Karyokinesis and Septum Formation during the Regeneration of Incomplete Cell Walls in Protoplasts of *Schizosaccharomyces japonicus* var. *versatilis*: a Time-lapse Microcinematographic Study

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Regeneration in flattened *Schizosaccharomyces japonicus* var. *versatilis* protoplasts was studied with regard to nuclear behaviour, septum formation and lateral wall regeneration. In the course of protoplast regeneration all stages of the cell cycle were realized. However, the time sequence was conserved only between nuclear division and the beginning of septum formation. From this it is suggested that the signal initiating septum formation was issued by the dividing nucleus. Its primary target was not the original cell wall. However, the wall, though incomplete, was an essential prerequisite for the signal to be put into operation, i.e. for septation to begin. Incomplete cell wall regeneration allowed cytokinesis but not reversion of protoplasts into cells with their genetically determined cylindrical cell shape.

INTRODUCTION

Interrelations of essential processes of the cell cycle have been studied in regenerating yeast protoplasts, in which karyokinesis takes place (Nečas *et al.*, 1961; Nečas, 1959; Havelková & Křípalová, 1980), but cytokinesis occurs only after cell walls have been regenerated (Nečas, 1971; Havelková, 1981; Nečas & Svoboda, 1981). However, the experimental study of the association of karyokinesis with septation is hindered by the fact that it is very difficult to detect nuclei *in vivo*. Nuclei are clearly visible in intact yeasts growing in 18–20% (w/v) gelatine nutrient medium (Müller, 1956; Robinow, 1975). However, nuclei become progressively less visible with increasing gelatin concentration. In *Saccharomyces cerevisiae* protoplasts regenerating in 30% gelatin (or 2%, w/v, agar) nuclei are practically invisible (Nečas, 1961; Svoboda, 1966; Gabriel, 1971). Nuclei can be visualized in flattened *S. cerevisiae* protoplasts grown on the surface of solid media (Nečas *et al.*, 1961), but these protoplasts never regenerate their complete cell walls nor divide nor revert to cells.

In *Schizosaccharomyces japonicus* var. *versatilis*, nuclei are distinctly visible under the phase-contrast microscope (Kopecká, 1975; Kopecká & Gabriel, 1978) despite the absence of gelatin. Therefore the relation between nuclear cycle events and cytokinesis was studied in the protoplasts of this yeast. Preliminary results of this work have been presented elsewhere (Gabriel, 1984).

METHODS

Strain. The yeast used in this study was *Schizosaccharomyces japonicus* var. *versatilis* CC-60-255 (sometimes referred to as *Schizosaccharomyces versatilis*), kindly provided by Professor H. J. Phaff of the University of California, Davis, USA. It was grown on 2% (w/v) malt extract agar, pH 5.5. Stock cultures were stored at 4 °C.

Protoplast preparation and cultivation. Protoplasts were prepared by means of digestive enzymes of *Helix pomatia* (Gabriel & Kopecká, 1983). The washed protoplasts were transferred to mannitol/stabilized malt extract medium. For microcinematographic observation, a small droplet of the protoplast suspension (approximately 10⁴ protoplasts) was put on a cover slip coated with a thin layer of malt extract agar. The cover slip was carefully placed on a

Fig. 1. Time-lapse microcinematography of growth and cell wall regeneration in flattened protoplasts of *S. japonicus* var. *versatilis*. Nuclei are indicated by simple arrows, septa by unfilled arrows, and regenerating wall by bold arrows. Phase-contrast microscopy. The bar markers represent 5 μ m in (a) to

slide such that the agar layer became sandwiched in between. Capillary forces together with the weight of the slip resulted in partial flattening of the protoplasts in which all cellular structures were situated in the focal plane.

Microcinematography. This was done with a 16 mm recording camera (RfS Meopta, Czechoslovakia) equipped with a time-recorder (Registimer B4, Meopta, Brno, Czechoslovakia), attached to a modified light microscope (Amplival, Carl Zeiss, Jena, GDR). Phase-contrast photomicrographs were taken with the same microscope through a yellow-green filter at a primary magnification of $\times 250$. The frequency of recording was about 1 picture every 4 s; exposure was 0.5 s on 16 mm inverse black and white film (Fomapan 21 DIN).

RESULTS

A total of 33 preparations were studied by time-lapse microcinematography and 21 specimens by serial microphotography. Probably due to differences in the degree of flattening, the timing of cell cycle steps (growth rate, onset of karyokinesis, rate of formation of wall structures, etc.) varied markedly with each protoplast preparation.

Karyokinesis. Karyokinesis took about 3 min in the protoplasts. A spherical nucleus (Fig. 1: *a*; *g*, III) became oval in shape (Fig. 1: *h*, III), then took a spindle-like shape (Fig. 1: *b*; *i*, III), formed a ribbon-like structure (Fig. 1: *j*, III), and divided rapidly into two daughter nuclei (Fig. 1: *c*; *k*, III; *l*, III) which remained connected by a thin bridge for a few tens of seconds. In resting

(*n*); the bar marker in (*o*) represents 2.5 μm . All the times shown are counted from the start of the incubation.

(*a*) Time 0 h 0 min: the start of incubation of a protoplast (P) containing one nucleus with a dark nucleolus. The protoplast was released from the cell wall (*w*). Some of the dark bodies in the cytoplasm are probably mitochondria (*m*). To the right of the protoplast there is a dying cell (*c*) with a cytoplasmic anucleate hernia (*h1*) penetrating through the digested wall.

(*b*) Time 1 h 12 min. The regenerating wall is visible on the surface of the protoplast. Small vacuoles (*v*) are formed in the cytoplasm. The nucleus is in metaphase.

(*c*) Time 1 h 37 min. Situation after nuclear division. Two daughter nuclei, with dark nucleoli, are apparent.

(*d*) Time 1 h 53 min. Septum formation begins along a plane approximately perpendicular to the axis of the nuclear division. Centripetal growth of the septum from the plasma membrane is evident.

(*e*) Time 2 h 8 min. The septum has closed during the preceding 15 min. Further thickening of the septum starts centripetally, i.e. from the periphery of the protoplast. The septum has divided the original protoplast into two uninucleate halves: protoplasts I and II. Protoplast II continues to grow; protoplast I does not grow.

(*f*) Time 3 h 28 min. In the growing protoplast II the dividing nucleus is visible. The septum has reached its final thickness.

(*g*) Time 4 h 4 min. A second septum has formed, which divides protoplast II into protoplasts II and III. An anucleate hernia (*h2*) has originated from protoplast I, through its incomplete cell wall.

(*h*) Time 5 h 0 min. Protoplast III grows; its nucleus is in prophase. The nucleolus is not clearly discernible. Inside the nucleus smaller dark spots are present. The second septum has reached its final thickness. Protoplast II has formed an anucleate hernia (*h3*) through its incomplete wall. The hernia probably remains in communication with the protoplast, because two further anucleate hernias (*h4*, *h5*) were later extruded by protoplast II (see *m*).

(*i*) Time 5 h 2 min 55 s. The nucleus in protoplast III is in metaphase.

(*j*) Time 5 h 2 min 59 s. The nucleus of the growing protoplast III is in anaphase.

(*k*) Time 5 h 3 min 3 s. The nucleus of the growing protoplast III is in late anaphase.

(*l*) Time 5 h 57 min. Daughter nuclei are well visible again in protoplast III. It continues to grow. Protoplasts I and II continue to regenerate a cell wall. Septum formation starts in protoplast III.

(*m*) Time 6 h 10 min. An asymmetrically placed septum divides protoplast III into an anucleate protoplast (IV) and a binucleate protoplast (III). Anucleate hernias *h4* and *h5* have formed through the incomplete wall of protoplast II.

(*n*) Time 9 h 15 min. Protoplast I, having a complete cell wall, starts to revert to a cell. Protoplast II has died. Protoplast III, with one visible nucleus and an incomplete wall, stagnates. The anucleate protoplast IV vacuolizes; it is separated from III by a multilayered septum. Protoplast V (produced by growth of protoplast III and separated from it physically) has completed nuclear division: two nuclei are present. An asymmetrically placed septum starts to grow centripetally and divides protoplast V into V (binucleate) and VI (anucleate) compartments.

(*o*) Time 22 h 0 min. Detail of regenerated protoplast I reverting to the cylindrical cell shape and reproducing by fission. An orientation mark for comparison with *a*, *d*, *g* and *m* is the original cell wall (*w*) of the yeast from which the protoplast was originally released.

nuclei darker spherical or cap-like areas corresponding to nucleoli could be distinguished from the lighter nuclear mass (Fig. 1: *a; c; g; l; m*). A few minutes before the onset of karyokinesis the sharp margins of the nucleoli disappeared.

Septation. The septum developed centripetally (Fig. 1: *d-f; m-n*). The time taken for closing of the septum ranged from 7 to 60 min. Even after the septum had apparently been completed, communication between the two cell compartments went on for some time. The septum first formed as a thin primary layer (Fig. 1: *d-e; m*), which then gradually thickened (Fig. 1: *d-f; g-h*), up to 1.5 μm in some cases, which is twice the normal septum thickness (0.6–0.7 μm in cells: unpublished data). The maximum thickness was usually attained by about 65 min after the beginning of septation. In some protoplasts multilayered septa were also formed (Fig. 1: *m-n*) between anucleate and binucleate compartments.

Relation of karyokinesis to septation. In some protoplasts the onset of karyokinesis was seen as early as 20 min after the beginning of incubation, in others as late as 150 min. Karyokinesis was followed by septum formation in the plane approximately perpendicular to the axis of nuclear division (Fig. 1: *d-g*) about 33 min (range ± 10 min) after division of the nucleus (in cells this lasted 23 ± 5 min: unpublished data). This period was very similar in all 328 successive divisions studied, even though the length of the nuclear cycle (from karyokinesis to karyokinesis) ranged between 2.5 and 9 h. The septum was produced earliest in primary binucleate protoplasts, i.e. those arising from cells that had accomplished karyokinesis but not septation before the protoplasts were prepared.

Relation of lateral wall regeneration and septation. Some of the protoplasts formed a thick cell wall over the whole surface after about 6 h (Fig. 1: *m, I*) and later reverted to cells (Fig. 1: *n, I; o, I*). The protoplasts which had only thin cell walls, both structurally and functionally incomplete, continued growing (Fig. 1: *b-d; e, II; f, II; g, III; l, III; m, III; n, V, VI*) in a multipolar or, more frequently, unipolar manner. Septation did not occur unless at least some cell wall regeneration had taken place. In primary binucleate protoplasts also, septation started after an incomplete wall was formed (after 1.5–3 h). The septum usually did not divide the protoplast into two equal halves (Fig. 1: *e; g; m*). Due to the formation of only an incomplete wall, the daughter protoplasts were of various shapes and, due to asymmetric positioning of septa, of differing sizes. They continuously produced anucleate ‘hernias’ through the incomplete wall (Fig. 1: *g, h2; h, h3; m, h4, h5*). No relation was found between the differing thickness of the wall and the asymmetric position of the septum. Only the protoplasts that formed complete cell walls and reverted to cells survived. The others gradually died during the next few divisions.

Asymmetric formation of the septum (Fig. 1: *m-n*) in growing protoplasts led to the production of both anucleate (Fig. 1: *m, IV; n, IV, VI*) and binucleate compartments (Fig. 1: *m, III; n, V*). The anucleate parts survived for several tens of hours, but did not form septa; they vacuolized (Fig. 1: *m, IV, h3; n, IV*) and gradually died. The secondary binucleate protoplasts went on growing and forming an incomplete wall. Both nuclei later divided in a synchronous fashion and the cell cycle was repeated. Their further fate was not studied in detail.

DISCUSSION

Time-lapse microcinematographic recording of the cytological processes in *S. japonicus* var *versatilis* revealed valuable information on their dynamics. However, the number of cells that could be analysed was limited. Therefore only those findings that appeared frequently enough to allow some degree of generalization will be discussed.

In the first place the results reported here throw new light on the relationship of cytokinesis to cell wall regeneration associated with reversion of the protoplast to a cell. Previous studies on yeast protoplast regeneration have been based on the assumption that protoplast reversion to a cell can occur only after the cell wall has been completely regenerated (Nečas, 1971; Nečas & Svoboda, 1981; Havelková, 1981). The analysis of behaviour of flattened protoplasts of *S. japonicus* var. *versatilis* showed that the incomplete wall on their surface, though incapable of

initiating reversion to a cell, was nevertheless a 'morphogenetically active' structure because it could induce the formation of a septum. Although septation was not associated with a genetically determined shape (i.e. cylindrical cell) it carried out a biological function such as the separation of duplicated cytoplasm and genomes. The resultant daughter protoplasts could divide for a limited period. In this organism cytokinesis is evidently dissociated from the genetically determined renewal of cell shape.

As flattened protoplasts of *S. japonicus* var. *versatilis* do not have a complete rigid wall, they attain various shapes. In our preparations septa were frequently found in atypical positions. Because in protoplasts growing in a polar manner the septum often formed near the 'growing pole' where the wall was thin, it could be concluded that the place for septum initiation was not determined by the wall thickness (i.e. by the degree of wall organization). The septum generally formed perpendicular to the axis of the dividing nucleus, as suggested by Girbardt (1979), but there were many variations. The latter may be accounted for by flattening of protoplasts, which might alter the space orientation of cytoskeletal components and thus affect the direction of the signal, in which microtubules and microfilaments are possibly involved (Margolis & Wilson, 1981).

Valuable data contributing to the elucidation of the control of cytokinesis have been gained through studies of the temperature-sensitive cell division cycle mutants of *Schizosaccharomyces pombe* in which mutations affect the septation process (Minet *et al.*, 1979; Streiblová, 1981). On the basis of these studies it was suggested that the induction of septation depends on the position of the dividing nucleus (Streiblová & Girbardt, 1979). In *S. japonicus* var. *versatilis* nuclei are clearly visible, so it was possible to study the relation between karyokinesis and septation *in vivo*. In all 328 cytokineses of flattened protoplasts having an incomplete wall, septation was always associated with the preceding karyokinesis. This relation was also demonstrated by the fact that the time between karyokinesis and the onset of septation was fairly constant (33 ± 10 min). It has been suggested (Girbardt, 1979; Minet *et al.*, 1979) that during karyokinesis a signal is issued to initiate septation. The signal might be received by the plasma membrane, the cytoskeletal components or the cell wall. Primary binucleate protoplasts lack one of these possible targets: the original cell wall. Upon incubation the protoplasts started to form septa but only after they had synthesized new wall structures. This unambiguously shows that the primary target for the signal initiating septation in protoplasts is not the original cell wall of the yeast cell, but some other cell structure. This suggests that though not a primary target for the septum initiating signal, regardless of whether it is complete or not, the wall is a necessary prerequisite for the formation (anchoring) of a septum.

The course of nuclear division in flattened protoplasts of *S. japonicus* var. *versatilis* is similar to that described for *S. pombe* cells by McCully & Robinow (1971). In some papers concerning multinuclear protoplasts (Nečas *et al.*, 1961; Havelková & Křipalová, 1980) asynchronous karyokineses have been reported. In the binuclear protoplasts of *S. japonicus* var. *versatilis* nuclei always divided in a synchronous fashion (unpublished data). However, one may assume that with the increasing number of nuclei in the protoplast errors are occurring in the control of karyokineses, and are expressed as asynchrony.

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