.e. nitrogen repression of sporulation, has been examined in detail by many authors (Durieu-Trautmann & Delavier-Klutcho, 1977; Piñon, 1977; Croes *et al.*, 1978; Fonzi *et al.*, 1979; Opheim, 1979; Vezinhet *et al.*, 1979; Delavier-Klutcho *et al.*, 1980), and some mutants released from the nitrogen repression have been isolated (Dawes, 1975; Piñon, 1977). The mechanism of the nitrogen repression, however, appeared to be very complicated. Piñon (1977) claimed that one of the effects of NH_4^+ was to disturb premeiotic DNA replication itself, but Croes *et al.* (1978) concluded that NH_4^+ interrupted sporulation prior to DNA synthesis. In the present study, NH_4^+ did not inhibit sporulation in large cells despite the occurrence of premeiotic DNA synthesis, and in small cells the transition point for NH_4^+ or methylamine preceded that for hydroxyurea. Hydroxyurea is an inhibitor of ribonucleotide reductase (Elford, 1968; Krakoff *et al.*, 1968), and is known to reversibly inhibit DNA synthesis in vegetative cells of yeast (Slater, 1973). Simchen *et al.* (1976) showed that hydroxyurea blocked premeiotic DNA synthesis in yeast. The transition point for hydroxyurea sensitivity may represent the completion of premeiotic DNA synthesis. Thus, in this strain, the inhibitory effect of NH_4^+ may not be directly related to premeiotic DNA replication itself. The transition point for cycloheximide sensitivity may represent the completion of protein synthesis required for sporulation. Protein synthesis has been thought to be necessary for sporulation up to the stage where the mature ascus is formed (Esposito *et al.*, 1969; Hopper *et al.*, 1974; Magee & Hopper, 1974). However, the present results show that protein synthesis required for sporulation ends prior to the formation of mature asci. The physiological processes affected by high temperature and ethanol in sporulating cells remain unknown. High

temperature and ethanol were, however, thought to affect sporulation-specific events, because cell division was not blocked but only delayed by these treatments. Isopropyl *N*-(3-chlorophenyl)-carbamate is known to disrupt the microtubule system (Hepler & Jackson, 1969). The present results show that this inhibitor blocks the development of sporulation when the spore wall becomes apparent after telophase II. This suggests that microtubules play some role in the formation of spore walls.

In general, both the change in NH_4^+ sensitivity and the transition to readiness stage have been thought to be sporulation-specific events and occur with progress through meiosis. In the present strain, however, it appeared that cells underwent these physiological changes as they grew into large cells. In conclusion, the present study suggests that some of the sporulation-specific events are directly related to the growth of cells, and cells can undergo such physiological changes with no direct coupling to meiosis which is initiated only upon exposure to sporulation conditions.

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