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A Further Two Mutants Defective in Initiation of the S Phase in the Yeast *Saccharomyces cerevisiae*

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Summary. Two new mutants *dbf3* and *4*, are specifically defective in DNA synthesis. When synchronous cultures of *dbf4* were transferred from the permissive to restrictive temperature before the start of the S phase, no DNA synthesis occurred. However when switched after the beginning of DNA replication, the cells completed that round of synthesis. *Dbf4* therefore resembles *cdc7*, a mutant believed to be required for initiation of DNA synthesis, and indeed *dbf4* acts at a point in the cell cycle close to *cdc7*, namely between *cdc4* and the final requirement for protein synthesis before the S phase. Like *dbf4*, cultures of *dbf3* transferred to restrictive conditions before the start of S showed little DNA synthesis. However a small burst did occur at a time roughly corresponding to when normal initiation would be expected. Cultures switched after this time completed the ongoing round of DNA synthesis.

Introduction

The previous paper (Johnston and Thomas 1982) described the isolation of new DNA synthesis mutants in yeast by the selection of mutants with a cell cycle phenotype. Mutants at four newly identified loci, *dbf1* to *4*, specifically synthesised little or no DNA at 37° C. From an analysis of the kinetics of DNA synthesis in asynchronous cultures of these mutants at the restrictive temperature it was clear that *dbf1* and *2* are involved in chain elongation. However the pattern of residual synthesis shown by *dbf3* and *4* could have resulted from a defect in initiation of DNA synthesis or, for example, from a 'leaky' defect in chain elongation. To distinguish between these possibilities requires the use of synchronised cells and such experiments are described here.

Materials and Methods

Strains and Cultural Conditions. The strains and media used are described in the previous paper. In addition, YM-1 medium was used, consisting of 0.5% Bacto yeast extract, 1% Bacto peptone, 0.67% yeast nitrogen base, 0.6% sodium hydroxide, 1.0% succinic acid, 1% glucose, pH 5.8.

Preparation of α-Hormone. This was prepared by the method of Bucking-Throm et al. (1973).

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Synchronisation of Cells. Cells of *a* mating type grown in YM-1 at 25° C to mid-log phase, were incubated for a time corresponding approximately to one division cycle (4 h) in the presence of α-factor. The hormone was then removed by filtration of the culture, followed by extensive washing of the cells with YM-1. Finally they were resuspended in fresh YM-1 at approximately 5×10^6 cells/ml.

Assay for DNA Synthesis. Cells were grown overnight in YM-1 containing 10 μCi (370 Kbq)/ml ^{3}H-adenine. After synchronisation and resuspension in fresh YM-1 containing ^{3}H-adenine at the same concentration, samples were removed at intervals. Incorporation into DNA was determined as described previously (Johnston and Game 1978).

Results

Synchronisation of Yeast Cells

Cells of the *a* mating type can be readily synchronised by arrest in the Gl phase of the cell cycle with the yeast pheromone α-factor (Bucking-Throm et al. 1973). Thus *dbf3* and *4* were treated with α-factor and the first round of DNA synthesis following removal of the hormone was studied in two ways.

Firstly, to confirm that these mutants were indeed defective in DNA synthesis, cells synchronised at 25° C were transferred to the restrictive temperature before the onset of DNA replication. Under these conditions, any cell which has a defect in either initiation or chain elongation should fail to synthesise any DNA at the restrictive temperature. In the second set of experiments, synchronised cells were transferred to 37° C after the start of DNA replication at the permissive temperature. This should distinguish defects in initiation, when cells should complete ongoing rounds of DNA synthesis, and chain elongation when synthesis should cease abruptly as indeed occurred with *dbf1* and *2* in such experiments (results not shown).

dbf4

When synchronised cells of *dbf4* at 25° C were shifted to 37° C before the onset of DNA replication, essentially no synthesis was observed (Fig. 1A). On the other hand, when cells were transferred in the middle of the S phase they completed the ongoing round of replication (Fig. 1B). These results indicate a specific involvement of *dbf4* in an event required for initiation of DNA replication, a conclu-

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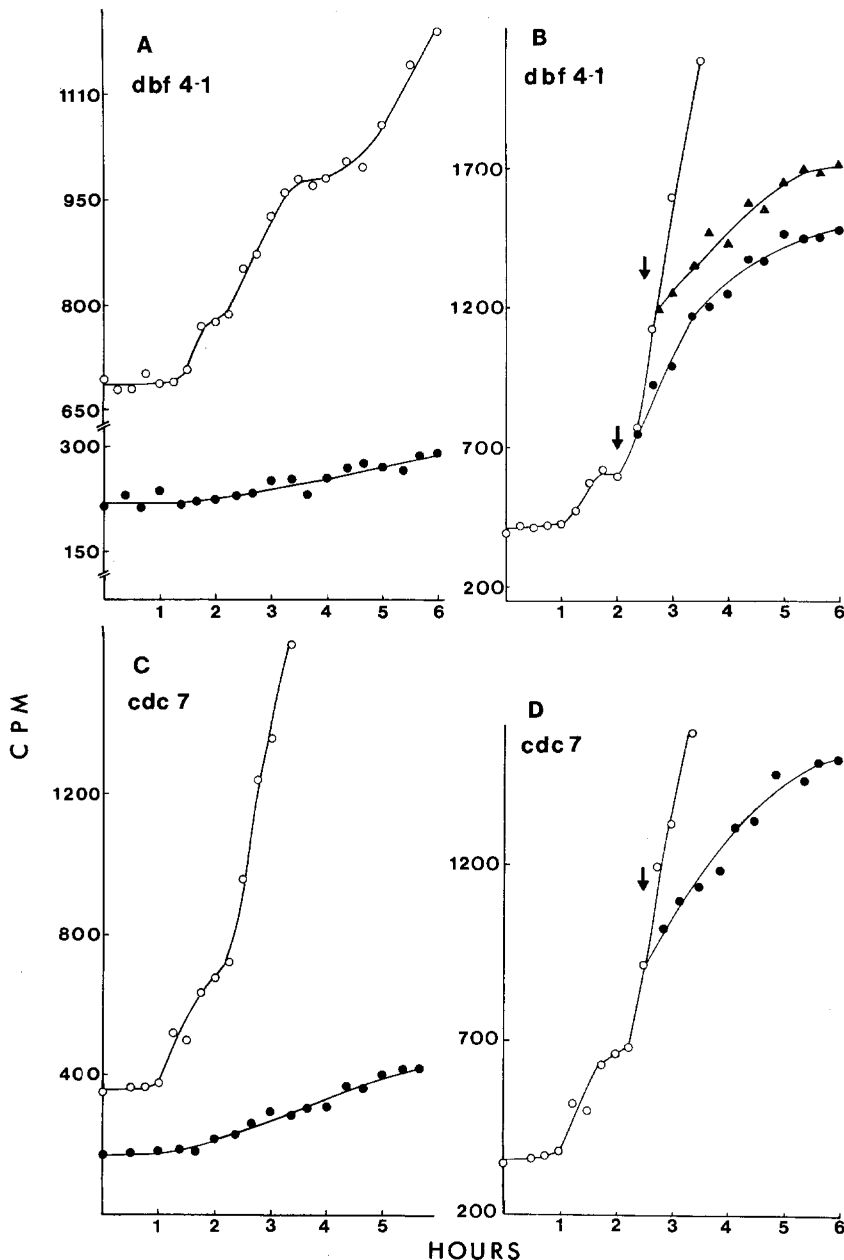

Fig. 1 A–D. Kinetics of DNA synthesis in synchronised cultures of *dbf4-1* and *cdc7*. Mid-log phase cells growing at 25° C in YM-1 containing ^{3}H-adenine were synchronised with α-factor and resuspended in fresh YM-1 containing the same concentration of label. In **A** and **C** the culture was split, one part being incubated at 25° C and the other part at 37° C. In **B** and **D** the cultures were incubated at 25° C and at times (arrowed) after the onset of DNA synthesis, a portion of the culture was transferred to 37° C. The cultures were sampled at intervals and analysed for incorporation of radioactivity into DNA. o—o incorporation at 25° C; ●—●, ▲—▲ incorporation at 37° C

sion which is supported by the similar behaviour of *cdc7* (Fig. 1 C and D), already diagnosed as a probable initiation mutant (Hartwell 1973).

In both mutants a small amount of continuous residual synthesis was observed when cultures were shifted before the onset of DNA replication, which probably represents mitochondrial DNA synthesis (see Discussion).

dbf3

When synchronised cells of *dbf3* were shifted to 37° C before the onset of DNA replication, a distinctive pattern of synthesis was observed. A short burst of incorporation occurred at a point approximately coincident with the initiation of DNA synthesis in a synchronous culture at the permissive temperature (Fig. 2A). This unusual pattern of synthesis was entirely reproducible: it was also observed in two separate experiments using cells derived from crosses of *dbf3* with two different wild type strains.

When cells were shifted to 37° C after the initiation of DNA replication an approximately complete round of synthesis occurred (Fig. 2B). In one experiment, however, a culture switched shortly after the start of DNA synthesis showed an abrupt cessation of replication.

Sequencing dbf4 with Respect to cdc Mutants Blocked in G1

Besides *dbf4* the products of *cdc28*, *4* and *7* are required in that order during G1 prior to the initiation of DNA synthesis (Hereford and Hartwell 1974). Consideration of the terminal cellular morphology of these mutants suggests that *dbf4* must act close to *cdc7*, as of the three mutants it alone forms dumbbells in common with *dbf4*. *Dbf4* therefore presumably acts at a point in G1 between the *cdc4* mediated step and the beginning of the S phase.

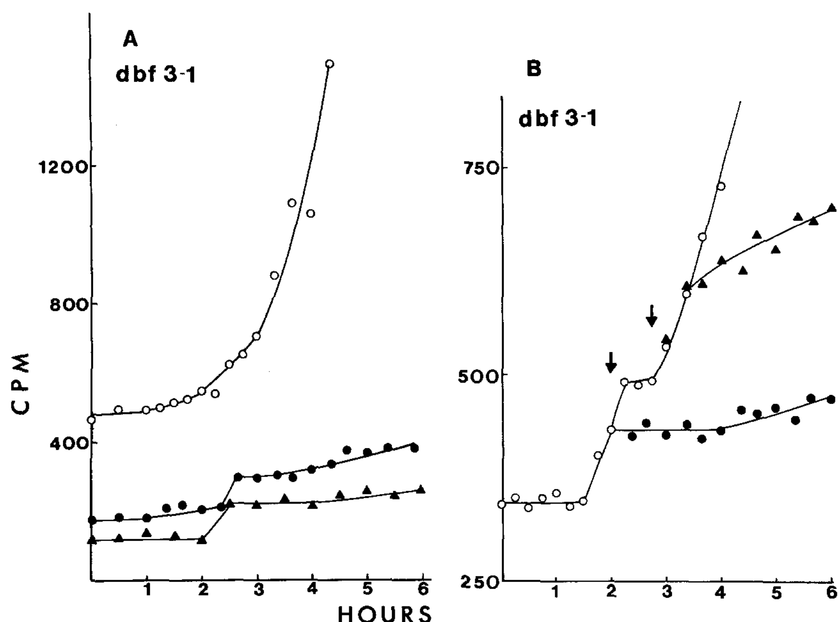

Fig. 2A, B. Kinetics of DNA synthesis in synchronised cultures of *dbf3-1*. Legend as in Fig. 1. The two DNA synthesis curves at 37° C in **A** are from two independent spore clones. o—o incorporation at 35° C; ●—●, ▲—▲ incorporation at 37° C

This can be examined by constructing a double mutant carrying both the *dbf4* and *cdc4* mutations and then determining the terminal cellular morphology formed by this strain at 37° C. Provided that a dependence relationship exists between the two mutants, under these conditions the double mutant should form the terminal phenotype associated with the earliest defect in G1.

Asynchronously growing cells of a *dbf4 cdc4* double mutant were therefore shifted to 37° C and at successive two hour intervals the cellular morphologies were examined microscopically. The majority of cells accummulated with the terminal morphology associated with a lesion in the *cdc4* gene (Table 1). This supports the notion that *dbf4*, like *cdc7* is required after *cdc4*.

Sequencing dbf4 with Respect to the Final Requirement for Protein Synthesis in G1

A further step defined in the G1 phase of the yeast cell cycle is the point at which all the proteins necessary for a round of DNA synthesis have been synthesised (Hereford and Hartwell 1974). This occurs late in G1 prior to the time of action of *cdc7* and *dbf4* has been sequenced with respect to this protein synthesis requirement.

Cells of *dbf4* were synchronised by α-factor at 25° C and after its removal, they were incubated for two hours at 37° C to align them at their temperature-sensitive block. The culture was then split three ways. One portion was left at 37° C and, as expected, no DNA synthesis occurred in this culture (see Fig. 1A). The other two cultures were shifted down to 25° C and to one the protein synthesis inhibitor, cycloheximide, was added. DNA replication was clearly not initiated in the absence of protein synthesis (Fig. 3). Thus *dbf4* acts after *cdc4* but before the final requirement for protein synthesis.

Discussion

This paper describes another two genes required for the initiation of the S phase in yeast. *Dbf4* resembles *cdc7* in that cells transferred to the restrictive temperature after the initiation of S are able to synthesise an approximately complete round of DNA, while a culture transferred before S fails to synthesise any nuclear DNA. The slight residual synthesis in both *dbf4* and *cdc7* under these circumstances propably represents mitochondrial DNA synthesis which is continuous throughout the yeast cell cycle (Williamson and Moustacchi 1971) and is unaffected by mutants blocked in the G1 phase of the cell cycle (Newlon and Fangman 1975).

In *dbf3* on the other hand, cultures transferred before the onset of S do show a small burst of synthesis, roughly coincident with the initiation of DNA synthesis in a synchronised culture at the permissive temperature. This is unlikely to be mitochondrial DNA synthesis for the reasons outlined above and is also unlikely to represent replication of the yeast plasmid, which occurs early in S, as this species comprises only some 2% of the total cellular DNA. This synthesis presumably, therefore, represents nuclear DNA.

Table 1. Terminal cellular morphology of the *cdc4 dbf4* double mutant at 37° C

Mutant	Time at 37° C (h)	A[a]	B	C	D
cdc4	0	52.0[b]	33.7	14.0	0
	2	34.7	25.7	24.5	14.9
	4	12.7	2.3	1.5	83.3
dbf4	0	47.7	33.5	18.7	0
	2	39.1	31.7	29.6	0
	4	33.8	4.8	61.0	0
cdc4 dbf4	0	36.3	51.7	11.7	0
	2	34.7	27.7	15.6	21.0
	4	9.0	6.5	7.8	76.6

[a] A – unbudded cells; B – cells with small buds; C – cells with large buds; D – cells with elongated buds
[b] Each figure represents the % of the total number of cells scored with phenotype A, B, C or D

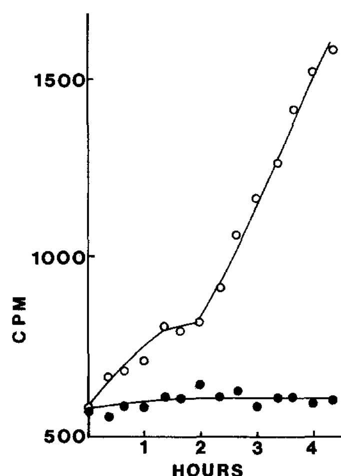

Fig. 3. Kinetics of DNA synthesis of *dbf4* in the presence of cycloheximide. Cells were synchronised with α-factor at 25° C and after its removal they were incubated at 37° C for 2 h and then transferred back to 25° C in the presence (●—●) or absence (o—o) of 100 μg/ml cycloheximide

This apparent early block in DNA synthesis may not necessarily be the result of a mutation directly in replication. Some events of the cell cycle form a dependent sequence and *dbf3* may therefore be defective in an event whose failure prevents replication as a secondary consequence, albeit shortly after synthesis has started. However, it is more probable that, for economic reasons, any regulatory effect on DNA synthesis would be at the beginning of the S phase and *dbf3* is therefore more likely to be defective in DNA synthesis itself.

However the exact nature of the defect remains unclear. Conceivably, *dfb3* may allow the initiation and completion of a small subset of replicons or, alternatively, all replicons may initiate and synthesise a small amount of DNA. This may occur if, for example, the mutant was defective in an enzyme involved in topological aspects of DNA replication such as a topoisomerase. Interestingly, in one experiment a culture transferred after the onset of DNA synthesis showed an immediate cessation of replication. This is rather difficult to interpret, however, since the cessation was abrupt and it would be expected to be more gradual, given the degree of asynchrony in α-hormone treated cells. In other cultures transferred after the beginning of S, *dbf3* resembled *dbf4* in that a more or less complete round of synthesis occurred.

The isolation of these two mutants brings to six the number of known steps which must be successfully completed prior to the initiation of DNA replication in *S. cerevisiae*. Of the four previously established steps (Hereford and Hartwell 1974), three are genetically defined, *cdc28*, *4* and *7*, and these function in that order during G1. The fourth step, located between *cdc4* and *7* is the point at which all proteins necessary for a round of DNA synthesis have been synthesised. This present study placed the site of action of the *dbf4* gene product after the *cdc4* step but before the final requirement for protein synthesis. As none of these mutants allows the initiation of S and since in *dbf3* a small amount of DNA synthesis occurred, it presumably acts after the other four mutants in the cell cycle.

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