REPLICATION OF THE NUCLEAR GENOME IN YEAST DOES NOT REQUIRE CONCOMITANT PROTEIN SYNTHESIS

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SUMMARY

Incorporation of ^{3}H-adenine into nuclear DNA in a short pulse in mid-S in a synchronised culture of Saccharomyces cerevisiae was unaffected by the presence of 100 µg/ml cycloheximide. However, colorimetric DNA analyses showed that entry into S was completely blocked by adding the drug at times earlier than about 10 min before initiation of replication. Cell autoradiography of cultures labelled in various regimes showed that at this time there is a cycloheximide-transition point at which the cell acquires the capacity to both initiate and complete a whole round of replication in the presence of 100 µg/ml of cycloheximide. Thus, all the proteins required for passage through one S period are made in advance of initiation.

Initiation of replication of the bacterial genome requires previous synthesis of proteins but, once in progress, a round of replication can be completed even in conditions which preclude further protein synthesis (1, 2).

The situation in eukaryotes is more complex. On the one hand, mitochondrial DNA (mitDNA) has been shown (at any rate in yeast) to be capable of replication for several generations in the absence of either cytoplasmic or mitochondrial protein synthesis (3, 4). In contrast, replication of eukaryotic nuclear DNA appears markedly sensitive to inhibition of protein synthesis by agents such as cycloheximide (CHI) or puromycin. There is mounting evidence that in many eukaryotes these inhibitors affect progress through the S period as well as the initiation of DNA replication (5 - 15). However, observations on the slime mould Physarum polycephalum (16, 17) are of particular interest. They showed that synthesis of the nuclear genome in this organism (which has 40 - 50

chromosomes) entails sequential replication of 10 "replicative units". Initiation of replication of each unit is sensitive to CHI, but once in progress, replication of a unit can be finished in the presence of the drug. Thus although the gross effect of adding CHI to cells engaged in replication is to halt their progress through S, it seems that this does not reflect a need for continuing protein synthesis for movement of the replicating fork along a stretch of DNA. Rather it stems from the way the replication of the chromosomes is organised and the fact that several initiations occur and are spread throughout the S period.

The results reported here show that the nuclear genome of the yeast Saccharomyces cerevisiae is organised in a much simpler manner. In this case the entire genome (about 17 small chromosomes (18, 19)) comprises a single replicative unit, all the proteins required for one complete round of replication being made shortly before the initiation of replication.

EXPERIMENTAL

Synchronously dividing cultures of Sacc. cerevisiae, strain NCYC 239 (20) were aerated at 25C in a semi-defined medium (21). Total DNA in culture samples was determined colorimetrically using a Schmidt/Tannhauser procedure (22). The specific activity of nuclear DNA from cells incubated in the presence of adenine-8-^{3}H (Amersham, 23 - 28C/mMole) was determined by extraction of the DNA (23) and isopycnic banding in CsCl/Distamycin gradients (24) to separate nuclear and mitochondrial components. The total DNA content of selected fractions containing either nuclear or mitochondrial DNA was estimated by analytical CsCl banding with a known amount of reference DNA (23) while the radioactive DNA content of the fractions was determined by a conventional alkaline digestion/acid precipitation routine using bovine serum albumen as carrier, and filtration on cellulose acetate membranes (Oxoid). Incorporation of ^{3}H-adenine into

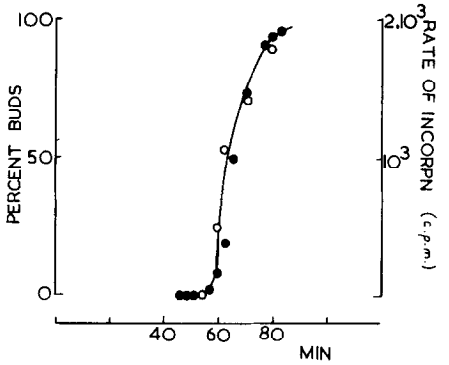
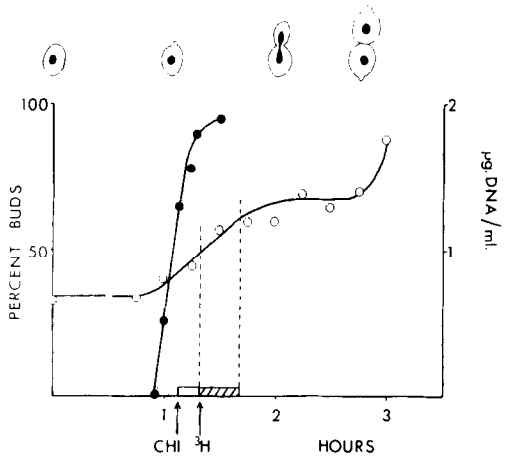


Fig. 1. Initiation of DNA replication in relation to bud formation in a synchronised culture. The culture was inoculated with ca 10^7 cells/ml and the emergence of buds followed microscopically. At intervals, 2 ml samples were labelled for 3 min with 5 µC/ml ^{3}H-adenine. The pulses were terminated by transferring the cells into ice-cold 0.2M perchloric acid, and the samples treated so as to remove non-DNA label (22). Aliquots of the samples were digested with DNAse (22) and the radioactivity present before and after digestion measured by extraction in 10% trichloroacetic acid for 20 min at 100 C, followed by scintillation counting. ●—● , percent young buds; O—O, radioactivity incorporated into DNA (corrected for DNAse-resistant background).

DNA in individual cells from cultures labelled for various periods was determined by cell autoradiography (22). Some of the grain counts were made by silver reflectance using a Leitz MPV microscope equipped for grain counting.

RESULTS AND DISCUSSION

The S period in the individual cell of this organism lasts about 30 minutes at 25C (22). In the synchronised system used here, nuclear DNA replication starts about 60 min after inoculation and is completed by about 150 min. By measuring the rate of incorporation of ^{3}H-adenine into DNA in short (3 min) pulses it was shown (Fig. 1) that the initial emergence of the bud in the synchronised individual cell coincides accurately with, and can therefore be used as a convenient indication of, the initiation of nuclear DNA replication. Figure 2 illustrates the effect of prior addition of CHI (100 µg/ml) on incorporation of ^{3}H-adenine into nuclear DNA in a 20 min pulse in the middle of the culture's S period.



	³H alone	CHI, ³H
specific activity nuclear DNA (c.p.m./µg.)	18.5×10^3	19.7×10^3

Fig. 2. Effect of CHI (100 µg/ml) on incorporation of ^{3}H-adenine into a synchronous culture in mid-S. ●—● , percent young buds (i.e., percent cells initiated S); ○—○, total DNA/ml culture. The essential morphological changes in this type of culture are illustrated diagrammatically. The interval between inoculation and the initial round of budding is referred to as a lag period (effectively G_0), which does not recur. The first cell cycle proper starts with S as the first buds appear, and ends around 3 hours when cell division occurs and the next S period starts. The bars indicate the periods for which separate portions of the culture were exposed to CHI followed by ^{3}H-adenine, or ^{3}H-adenine alone.

This concentration of CHI prevents cell division almost immediately and completely blocks incorporation of ^{35}S-methionine into protein less than 5 minutes after addition (25). As shown in Fig. 2, exposure to CHI about 10 minutes before the pulse was almost without effect on the specific activity of the nuclear DNA, that obtained in the presence of CHI being in fact about 7% greater than in its absence. The budding data shows that the majority of the cells had already initiated DNA replication by the time the inhibitor was added. It is concluded, therefore, that the propagation of replication in cells already initiated is essentially unaffected by complete inhibition of concomitant protein synthesis.

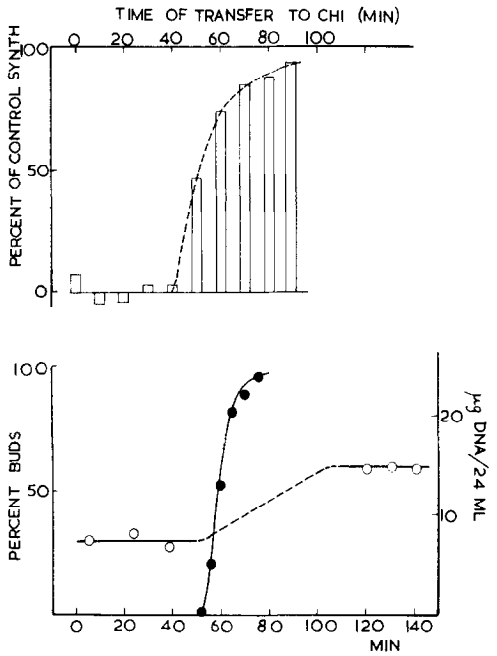


Fig. 3. The effect on DNA synthesis of exposing synchronous cells to CHI (100 μg/ml) at different times during the initial lag phase and first S period. ●—● , percent buds (initiations) in the master culture; ○—○ , total DNA per 24 ml culture (the broken line indicates the usual time course of DNA synthesis in this system). The histogram bars show the amounts of DNA synthesised is separate portions of the culture transferred to CHI at the indicated times and incubated to 140 min. The ordinate in this case is the amount of DNA synthesised as a percentage of the amount synthesised in the master culture in the course of the S period.

Figure 3 illustrates the effect of adding CHI at different times to a synchronised culture. The initiation of DNA synthesis in an untreated control culture was followed by counting emerging buds, and the total amount of DNA synthesised during one S period was assessed by colorimetric analyses on samples before and after S. The time course of the synthesis was not followed in detail but the usual time course in this type of culture is highly reproducible and is indicated in the Figure. At various intervals during the lag phase and early S period, separate portions of the culture were withdrawn and incubated with 100 μg/ml CHI until 140 min. These cultures were then analysed for total DNA and the amounts each one synthesised are recorded in Fig. 3 as per-

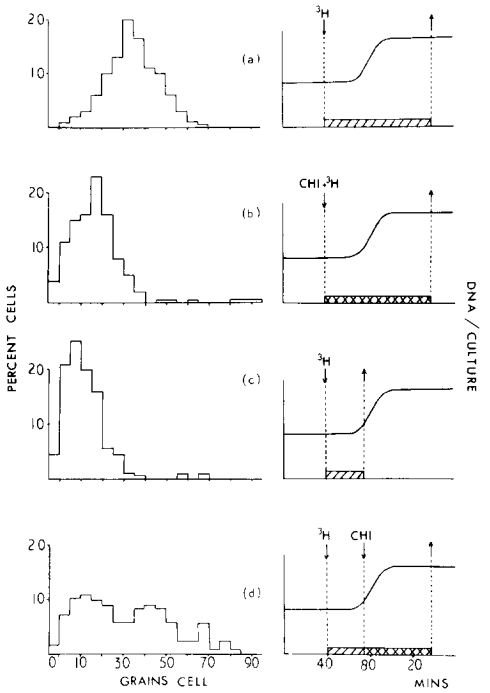


Fig. 4. Frequency distributions of DNA grain counts over individual cells following the various labelling routines illustrated diagrammatically on the right. Approximately 200 cells were counted in each preparation.

centages of the total amount synthesised in the control. Although some mitDNA is synthesised in lag and S phase cells (see below and (26)) the amount synthesised is too small to be detected by ordinary colorimetric analyses for total DNA, and the data in Fig. 3 are to be interpreted in terms of nuclear DNA synthesis only.

It will be seen that the first cells to enter the S period initiated replication at about 50 min, and 90% of the population had initiated by about 75 min. Culture samples transferred to CHI at times up to and including 40 min failed to make any DNA at all, and it is concluded that early synthesis of some proteins is required for entry into S to occur. However, transfer into CHI at 50 min permitted the synthesis of 47% of the amount of DNA made in the control. Since no initiations had taken place in this sample before exposure to the drug, it is clear that between 40

and 50 min, at least some of the cells in the culture had become 'resistant' to CHl in the sense of being able to initiate DNA in the presence of the drug. With the results of Fig. 2 in mind, it seemed possible that these individuals would be able to complete a round of replication whilst their fellows who had not yet acquired this 'resistance' to CHl would be unable to enter S at all. The correctness of this view was confirmed by an autoradiographic analyses of incorporation at the cellular level in a CHl-treated culture. The protocol of the experiment and the results are illustrated in Fig. 4.

A synchronous culture had 50 µC/ml ^{3}H-adenine added at 40 min, i.e., prior to the start of S. Initiation started in this particular culture at 60-65 min and was 80% complete by 95 min. The culture was sampled at 75 min (when about a quarter of the cells had initiated) and again at 140 min (after completion of replication). In addition, separate portions of the labelled culture were taken at 40 and 75 min and incubated in the presence of 100 µg/ml CHl until 140 min. All samples were then examined by cell autoradiography.

Labelling for one complete round of replication (40-140 min) in the absence of CHl gave a uni-modal, symmetrical frequency distribution of grain counts with an average of around 35 grains per cell (Fig. 4a). However, if CHl was present during this period, the average incorporation amounted to only about 15 grains per cell (Fig. 4b), and separate measurements of the specific activities of the mitochondrial and nuclear DNAs of cells labelled in this way showed that more than 92% of the grains recorded in Fig. 4b were due to label in the mitochondrial component. The distribution in Fig. 4b thus represents the incorporation occurring in cells whose entry into S was blocked by the presence of CHl.

The distribution in Fig. 4c shows the result of labelling from 40-75 min without CHl. Since overall replication of DNA had started around 65

min, the incorporation in this case must have been partly due to nuclear synthesis. However, there was an average of only 10 grains/cell, and comparison with the fully labelled control cells (Fig. 4a) shows that few, if any, cells had completed S by 75 min. However, when CHl was added at 75 min and incubation continued to 140 min, the bi-modal distribution shown in Fig. 4d was obtained, with modes around 12 and 42 grains/cell. Since the more radioactive cells were not present when CHl was added at 75 min, their label must have resulted largely from incorporation in the presence of the drug. Moreover, the final extent of their labelling was comparable with that in the fully replicated control (Fig. 4a) which implies that these cells had in fact gone on to complete a round of replication in the presence of the drug. The less heavily labelled group, on the other hand, probably did not enter S at all, for their radioactivity was comparable with that of the cells in Fig. 4b and was therefore presumably attributable to mitDNA.

The simplest interpretation of these results is that there exists a sharp CHl-transition point in the cycle of the individual cell, rather less than 10 min before the initiation of nuclear DNA replication, at which the cell acquires the capacity to initiate in the presence of 100 µg/ml CHl. Once it has passed the transition point, moreover, the whole genome can be replicated in the presence of the drug. In this light the data in the upper portion of Fig. 3, showing the amounts of DNA synthesised in cells transferred into CHl at different times, is interpreted as showing the passage of the culture through the transition point. It is entirely consistent with this view that the curve denoting this transition point runs approximately parallel to the curve for initiation rather than to the time course of DNA synthesis. It may also be noted that in more detailed experiments to be reported elsewhere, the proportions of cells in the heavily and lightly labelled groups of Fig. 4d varied with time of transfer to CHl in exactly the way that would be predicted by this model; the bi-modal

nature of the distribution remained apparent, but the proportion of cells in the more heavily labelled group increased in accord with passage of the culture through the transition point.

The nature of the transition point is not known. It should be mentioned, however, that an exactly similar transition point has been observed about 10 min before the next S period, so it is not therefore a feature only of the initial lag phase. The most likely interpretation is that it represents a point in the cell cycle by which all the proteins required for one complete round of replication have been made. Its existence does not preclude the possibility of multiple initiation events or sites in the yeast genome. However, our observations certainly suggest that replication of the chromosomes in this organism is controlled in a different and possibly less complex manner from that of other eukaryotes.

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