REGULATION OF WALL SYNTHESIS DURING *SACCHAROMYCES CEREVISIAE* CELL CYCLE

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1. Introduction

In recent years, studies on specific enzyme formation during the cell cycle have proved very useful for investigating the regulatory mechanisms operating in dividing cells. The correlation between cell cycle and activities of several enzymes has been interpreted as the "linear reading" of the structural genes involved [1, 2].

Although there are many studies on the biosynthesis of wall components in *Saccharomyces cerevisiae*, the pattern of formation of these macromolecules during the cell cycle is largely unknown and knowledge on this topic would provide information about the regulatory mechanisms controlling the pathways involved in the formation of yeast cell wall.

Recently, Cabib and Farkas [3] have shown that chitin, the specific component of the septum between mother and daugther cells, is synthesized during a limited portion of the cell cycle. A periodic synthesis has also been suggested for glucan, one of the two main yeast wall polymers [4], but not for mannan, the other main polymer [5]. The interest in mannan polymers is due to the fact that they are a group of glycoproteins, some of which show catalytic activity [6]. In this context it may be emphasized that one of the catalytic mannan-containing proteins, invertase, is possibly synthesized in a periodic manner since the activity of the enzyme varies through the cell cycle [7].

The aim of the present study was to investigate the biosynthesis of mannan and alkali- and acid-insoluble glucan during the division of *S. cerevisiae*. Our results showed that both yeast wall components are synthesized continuously through the entire cell cycle. This information together with the data already published [8] suggest that the continuous synthesis is probably due to the slow turnover of the enzymes involved.

2. Materials and methods

S. cerevisiae LK2G12 was obtained from Dr. C. Lindegren, Carbondale, Ill., USA and grown in the following medium (g/ℓ): glucose 20, yeast extract 3. *S. cerevisiae* 369 D-1, a temperature-sensitive mutant was obtained from Dr. L.H. Hartwell, Derpartment of Genetics, University of Washington, USA. The characteristics and growth conditions of this mutant have previously been described [9].

2.1. *Synchronization of cells*

Two methods have been used to obtain synchronous cultures of yeast cells. i) An asynchronous, exponentially growing population of *S. cerevisiae* LK2G12 (20–25 mg) was collected by centrifugation, chilled by immersing in ice-water, suspended in 2 ml of growth medium and layered on the top of a discontinous Ficoll density gradient (Pharmacia, Uppsala, Sweden). The gradient was formed by the following solutions of Ficoll, 10 ml 29% (w/w), 30 ml 26.5% (w/w) and 5 ml 26% (w/w). The gradient was centrifuged at 27 000 *g* for 120 min at 4°C in a Spinco L265B ultracentrifuge. Three to five percent of the total cells were located at the interface between 26–26.5% Ficoll. The cells of this band were removed from the tube with a peristaltic pump, washed three times and inoculated into growth medium. ii) An exponentially growing, asynchronous cul-

ture of *S. cerevisiae* 369 D-1 cells was transferred from the permissible (25°C) to the non-permissible temperature (37°C) for 120 min. On returning to 25°C in a fresh medium, the cells divide synchronously.

2.2. *Monitoring cell number and budding*

Samples of the growing cells were removed at different times and fixed with a solution of NaCl containing 3.7% formaldehyde. After 10 min all samples were sonicated for 5 sec to disperse cells clumps. Cell number and budding were then measured by visual inspection and counting in a Zeiss phase-contrast microscope.

2.3. *Nucleic acids syntheses*

RNA and DNA syntheses were measured by the incorporation of [^{14}C]uridine [10]. For RNA synthesis, a sample of cell suspension (1 ml) was removed from the culture and added to cold 10% (w/v) trichloroacetic acid (1 ml) containing 100 μg DNA (from calf thymus). After 20 min at 0°C, cells were collected on Whatman GF/C glass fiber filter paper, washed with cold 5% trichloroacetic (50 ml) and dried [11]. For DNA synthesis, a sample of cells suspension (1 ml) was removed from the culture and added to 2 N NaOH (1 ml). RNA was hydrolyzed by incubation of the alkaline suspension at 80°C for 60 min [12], the residue chilled and carrier DNA (100 μg) and 50% trichloroacetic acid (1 ml) added. The precipitates obtained were collected on a glass fiber filter and washed as before. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

2.4. *Mannan and glucan determination*

A population of synchronized cells was incubated in the appropriate growth medium supplemented with [^{14}C]glucose (specific activity 360 nCi/nmole). At intervals, samples (5 ml) were removed and cold 10% trichloroacetic acid (5 ml) added. After 15 min the suspension was centrifuged at 2000 *g* for 10 min and the pellet transferred to ampoules containing 2 N NaOH (3 ml). The extraction of mannan and alkali- and acid-insoluble glucan, and determination of the radioactivity incorporated, was carried out following the procedure previously described [11].

3. Results

When an asynchronous culture of growing *S. cerevisiae* LK2G12 is centrifuged at 27 000 *g* for 120 min in a continous Ficoll gradient (26–29%, w/w) the cells are widely distributed along the gradient. Examination of the different bands from such a gradient indicated that the fractionation of cells was correlated with the position of the cell in the division cycle. The cells of lightest density were mostly those with very large buds, whereas the cells with greatest density were those with small buds.

In subsequent experiments we used discontinous gradients. This facilitated isolation of cell bands and reduced diffusion to a minimum. When exponentially growing *S. cerevisiae* LK2G12 was centrifuged under the conditions described in Materials and methods most of the cells at the interface between 26–26.5% (w/w) of Ficoll has a very large bud. A few cells (5–10%) without a bud could also be found. Electron microscopy of the cell present in this band indicated that most of them were at the point in which the septum between mother and daughter had already been laid down. The unbudded cells seemed to be the products of cell separation.

Biochemical events in a single cell can be deduced by following the conduct of a synchronously growing culture but this kind of investigation has been hindered by the difficulty encountered in preparing large batches of cultures. To overcome this obstacle, we investigated the behavior of a temperature-sensitive mutant [9]. This mutant, *S. cerevisiae* 369 D-1, is defective in a gene function needed at a specific stage of the cell cycle. When an exponentially growing population of these cells was transferred at 37°C, they stopped growing at the "execution point", probably at the time of DNA replication they carried a tiny bud. Viability of the cells depended on the duration of incubation at the non-permissible temperature (fig. 1) and was in agreement with that previously described [9]. After incubation at 37°C for 120 min, the residual viable cells grew synchronously when they were transferred to a fresh medium at 25°C. This afforded a practical method for preparing large amounts of synchronized cells with a minimum of operations and avoided the use of gradients.

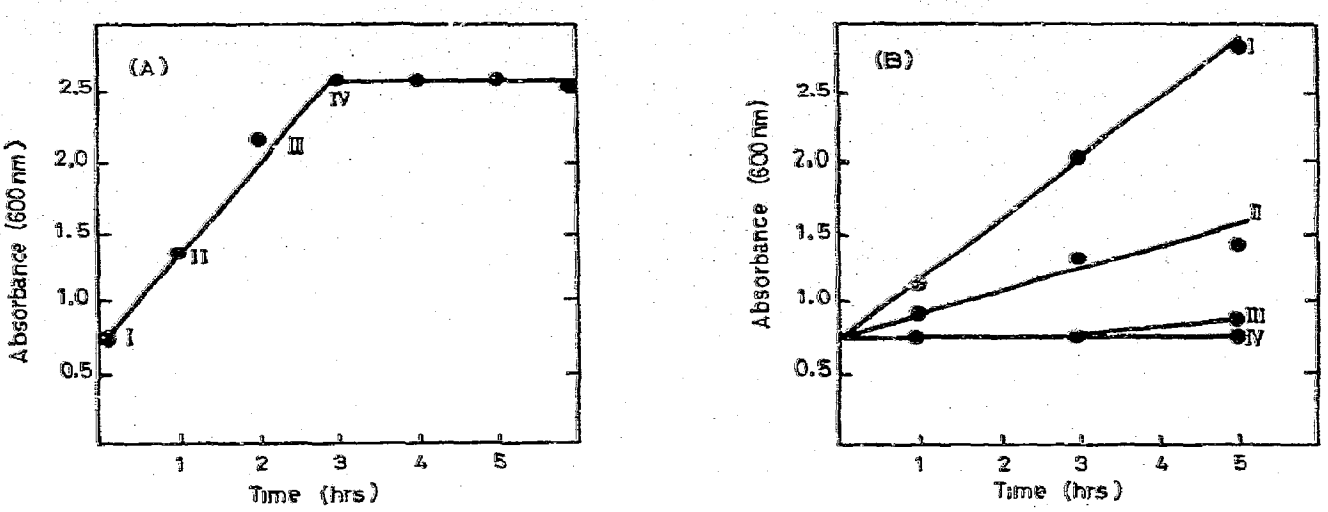

Fig. 1. Viability of *S. cerevisiae* 369 D-1 cells at the non-permissible temperature. An exponentially growing population of cells was incubated at non-permissible temperature (37°C) (A). Samples were removed at different times (I, II, III, IV) and incubated in a fresh medium at 25°C (B).

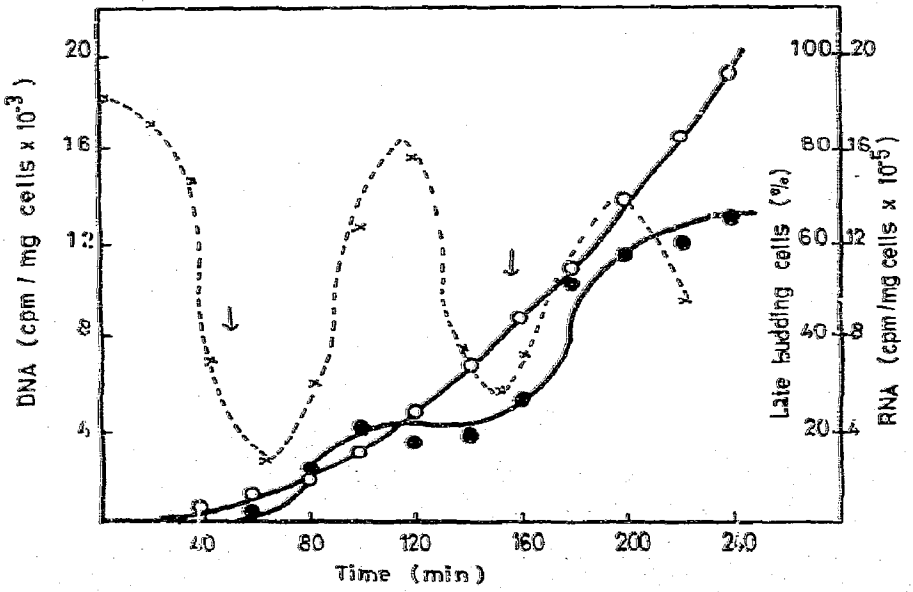

Fig. 2. Timing of budding, DNA synthesis and RNA synthesis in synchronous cultures of *S. cerevisiae* LK2G12. Cells (5 mg) collected from the Ficoll gradient were suspended in a medium (100 ml) at 28°C containing 3 μCi of [^{14}C]uridine (specific activity 0.71 nCi/nmole). Samples (1 ml) were withdrawn at the times indicated and budding (×), radioactivity incorporated into RNA (○) and DNA (●) determined as indicated in Materials and methods. Arrows indicate budding initiation.

Synthesis of macromolecules such as DNA and specific proteins during the cell cycle can be used as biochemical markers for cell growing synchronously. Autoradiography and chemical measurements have shown that DNA is synthesized periodically through the cell cycle of *S. cerevisiae* [13]. This finding affords a useful marker for studying the degree of synchrony obtained by both methods. The behavior of *S. cerevisiae* LK2G12 synchronized by gradient centrifugation is summarized in fig. 2. Cells at the interface between

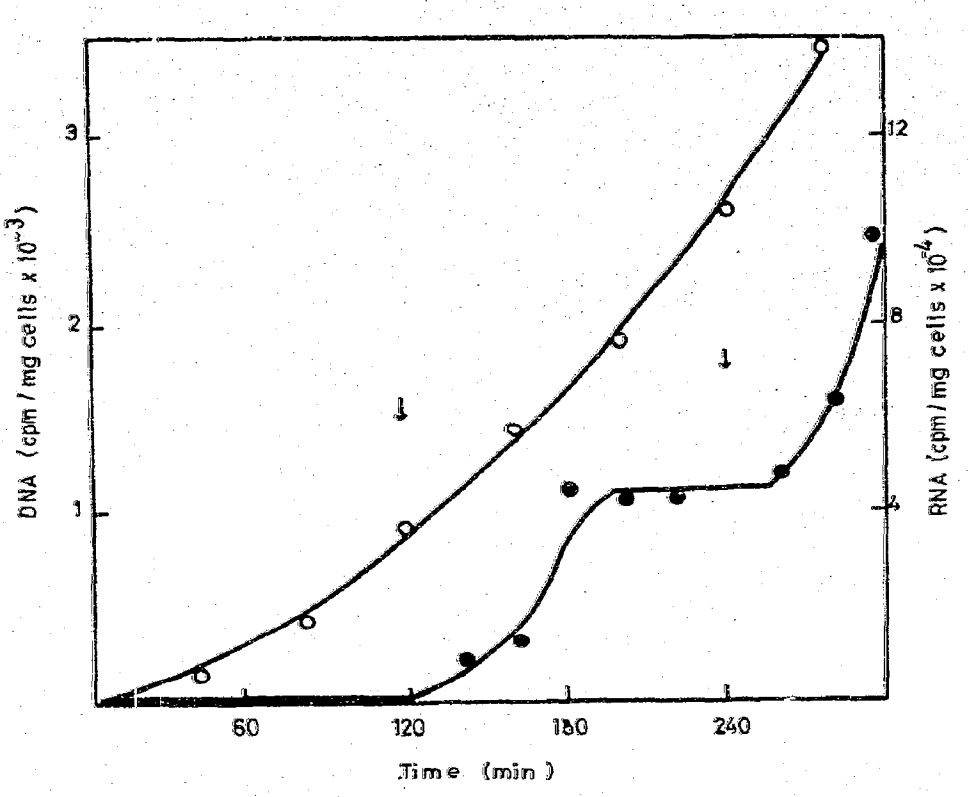

Fig. 3. Timing of DNA and RNA synthesis in synchronous cultures of *S. cerevisiae* 369 D-1. Cells previously treated at 37°C during 120 min were incubated in a fresh medium containing 3 μCi of [^{14}C]uridine (specific activity 0.7 nCi/nmole) at 25°C. Samples (1 ml) were withdrawn at the times indicated and radioactivity incorporated in RNA (○) and DNA (●) determined. Arrows indicate budding initiation.

26–26.5% (w/w) Ficoll had a large bud and, after inoculation into a fresh medium, separated and began to bud again. As growth went on, a progressive decay of synchrony was observed. The rate of synthesis of DNA began just after budding initiation and continued for about a quarter of the total cycle. This period defines the interval of total DNA replication in the culture. RNA synthesis, by contrast, occurred throughout the cell cycle at a constant rate.

The same pattern of nucleic acid synthesis was observed with the temperature-sensitive *S. cerevisiae* 369 D-1 (fig. 3). In this case, the generation time was longer, but as before, DNA synthesis was periodic while RNA synthesis was continuous. Our results indicated that we obtained synchronous cultures with both *S. cerevisiae* strains although different methods were used for both cultures. Moreover, the normal periodic DNA synthesis and also [^{14}C]glucose incorporation into the cells (unpublished data) by temperature-treated *S. cerevisiae* 369 D-1 suggested that "balanced growth" [14] had taken place and that this method of synchronization can be used in biochemical determinations.

Incorporation of radioactivity from [^{14}C]glucose into yeast cells and into the two main polymers of the w[illegible]s shown in fig. 4. It is evident that under our experimental conditions radioactivity increased exponentially through the whole incubation time (fig. 4A). The results summarized in fig. 4B are consistent with the fact that both mannan and alkali- and acid-insoluble glucan were also synthesized exponentially through the entire cell division cycle. We emphasize that the rate of synthesis was constant at all stages of the cell cycle and specific to each polymer. This experiment was repeated with *S. cerevisiae* 369 D-1 and synthesis of glucan and mannan proceded in the same manner.

Although the results seem clear, it was possible that the radioactivity that accumulated after a long

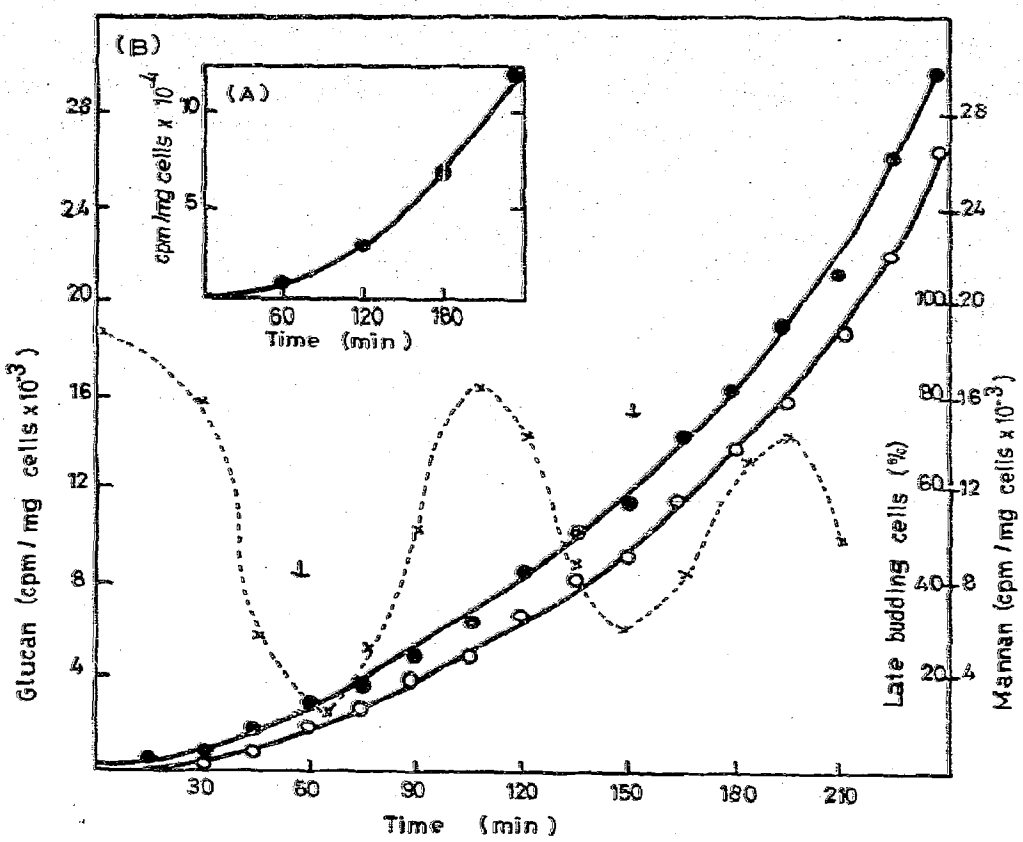

Fig. 4. Timing of glucan and mannan synthesis in synchronous cultures of *S. cerevisiae* LK2G12. Cells (5 mg) collected from the discontinous gradient of Ficoll were suspended in a medium (100 ml) containing 5 μCi of [^{14}C]glucose (specific activity, 3×10^{-3} nCi/nmole) at 28°C. Samples (5 ml) were withdrawn at the times indicated and radioactivity incorporated into whole cells (A), mannan (B, ○) and alkali- and acid-insoluble glucan (B, ●) determined. Arrows indicate budding initiation and (×) percentage of late budding cells.

Table 1
Yeast cell cycle: synthesis of glucan and mannan.

Cell cycle fraction	Radioactivity incorporated (cpm/mg cells)		Glucan (cpm/mg cells) / Mannan (cpm/mg cells)
	Glucan	Mannan	
0	2344	1600	1.46
0.2	3000	2200	1.36
0.4	4100	2800	1.46
0.6	5800	4000	1.45
0.8	9000	6200	1.45
1.0	17200	11400	1.50

Synchronized cells of *S. cerevisiae* 369 D-1 were incubated at 25°C. Samples were collected at different points of time during the cell cycle (0, 0.2, 0.4, 0.6, 0.8, 1.0) and grown with [^{14}C]-glucose (specific activity 360 nCi/nmole) for 10 min. Wall polymers were extracted and the radioactivity measured [11].

incubation might have masked any small variations in the rates of the synthesis at these times. To determine small variations, we have measured the instantaneous synthesis of both polymers by following incorporation of radioactivity into cell samples at different stages of the cell cycle (table 1). The ratio of radioactivity incorporated into glucan to that incorporated into mannan remained constant during all stages of the division cycle. The increase of total radioactivity incorporated in both cases (table 1) was the result of an increase in the specific activity of the [^{14}C]glucose in the different samples due to the uptake of "cold" glucose by the growing cells.

4. Discussion

The *S. cerevisiae* wall is built largely by a β-linked glucan and several glycoproteins whose carbohydrate moiety is generally known as yeast mannan. In this paper we report some aspects of the synthesis of these two polymers during the cell cycle of the yeast.

The results summarized in fig. 2 show that *S. cerevisiae* LK2G12 cells exhibit fluctuations in density during the division cycle. This has also been shown in other strains [10, 15]. Cell density fluctuations seem to be the result of the changing ratio of mass/volume during the cell cycle [16—18]. Wiemken et al. [15] have demonstrated the existence of vacuolar changes in yeast during the cell cycle but an understanding of these changes at the molecular level require further work.

The synthesis of DNA and the normal incorporation of glucose in synchronized *S. cerevisiae* 369 D-1 (fig. 3) suggested that "balanced growth" [14] was taking place. We think that the method used affords an easy way of preparing large amounts of synchronized cells for biochemical studies.

Investigations on the biosynthesis of mannan and alkali- and acid-insoluble glucan, the two main *S. cerevisiae* wall components, have been carried out in synchronized cultures of both yeast strains. The results (fig. 4 and table 1) indicated that both polymers were synthesized exponentially through out the entire cell division cycle. Moreover, the rate of synthesis, though specific for each polymer, was constant for all stages of the cycle (table 1).

These findings indicated that either i) the genes involved in the synthesis of the RNA messengers of the glucan and mannan-synthetases or the mannoproteins were always available for transcription or ii) these RNA messengers, or the glucan and mannan synthetases had a slow decay, or iii) the number of the genes involved is large and scattered throughout the whole cell genome. At the present it is impossible to decide between these hypotheses but it has recently been suggested that the RNA messengers of the acid phosphatase and invertase, two of the catalytic mannoproteins of the wall, have a relatively long life [19]. Similarly we have been able to show by inhibiting protein synthesis with cycloheximide that the glucan and mannan synthetases have also a very low turnover [8].

With this in mind and independently of any control at the transcription level, the existence of RNA messengers and synthetases of low decay would result in the continuous synthesis of both glucan and mannan throughout the whole cell cycle.

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