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De novo synthesis of budding yeast DNA polymerase α and *POL1* transcription at the G_1/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 (polα) in *Saccharomyces cerevisiae*, is transiently transcribed during the cell cycle at the G_1/S phase boundary. Here we show that yeast polα 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 polα 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 polα is not rate limiting and *de novo* synthesis of the enzyme is not required for entrance into S phase.**

Eukaryotic DNA polymerase α (polα), 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 polα in *Saccharomyces cerevisiae* (*POL1*) is transiently transcribed during the cell cycle at the G_1/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 polα, RF-A, proliferating cell nuclear antigen (PCNA), DNA ligase (13, 16–19), and *Schizosaccharomyces pombe* polα, 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 polα in *Saccharomyces cerevisiae* is rate limiting and periodic transcription of the *POL1* gene is necessary for entrance into S phase. We show that yeast polα is present at every stage of the cell cycle and its level only slightly increases following the peak of *POL1* mRNA at the G_1/S phase boundary. To monitor the requirement for *de novo* synthesis of polα, 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 G_1 and the amount of polα drops below the physiological level, cells can still undergo several division cycles. These findings indicate that polα 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 (*MATa 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 *pol1* 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 *ARS1 TRP1 CEN6* plasmid carrying a *POL1* fragment spanning position −589 to position +4946 and containing the entire *POL1* promoter (8). Plasmid pMA2 was constructed by cloning a *POL1* fragment, spanning position −8 to position +4946 with respect to the translation initiation codon, into the *Bam*HI 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 4×10^8 cells collected at different times from logarithmically or synchronously growing yeast cultures. Cells were washed with 20% trichloracetic acid (TCA) in order to prevent proteolysis and resuspended in 200 μl of 20% TCA at room

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Abbreviations: polα, polymerase α; mAb, monoclonal antibody; DAPI, 4′,6-diamidino-2-phenylindole.

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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 *POL1*, *PRI2*, and H2A transcripts have been described (8). The nuclear localization of polα was determined by *in situ* indirect immunofluorescence (29) using anti-yeast 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. 1*b*) on crude extracts prepared to minimize proteolysis from α-factor-synchronized yeast cultures (Fig. 1*a*). The p180 polα polypeptide was present at every stage of the cell cycle and its level almost doubled following the increase of the *POL1* transcript (Fig. 1*b*: 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 *POL1–lacZ* fusion for β-galactosidase activity (data not shown). Therefore, the 30-fold increase of *POL1* mRNA level at the G_1/S transition (Fig. 1*c*) 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. 1*c*: time points, 160–180 min; ref. 22). Although the fluctuation of the *POL1* 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 *POL1* chromosomal locus and the *POL1* gene under the control of the *GAL1* promoter on a centromeric

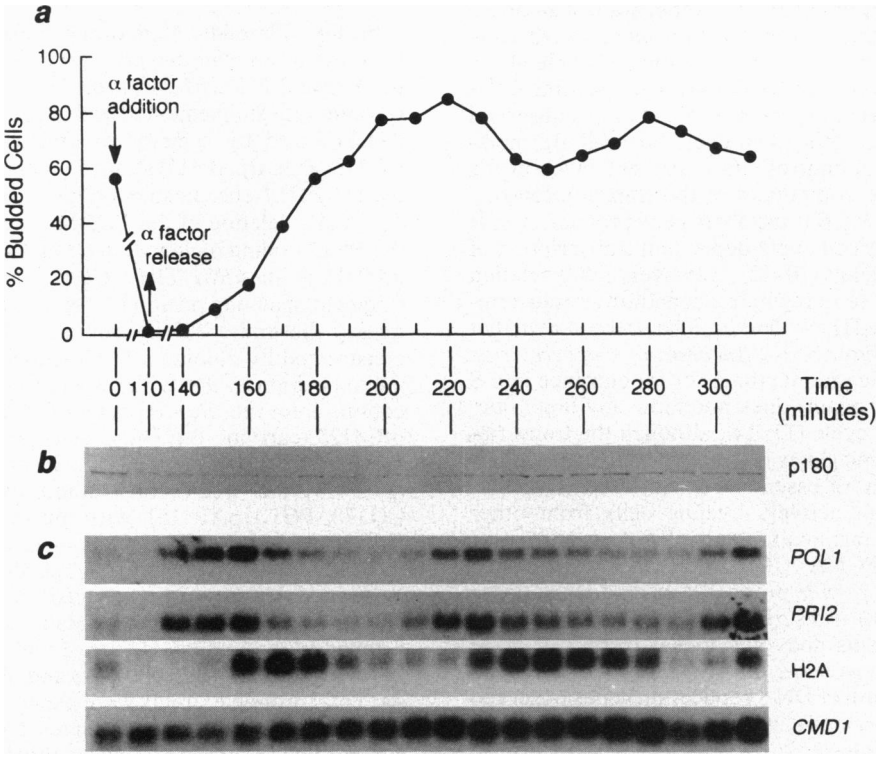


FIG. 1. The level of the polα polypeptide increases nearly 2-fold following the periodic increase in the level of the *POL1* transcript at the G_1/S boundary. A logarithmically growing culture (7×10^6 cells per ml) of strain CG378 was synchronized by α-factor treatment. Times of addition and removal 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 *POL1*, *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.

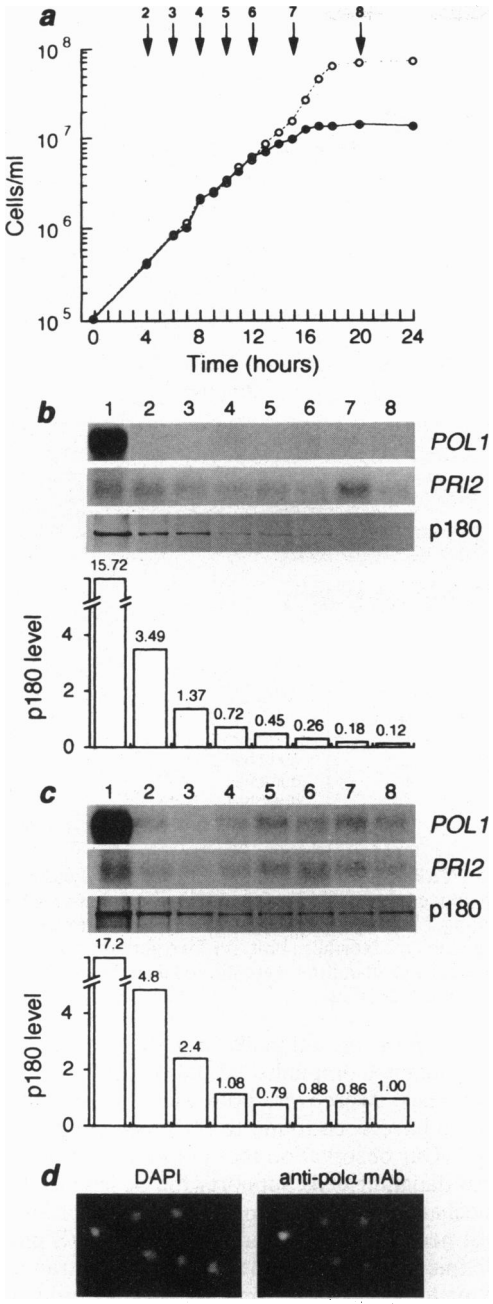


FIG. 2. Expression of *POL1* driven by the *GAL1* promoter gives rise to a functional polα polypeptide that can be used for several cell divisions. (*a*) Growth rate of strains CG378ΔPOL1[pMA2] (●) and CG378ΔPOL1[pMA2][pAP415] (○) after shift from induced (galactose-containing medium) to repressed (glucose-containing medium) conditions. Cells of both strains were grown at 28°C under selective conditions in synthetic medium containing 2% galactose to a concentration of 2×10^6 cells per ml. Aliquots of 3×10^8 cells were taken for Western and Northern blotting analysis (sample 1, *b* and *c*), whereas the remaining cells were filtered, washed, and resuspended at 1×10^5 cells per ml in synthetic medium containing 2% glucose (time zero). Growth rate was monitored by cell counting, and aliquots of 4×10^8 cells were withdrawn at the times indicated by arrows (samples 2–8). (*b* and *c*) Level of the *POL1* and *PRI2* mRNA and amount of the p180 polα polypeptide in strain CG378ΔPOL1[pMA2] (*b*) and in strain CG378ΔPOL1[pMA2][pAP415] (*c*). Five micrograms of total RNA and 25 μg of total proteins prepared from samples 1–8 were loaded in each lane and analyzed by Northern and Western blotting. The level of p180 was evaluated by densitometric scanning and its amount is plotted relative to that of a Ponceau S-stained band on the Western blot used as a loading control. (*d*) The nuclear localization of polα in the CG378ΔPOL1[pMA2] culture grown under induced conditions (sample 1 in *b*) was determined by *in situ* indirect immunofluorescence. DAPI was used to visualize the nucleus in the same cells.

plasmid (*Materials and Methods*). The isogenic haploid strain CG378ΔPOL1[pMA2][pAP415], containing also the normally regulated *POL1* gene, was used as a control.

In the two strains described above, galactose-induced *POL1* transcription gave rise to a large increase in the level of *POL1* mRNA (Fig. 2 *b* and *c*). Correspondingly, the amount of the p180 polα polypeptide was ≈16-fold higher than that found in yeast cells expressing the *POL1* gene under the control of its own promoter (Fig. 2 *b* and *c*). All p180 polα polypeptide was localized in its correct subcellular compartment, the nucleus (Fig. 2*d*). Neither constitutive nor induced overexpression of *POL1* leads to any evident growth defect (this work and unpublished observations), probably because the *in vivo* activity of polα is mediated by its stoichiometric assembly with the other polypeptides of the polα–primase complex, whose expression is normally regulated [see the level of the *PRI2* gene transcript, encoding the p58 primase subunit (32), in Fig. 2 *b* and *c*, and its fluctuation in Fig. 3*b*].

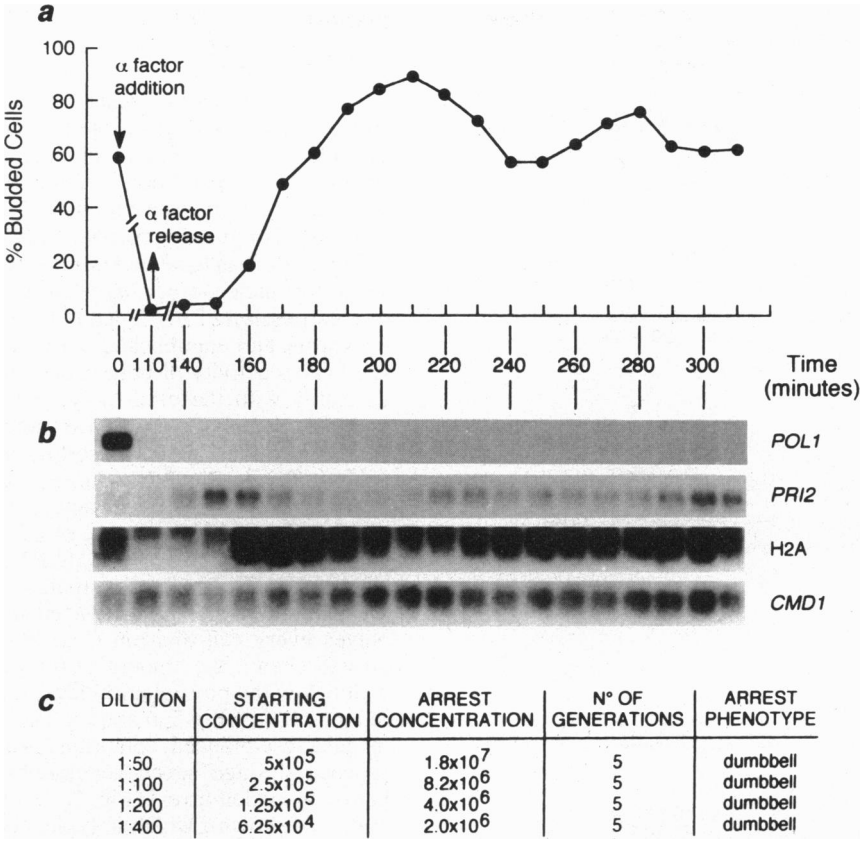
A metabolic shift from galactose to glucose does not immediately affect the growth rate of strain CG378ΔPOL1[pMA2] (Fig. 2*a*), although the *POL1* mRNA becomes undetectable already 10 min after the shift (data not shown). After the shift, the amount of polα synthesized under induced conditions halves every cell division (Fig. 2*b*), but could support cell growth even if the amount of the p180 polypeptide dropped well below the physiological level (compare the level of p180 in lanes 4–8 of Fig. 2 *b* and *c*). The pool of polα synthesized in galactose-induced conditions was sufficient for cells to proceed through seven generations after shift to glucose, before a sudden arrest (Fig. 2*a*) with a dumbbell phenotype (data not shown), which is typical of yeast cells impaired in essential functions required for DNA replication (5). Therefore, it appears that the level of normally expressed polα is higher than that required for a single cell generation, and the enzyme synthesized under galactose-induced conditions is very stable and can be used for several cell divisions.

Entrance into S Phase Does Not Require *de Novo* Synthesis of polα. To further test whether *de novo* synthesis of the enzyme was necessary for cells to undergo DNA replication within the same cell cycle, CG378ΔPOL1[pMA2] cultures grown in galactose medium were treated with α-factor under glucose-repressed conditions. As is shown in Fig. 3*a*, cells recovered synchronously from α-factor treatment, allowing proper periodic transcription of transiently expressed genes (see the level of *PRI2* and H2A transcripts in Fig. 3*b*), while the *GAL1*-driven *POL1* transcription was turned off. After two synchronous divisions (time 290 min in Fig. 3*a*), cells diluted in glucose medium were able to divide for five more generations, before quickly arresting as dumbbell-shaped cells independently on the starting cell concentration (Fig. 3*c*), thus showing that the arrest phenotype was not due to a lack of nutrients. Neither the *POL1* transcript nor the polα polypeptide was detectable in arrested cells, but the same cells underwent a total of seven divisions after α-factor release. Therefore, newly synthesized polα is not required for cells to enter S phase, and the pool of enzyme produced under induced conditions can be used for several generations.

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



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DILUTION	STARTING CONCENTRATION	ARREST CONCENTRATION	N° OF GENERATIONS	ARREST PHENOTYPE
1:50	$5x10^5$	$1.8x10^7$	5	dumbbell
1:100	$2.5x10^5$	$8.2x10^6$	5	dumbbell
1:200	$1.25x10^5$	$4.0x10^6$	5	dumbbell
1:400	$6.25x10^4$	$2.0x10^6$	5	dumbbell

FIG. 3. *De novo* synthesis of polα is not required for entrance into S phase. A CG378ΔPOL1[pMA2] culture was grown in synthetic medium containing 2% galactose to a concentration of 7×10^6 cells per ml. Glucose (2% final concentration) and α-factor were added at time zero. After 120 min, cells were filtered, washed, and resuspended in synthetic medium containing 2% glucose and aliquots of the culture were analyzed at the times indicated. (*a*) Budding profile. (*b*) Five micrograms of total RNA was analyzed on a Northern blot. (*c*) Two generations after α-factor release (sample 290 min at a concentration of 2.5×10^7 cells per ml), aliquots of the synchronized culture were diluted as indicated in 2% glucose synthetic medium, and the cell number and morphology were followed until cells stopped dividing.

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 G_1/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 G_1/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 totipotential, 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 polα 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 G_2/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 G_1/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 G_1 (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 G_1/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.