De novo synthesis of budding yeast DNA polymerase α and POL1 transcription at the G1/S boundary are not required for entrance into S phase

(Saccharomyces cerevisiae/transcriptional control/cell cycle)

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ABSTRACT The POL1 gene, encoding DNA polymerase α (pola) in Saccharomyces cerevisiae, is transiently transcribed during the cell cycle at the G1/S phase boundary. Here we show that yeast pola is present at every stage of the cell cycle, and its level only slightly increases following the peak of POL1 transcription. POL1 mRNA synthesis driven by a GAL1 promoter can be completely abolished without affecting the growth rate of logarithmically growing yeast cultures for several cell divisions, although the amount of the pola polypeptide drops below the physiological level. Moreover, α-factor-arrested cells can enter S phase and divide synchronously even if POL1 transcription is abolished. These results indicate that the level of yeast pola is not rate limiting and de novo synthesis of the enzyme is not required for entrance into S phase.

Eukaryotic DNA polymerase α (pola), together with the tightly bound DNA primase, plays an essential role in lagging strand synthesis and initiation of DNA replication at an origin (1–3). The gene encoding pola in Saccharomyces cerevisiae (POL1) is transiently transcribed during the cell cycle at the G1/S phase boundary (4) concomitantly with several DNA synthesis genes (for a review, see refs. 5 and 6). A conserved promoter sequence, the Mlu I cell cycle box (MCB), mediates this transcriptional control and is present twice in the POL1 gene (7–9). One component of the transcription factor(s) that binds to the MCB is the SWI6 gene product, whose function is relevant for cell cycle-dependent transcription of yeast DNA synthesis genes (10–12). However, SWI6 deletion is not lethal but leads to deregulated constitutive transcription of these genes (10, 11). Moreover, it has been shown that the level of proteins required for Saccharomyces cerevisiae DNA replication (replication factor A) or for entrance into S phase (CDC46 gene product) does not show any large fluctuation during the cell cycle (13, 14), although the transcription of the corresponding genes is clearly periodic (14, 15). A nearly constant amount of essential replication proteins has been observed also in actively cycling cells from other eukaryotes, including mammalian pola, RF-A, proliferating cell nuclear antigen (PCNA), DNA ligase (13, 16–19), and Schizosaccharomyces pombe pola, PCNA, and DNA ligase (20–22). However, the transcription of the corresponding genes in these organisms does not appear to be cell cycle regulated. These findings leave uncertain as to whether the transcriptional activation of DNA replication genes observed in Saccharomyces cerevisiae is required for the onset of DNA replication in S phase. Our goal in the present work was to establish whether the amount of pola in Saccharomyces cerevisiae is rate limiting and periodic transcription of the POL1 gene is necessary for entrance into S phase. We show that yeast pola is present at every stage of the cell cycle and its level only slightly increases following the peak of POL1 mRNA at the G1/S phase boundary. To monitor the requirement for de novo synthesis of pola, we fused the POL1 gene to the repressible GAL1 promoter (23) and blocked POL1 mRNA synthesis in asynchronously and synchronously growing cells containing this construct as the only source of the enzyme. We observed that even when POL1 transcription is abolished in G1 and the amount of pola drops below the physiological level, cells can still undergo several division cycles. These findings indicate that pola remains functional when inherited by daughter cells, and de novo synthesis of the enzyme is not essential for initiation of DNA replication within the same cell cycle.

MATERIALS AND METHODS

Strains, Plasmids, Media, and Yeast Cell Synchronization. Cultures of Saccharomyces cerevisiae strain CG378 (MATα ade5 leu2-3,112 trp1-289 ura3-52) (24), grown in synthetic medium (25) supplemented with the required nutrients, were synchronized by α-factor treatment as described (8). The CG378ΔPOL1[pAP415] strain has been constructed by replacing the POL1 chromosomal copy (25) with a null pola allele carrying a deletion of the 2325-bp Hpa I fragment internal to the POL1 coding region (7) in strain CG378[pAP415]. Plasmid pAP415 is an ARSI TRP1 CEN6 plasmid carrying a POL1 fragment spanning position −589 to position +496 and containing the entire POL1 promoter (8). Plasmid pMA2 was constructed by cloning a POL1 fragment, spanning position −8 to position +496 with respect to the translation initiation codon, into the BamHI site of the centromeric plasmid pBM125 (carrying the URA3 selectable marker) (23), downstream of the GAL1 promoter. Strain CG378ΔPOL1[pMA2] [pAP415] has been constructed by transforming strain CG378ΔPOL1[pAP415] with plasmid pMA2 and strain CG378ΔPOL1[pMA2] was obtained from the previous strain by standard plasmid shuffling procedures (25). These strains have been grown under induced (galactose-containing medium) or repressed (glucose-containing medium) conditions as detailed in the legends to Figs. 2 and 3.

Preparation of Yeast Extracts and Western Blotting Analysis. Total protein extracts were usually prepared from 4 × 10⁸ cells collected at different times from logarithmically or synchronously growing yeast cultures. Cells were washed with 20% trichloroacetic acid (TCA) in order to prevent proteolysis and resuspended in 200 μl of 20% TCA at room temperature. The protein concentration was determined by the method of Lowry et al. (10). The gel was prepared in the presence of 2% SDS to avoid the overdigestion of the proteins. The pola antibody was a kind gift of M. Foiani. The pola antibody was affinity purified on a pola column (5) and was affinity purified on a pola column (5) and was used at a dilution of 1:2000.

The abbreviations used are: pola, polymerase α; mAb, monoclonal antibody; DAPI, 4′-6-diamidino-2-phenylindole.‡ To whom reprint requests should be addressed.
temperature. After addition of the same volume of glass beads, cells were disrupted by spinning in a Vortex for 2 min. Glass beads were washed twice with 200 μl of 5% TCA and the resulting extract was spun for 10 min at 3000 rpm in a Microfuge at room temperature. The pellet was resuspended in 200 μl of Laemmli buffer (26), neutralized by adding 100 μl of 1 M Tris base, boiled for 3 min, and finally clarified by centrifugation as described above. Aliquots (25 μg) of the extract, as determined by a Bio-Rad protein assay (26), were analyzed by polyacrylamide gel electrophoresis in the presence of SDS, and the detection of the immunoreactive polypeptides was carried out as described (27). Anti-polα monoclonal antibodies (mAbs) (28) isolated from ascites fluids were used as the primary antibodies at a 1:2000 dilution.

Miscellaneous Procedures. Extraction of total yeast RNA, Northern blotting analysis, and DNA probes used to monitor the POLI, PRI2, and H2A transcripts have been described (8). The nuclear localization of polα was determined by in situ indirect immunofluorescence (29) using anti-polα mAb (24) as the primary antibody and a rhodamine-conjugated goat anti-mouse IgG (Sigma) as the secondary antibody. The DNA binding dye 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize the nucleus (29).

RESULTS

Level of polα in Synchronous Yeast Cultures. Yeast polα is a 180-kDa polypeptide (p180) tightly bound to DNA primase in a four-subunit complex (28, 30). Because p180 is highly susceptible to proteolysis (28), we monitored the level of this polypeptide by Western blot analysis (Fig. 1b) on crude extracts prepared to minimize proteolysis from α-factor-synchronized yeast cultures (Fig. 1a). The p180 polα polypeptide was present at every stage of the cell cycle and its level almost doubled following the increase of the POLI transcript (Fig. 1b: time points, 170–190 and 240–260 min). By densitometric scanning, we calculated that the increase in the level of p180 at the time points indicated, compared to that found in the preceding samples (time points, 140–160 and 210–230 min), was 1.68-fold and 1.48-fold, respectively. A similar result was observed by assaying polα activity during the cell cycle in immunoprecipitates obtained with nonneutralizing anti-polα mAbs and by testing an in-frame POLI–lacZ fusion for β-galactosidase activity (data not shown). Therefore, the 30-fold increase of POLI mRNA level at the G1/S transition (Fig. 1c) results, at the most, in a doubling of the amount of the corresponding gene product. The highest p180 level was found to be coincident with the periodic increase of histone H2A mRNA in S phase (Fig. 1c: time points, 160–180 min; ref. 22). Although the fluctuation of the POLI mRNA is typical of highly unstable regulated transcripts, the polα polypeptide is quite stable. Consistent with this observation, the half-life of p180 determined by pulse-chase experiments is >4 hr (unpublished observation). Similarly, other yeast DNA replication factors are stable proteins even if the level of the corresponding transcripts fluctuates periodically during the cell cycle (13, 14). These data raise the questions as to whether the amount of replication proteins is limiting and if their synthesis is required for the next round of DNA replication.

The Level of Yeast polα Is Higher than That Required for a Single Cell Generation, and the Enzyme Is Functional when Inherited by Daughter Cells. To test whether de novo synthesis of polα is necessary to support cell division, we used the haploid strain CG378ΔPOL1[pMA2], carrying a lethal deletion of the POLI chromosomal locus and the POLI gene under the control of the GAL1 promoter on a centromeric

Fig. 1. The level of the polα polypeptide increases nearly 2-fold following the periodic increase in the level of the POLI transcript at the G1/S boundary. A logarithmically growing culture (7 × 10⁶ cells per ml) of strain CG378 was synchronized by α-factor treatment. Times of addition and removal of α-factor are indicated by arrows, and samples were taken at the indicated times. (a) Budding profile. (b) Western blot probed with anti-polα mAbs. Each lane contained 25 μg of total protein extracted from cells at the indicated times. (c) Five micrograms of total RNA per lane was used to monitor the fluctuation of POLI, PRI2, and H2A histone gene transcripts during the cell cycle (8) by Northern blot analysis. The constitutively expressed calmodulin mRNA (31) was used as a loading control.
DISCUSSION

DNA replication is coupled to cell cycle progression, but the molecular mechanisms turning replication on and off and preventing rereplication within the same cell cycle are still poorly understood (for a recent review, see ref. 33).

The coordinated periodic transcription of DNA synthesis genes in budding yeast has been generally interpreted as a

(sample 1 in b) was determined by in situ indirect immunofluorescence. DAPI was used to visualize the nucleus in the same cells.
means to provide a timely supply of the necessary proteins (6, 34). However, in other eukaryotic organisms, the transcription of DNA replication genes is not so severely controlled during the cell cycle (3, 16, 18, 22). Our finding that polα is a stable protein and that the G1/S increase of POL1 mRNA results, at the most, in a doubling of the amount of protein, is in agreement with that observed for other DNA replication enzymes, both in yeast and higher eukaryotic cells (13, 16–22). Moreover, the effect of blocking POL1 transcription in logarithmically and synchronously growing cells clearly indicates that polα is present in an excess amount in yeast cells, and it can be inherited and used by daughter cells to proceed through S phase even in the absence of a pool of newly synthesized enzyme. Therefore, the onset of DNA synthesis is not coupled to the transcriptional activation of the POL1 gene at the G1/S phase boundary. Periodic transcription might still be relevant to provide a timely supply of limiting or labile key protein factors required to enter S phase (35–38). Moreover, coordinated activation of the DNA synthesis genes in budding yeast might represent a fine-tuning control mechanism that optimizes the efficiency of S phase rather than its timing (33, 34). Such a mechanism likely represents a selective advantage for a totipotent, rapidly growing unicellular eukaryote.

On the other hand, the in vivo function of stable DNA replication proteins might be modulated by several regulatory mechanisms, including posttranslational modifications carried out by one of the numerous kinases or phosphatases found in eukaryotic cells (39, 40), or association with some key regulatory factors. Our recent finding that the p86 protein, which is tightly bound to the p180 pola polypeptide (30, 41), is posttranslationally modified in a cell cycle-dependent manner (unpublished data) is in agreement with this hypothesis. Moreover, polα and the p86 homolog in human cells have been found to be hyperphosphorylated at G2/M (42). Our observation that polα can be inherited from mother to daughter cells supports the possibility that regulatory mechanisms possibly controlling entrance into S phase might not necessarily be restricted to the G1/S phase transition. In this view, the switches controlling entrance into the S phase might be linked not only to passage through "start" or the restriction point in G1 (33) but also to other cell cycle events, such as mitosis. This mechanism would provide the cell with a larger time interval to modulate the activity of DNA synthesis factors, their intracellular localization, or proper assembly. This hypothesis is in agreement with the implications of the "licensing factor" model (43), suggesting that nuclear membrane breakdown is required for entrance into the nucleus of a positive inducer of DNA replication synthesized during the previous cell cycle, thus coupling mitotic events with initiation of DNA replication.

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Biochemistry. In the article "De novo synthesis of budding yeast DNA polymerase α and POL1 transcription at the G1/S boundary are not required for entrance into S phase" by Marco Muzi Falconi, Anna Piseri, Marina Ferrari, Giovanna Lucchini, Paolo Plevani, and Marco Foiani, which appeared in number 22, November 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 10519–10523), the following correction should be noted. Due to an error in the Proceedings office, one of the authors indicated for reprint requests was incorrect. Reprint requests should be addressed to Marco Foiani or Paolo Plevani.

Immunology. In the article "Prevention of experimental autoimmune myasthenia gravis by manipulation of the immune network with a complementary peptide for the acetylcholine receptor" by Shigeru Araga, Robert D. LeBoeuf, and J. Edwin Blalock, which appeared in number 18, September 15, 1993, of Proc. Natl. Acad. Sci. USA (90, 8747–8751), the authors request that the following correction be noted. The monoclonal antibody, TCM 240, was incorrectly reported to be the IgG2b/κ isotype. The correct isotype is IgG1/κ. This error in no way alters the conclusions or results of experiments performed with TCM 240.