Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 9025–9029, December 1987
Cell Biology

Periodic mitotic events induced in the absence of DNA replication

(cell cycle regulation/premature chromosome condensation/caffeine/tsBN2)

ROBERT SCHLEGEL* AND ARTHUR B. PARDEE

Department of Pharmacology, Harvard Medical School, and Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115

Contributed by Arthur B. Pardee, August 26, 1987

ABSTRACT **We have discovered and report here a means of separating a mitotic "subcycle" from the G_1- and S-phase events of the mammalian cell cycle. Time-lapse videomicroscopy of Syrian hamster fibroblast (BHK) cells revealed that caffeine could induce multiple entries into mitosis while cells were blocked in DNA synthesis. As with normal mitoses, the abundance of mitosis-specific phosphoproteins was coupled with the condensation of chromatin. The BHK temperature-sensitive mutant tsBN2 also completed multiple entries into mitosis while arrested during DNA replication and raised to the restrictive temperature. Periodic mitotic events occurred even when BHK cells were exposed to low concentrations of serum or cycloheximide, conditions that prevent the cycling of BHK cells by blocking their entry into S phase. These results suggest that an oscillation governing the activation and inactivation of mitotic factors can be generated in mammalian cells and uncoupled from the G_1 and DNA replication events of the normal cell cycle. This system will be useful for examining the molecular nature of mitotic factors.**

The mammalian cell cycle is classically viewed as an interdependent sequence of biochemical events. The completion of DNA replication has therefore been considered a prerequisite for the initiation of mitosis (1). This view was supported by the studies of Rao and Johnson (2), which showed that mitosis was delayed in G_2-phase cells that were fused with S-phase cells. Both nuclei underwent mitosis simultaneously, but only after the S-phase nucleus had completed DNA replication.

Two examples of alterations in the temporal sequence of cell cycle events have been reported in mammalian cells. Chromosome condensation, nuclear envelope breakdown, and other mitotic events occur at the restrictive temperature in the BHK temperature-sensitive mutant tsBN2 (3) and in normal BHK cells that have been arrested in S phase and exposed to caffeine (4, 5). These studies showed that one final mitosis can be uncoupled from the completion of DNA replication. Both exposure to caffeine and suppression of DNA replication are required for this premature induction of mitosis in normal BHK cells (4). Experiments with inhibitors suggest that these conditions permit the accumulation of mitosis-inducing mRNA(s) and their protein(s) (5). The present report extends these observations by providing evidence that mitotic events can be made to occur periodically, independent of G_1- and S-phase events. We propose that an oscillator controlling the entry into and exit from mitosis can be uncoupled from the normal cell cycle and examine its regulation during growth-inhibiting conditions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Culture and Synchronization. Syrian Hamster fibroblast (BHK) cells were grown and synchronized in early S phase as described (4). Briefly, cells were plated at 1×10^5 cells per 60-mm dish and grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum for 24 hr. Synchrony in early S phase was achieved by incubation in isoleucine-deficient DMEM/5% dialyzed fetal calf serum for 30–32 hr (6, 7) followed by DMEM/10% fetal calf serum containing 2.5 mM hydroxyurea for 16 hr. The same procedure was used to synchronize the BHK temperature-sensitive mutant, tsBN2, except that cells were grown at 33°C and remained in isoleucine-deficient medium for 55 hr followed by complete medium containing 2.5 mM hydroxyurea for 17 hr.

Time-Lapse Videomicroscopy. At the start of each experiment, the 60-mm dish was placed into a controlled 37°C 10% CO_2 viewing chamber attached to a Leitz inverted microscope. Videomicroscopy was conducted with a Gyyr time-lapse recorder and an RCA video camera. Individual cells under all conditions were monitored for at least 23 hr at magnifications ranging from ×63 to ×320. The number and timing of cells displaying one, two, three, or four mitotic events were recorded. Greater than 50% of total cell populations underwent one or more mitotic events under all conditions.

Indirect Immunofluorescence. Monoclonal antibody MPM-2 (8) and rhodamine-conjugated goat anti-mouse secondary antibody were diluted 1:500 and 1:1000, respectively, in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Methanol-fixed cells were preincubated at 37°C with DMEM/10% fetal calf serum for 30 min; incubated with MPM-2 for 4 hr; rinsed successively with DMEM/10% fetal calf serum, PBS, and distilled water; air-dried; incubated with the fluorescent secondary antibody for 1 hr; and rinsed as described above. DNA was counterstained with Hoechst 33242 (1 μg/ml for 10 min).

RESULTS

In the presence of 5 mM caffeine, synchronized BHK cells entered mitosis up to four times while DNA synthesis was arrested. As documented by time-lapse videomicroscopy (Fig. 1), disappearance of the nucleoli and breakdown of the nuclear envelope could be seen as the cells "rounded up" during the mitotic response (Fig. 1 *a–c*). Approximately 2 hr later, the cells began to assume their original flat morphology, reform the nucleoli, and reassemble the nuclear envelope around the fragmented chromatin to form numerous micronuclei (Fig. 1*d*). This cycle was repeated three times by the two cells indicated by arrows (Fig. 1 *e–i*). The average cycling time was 7.7 ± 0.6 hr (Table 1), somewhat shorter than the

*Present address: Laboratory of Toxicology, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115.

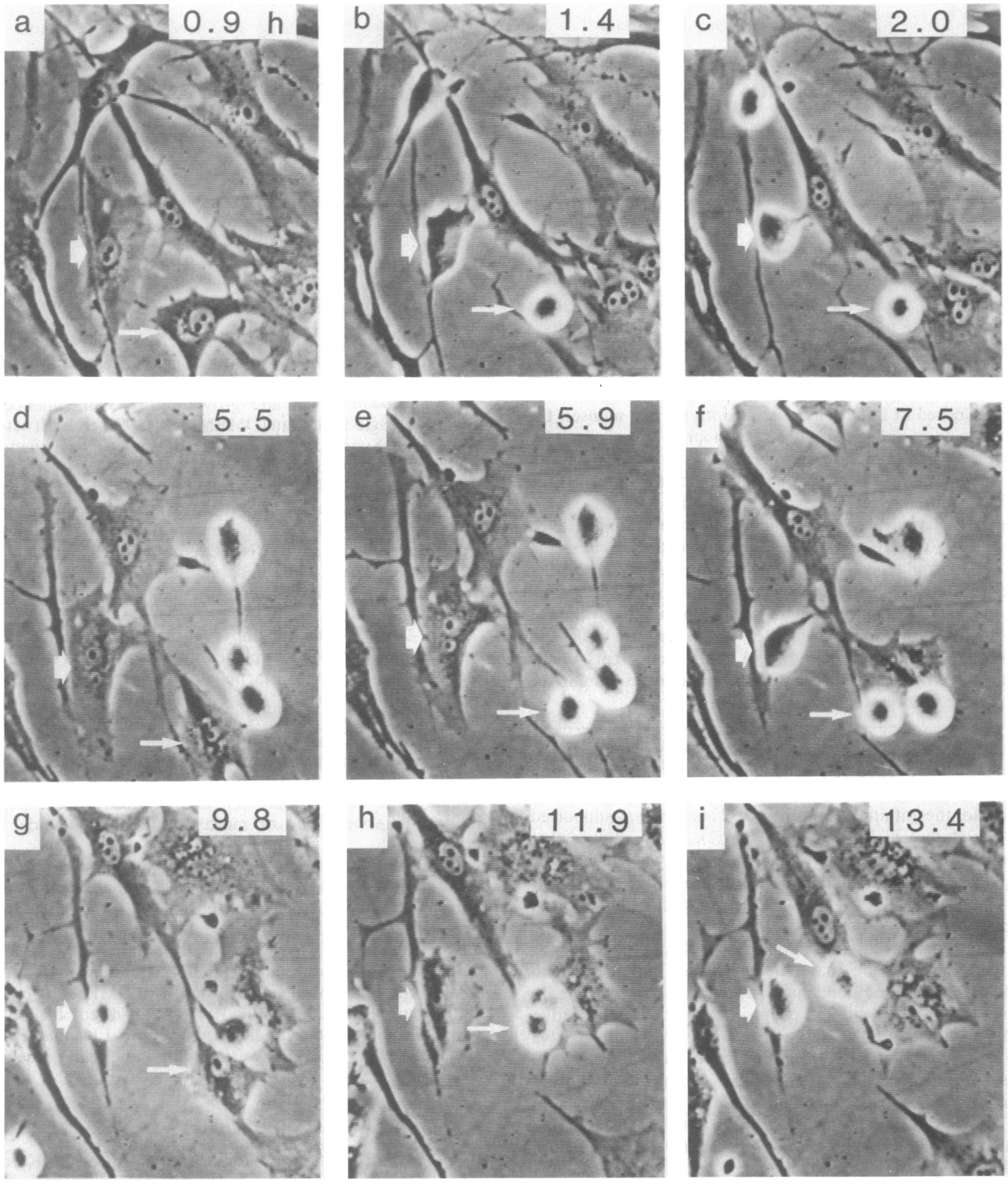

FIG. 1. Time-lapse videomicroscopy of caffeine-induced mitotic events in BHK cells arrested in early S phase. Cells were plated and synchronized in early S phase as described in *Materials and Methods*. Caffeine (5 mM) was then added (t = 0) and videomicroscopy was conducted at ×290 magnification. Photographs were taken from the video monitor at times (hours) after caffeine addition shown in the upper right corner. Arrows show the progression of two different cells, each undergoing three mitotic cycles.

doubling time of 11.3 ± 1.0 hr for cells that were released from S-phase arrest and allowed to grow in the absence of caffeine. Cell division did not occur, indicating that the conditions used in these experiments uncoupled nuclear mitotic events and cytokinesis, as also seen with drugs such as the cytochalasins (9, 10).

The condensation state of the chromatin and the levels of mitosis-specific phosphoproteins are intimately related to the onset of mitosis. These events were therefore examined during this caffeine-induced mitotic "cycle." S-phase arrested cells were exposed to 5 mM caffeine for 8 hr. Fig. 2 (*Left*) shows Hoechst-stained DNA in the nuclei of three cells that typify the sequence of uncondensed (U), condensed and fragmented (C), and decondensed and micronucleated (D) states of the chromatin. In these same cells (Fig. 2 *Right*), the synthesis of mitosis-specific phosphoproteins was examined

Table 1. Effect of various growth conditions on mitotic cycles as monitored by time-lapse videomicroscopy

Conditions	Cells with given no. of mitotic cycles 1	2	3	4	Average cycling time ± SEM, hr
BHK cells					
With caffeine and hydroxyurea					
FCS (10%)	1	9	3	1	7.7 ± 0.6
FCS (0.5%)	1	5	3	0	6.6 ± 0.6
CHM (0.1 μg/ml)	7	16	3	0	10.6 ± 0.8
Without caffeine and hydroxyurea					
FCS (10%)	0	18/18*	ND	ND	11.3 ± 1.0
FCS (0.5%)	13	0/23*	0	0	NA
CHM (0.1 μg/ml)	8	0/12*	0	0	NA
tsBN2 cells (39°C)					
FCS (10%)	5	3	2	0	7.7 ± 0.7

BHK and tsBN2 cells were synchronized, arrested in early S phase, and prepared for videomicroscopy as described in *Materials and Methods*. At $t = 0$, BHK cells were treated with caffeine (5 mM) with or without the simultaneous addition of cycloheximide (CHM; 0.1 μg/ml) or reduction of fetal calf serum (FCS) to 0.5%. Controls were released from hydroxyurea arrest with or without the addition of cycloheximide (0.1 μg/ml) or reduction of FCS to 0.5%. tsBN2 cells were raised to 39°C at the start of videomicroscopy. NA, not applicable; ND, not determined.

*Ratio of the number of second mitoses occurring in daughter cells that remained in view from the first mitosis.

by indirect immunofluorescence with a monoclonal antibody that recognizes a family of such proteins (8). The level of these phosphoproteins increased during condensation and subsided after decondensation of the chromatin.

An independent cycling of mitotic factors can be generated genetically as well as with caffeine. In tsBN2 cells, mitosis was initiated up to three times at the restrictive temperature of 39°C (which was more effective than 40.5°C), while the cells remained arrested in S phase (Table 1). As reported earlier by Nishimoto *et al.* (11), no mitoses occurred at the permissive temperature (33°C) in tsBN2 cells that were arrested in S phase. Approximately 40–50% of BHK cells exposed to caffeine or tsBN2 cells raised to the restrictive temperature did not display these events (4, 11), and only those that underwent at least one mitotic event are reported in Table 1.

Requirements for the passage of normal cells through G_1 phase proved not to be essential for multiple mitotic events. Little inhibition of the effect was seen when cells treated with hydroxyurea and caffeine were simultaneously shifted to low serum or exposed to low cycloheximide concentrations, conditions that arrest exponentially growing cells prior to DNA replication (Table 1). Shifting hydroxyurea-blocked cells into 0.5% fetal calf serum at the time of caffeine addition produced an average mitosis cycling time similar to that seen with these cells maintained in 10% fetal calf serum. Addition of cycloheximide at 0.1 μg/ml (which reduced protein synthesis to 47% of control, as measured by [^{35}S]methionine incorporation) together with caffeine also permitted several cycles, lengthened to 10.6 ± 0.8 hr. Controls that were shifted to either 0.5% fetal calf serum or had cycloheximide (0.1 μg/ml) added after their release from S-phase arrest (and in the absence of caffeine) ceased cycling after completing only one mitosis within 27 hr.

DISCUSSION

The cycling of mitotic events in our experiments might be transcriptionally and/or posttranscriptionally regulated. Both of these mechanisms appear important for controlling normal mitosis and meiosis. It has been known for more than 20 years that certain uncharacterized mRNA transcripts must be synthesized shortly before mitosis in mammalian cells (12, 13). More specifically, the level of a transcript required for mitosis in *Aspergillus nidulans (nimA)* has been found to be cell-cycle regulated (14). Selective translation of preexisting mRNA can also control the onset of meiosis as seen in many embryo systems (15–17).

Our finding of multiple mitotic events in mammalian cells is related to earlier experiments conducted with cycloheximide-treated *Xenopus* eggs, in which repeated injections of maturation promoting factors followed by their spontaneous inactivation repeatedly induced mitosis and subsequent return to interphase (18). Our experiments presumably produced a similar, but endogenously generated, cycling of mitotic factors.

A possible model for our results is depicted in Fig. 3. RNA needed for mitosis can be synthesized during S-phase arrest (4, 5, 11), and this RNA appears to become very labile if DNA replication is allowed to resume (5). Exposure to caffeine (and perhaps raising tsBN2 to 39°C) increases the stability of the mitosis-inducing proteins (5) and allows them to reach critical levels and trigger mitosis.

The elevated levels of mitosis-specific phosphoproteins seen during caffeine-induced mitotic events are consistent

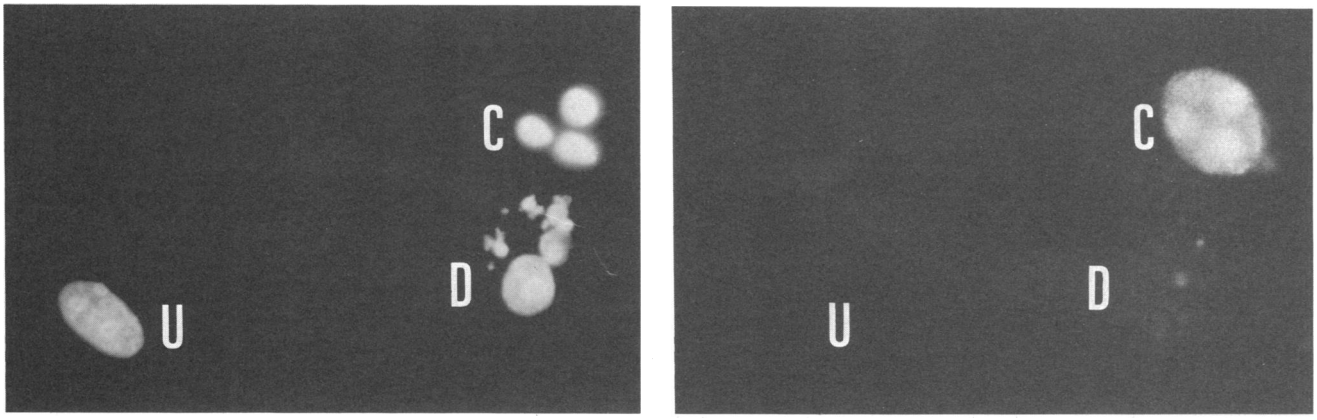

FIG. 2. Chromatin condensation and expression of mitosis-specific phosphoproteins during caffeine-induced mitotic cycles. BHK cells were synchronized and arrested in early S phase as described in *Materials and Methods*. Eight hours after caffeine (5 mM) addition, cells were fixed in absolute methanol. (*Left*) Hoechst 33242 staining shows the uncondensed (U), condensed and fragmented (C), and decondensed and micronucleated (D) chromatin states. (×2475.) (*Right*) Indirect immunofluorescence with monoclonal antibody MPM-2, which recognizes a family of mitosis-specific phosphoproteins (8). Cells in *Left* and *Right* are the same.

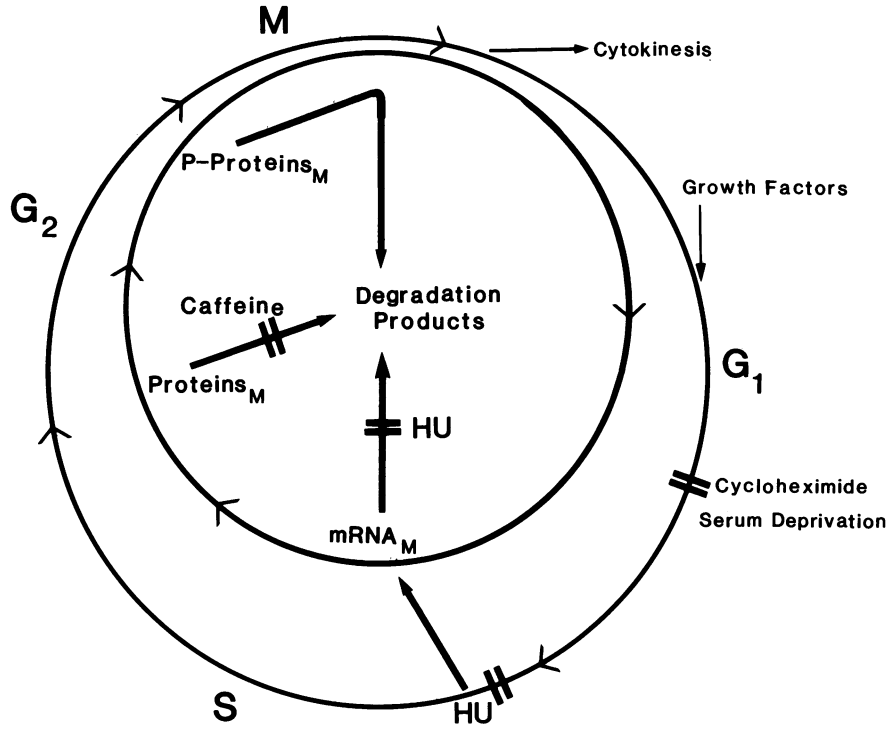

FIG. 3. Mitotic cycle induced by caffeine in cells arrested in S phase. Outer circle depicts the normal cell cycle. Inner circle represents the mitotic "minicycle" induced by caffeine in cells arrested in S phase. $mRNA_M$, $Proteins_M$, and P-$Proteins_M$ are mitosis-related mRNA, proteins, and phosphoproteins, respectively. HU, hydroxyurea.

with earlier studies supporting an important role for post-translational phosphorylation in the regulation of mitosis and meiosis. This earlier evidence includes the increased activity of protein kinases in mitotic mammalian cells (19, 20), the identification of kinases that regulate mitosis in *Schizosaccharomyces pombe* (21–23), the activation of maturation promoting factors in cycloheximide-treated *Xenopus* oocytes (24, 25), and the phosphorylation and dephosphorylation of many constitutively and previously synthesized proteins in mammalian cells such as lamin, histone, high mobility group, nuclear matrix, intermediate filament, and nonhistone proteins (see ref. 26 for review).

Caffeine-induced mitosis is succeeded by events that lead to the inactivation of the mitosis-inducing factors, as evidenced by the loss of mitosis-specific phosphoproteins and the return to interphase morphology. A similar mechanism of periodic destruction near the end of each mitosis has been shown to operate in the mitosis-inducing cyclin A protein of clam embryos (27) as well as the cyclin proteins of sea urchin embryos (28). In mammalian cells, the degradation of mitotic factors after the completion of mitosis is supported by early cell fusion studies (2) and by microinjection experiments with extracts from various stages of the cell cycle to induce oocyte maturation (29, 30). These findings suggest that protein degradation is also important in mitotic control.

Normal cycling cells initiate mitosis only once after completing DNA replication. This report demonstrates that mitosis can be initiated periodically in mammalian cells whose DNA synthesis is stopped after initiation of S phase. This arrest may elevate the levels of mitosis-related RNAs for prolonged periods, thereby permitting multiple mitotic events to occur. Conditions that prevent normal cell proliferation by blocking the onset of S phase (low serum or cycloheximide exposure) would therefore not be expected to prevent mitotic events in these cells.

We thank M. Rider for manuscript preparation. Special thanks to Dr. L. Chen and Dr. J. Rheinwald for use of their time-lapse equipment and for helpful suggestions, Dr. F. Davis and Dr. P. Rao for their gift of monoclonal antibody MPM-2, and Dr. C. Basilico for the tsBN2 cell line. This work was supported by National Institutes of Health Grant CA 22427 to A.B.P. and by Environmental Protection Agency Grant CR-812699-01-0 to the Interdisciplinary Programs in Health (Harvard School of Public Health).

1. Baserga, R. (1976) *Multiplication and Division in Mammalian Cells* (Dekker, New York), pp. 39–40.
2. Rao, P. N. & Johnson, R. T. (1970) *Nature (London)* **225,** 159–164.
3. Nishimoto, T., Eilen, E. & Basilico, C. (1978) *Cell* **15,** 475–483.
4. Schlegel, R. & Pardee, A. B. (1986) *Science* **232,** 1264–1266.
5. Schlegel, R. & Pardee, A. B. (1987) *J. Cell. Physiol.* **131,** 85–91.
6. Tobey, R. A. & Crissman, H. A. (1972) *Exp. Cell Res.* **75,** 460–464.
7. Hamlin, J. L. & Pardee, A. B. (1976) *Exp. Cell Res.* **100,** 265–275.
8. Davis, F. M., Tsao, T. Y., Fowler, S. K. & Rao, P. N. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2926–2930.
9. Carter, S. B. (1967) *Nature (London)* **213,** 261–264.
10. Krishan, A. (1972) *J. Cell Biol.* **54,** 657–664.
11. Nishimoto, T., Ishida, R., Ajiro, K., Yamamoto, S. & Takahashi, T. (1981) *J. Cell. Physiol.* **109,** 299–308.
12. Kishimoto, S. & Lieberman, I. (1964) *Exp. Cell Res.* **36,** 92–101.
13. Tobey, R. A., Petersen, D. F., Anderson, E. C. & Puck, T. T. (1966) *Biophys. J.* **6,** 567–581.
14. Osmani, S. A., May, G. S. & Morris, N. R. (1987) *J. Cell Biol.* **104,** 1495–1504.
15. Rosenthal, E. T., Hunt, T. & Ruderman, J. V. (1980) *Cell* **20,** 487–494.
16. Gross, P. R., Malkin, L. I. & Moyer, W. A. (1964) *Proc. Natl. Acad. Sci. USA* **51,** 407–414.
17. Smith, L. D. & Ecker, R. E. (1965) *Science* **150,** 777–779.
18. Newport, J. W. & Kirschner, M. W. (1984) *Cell* **37,** 731–742.
19. Halleck, M. S., Lumley-Sapanski, K., Reed, J. A., Iyer, A. P., Mastro, A. M. & Schlegel, R. A. (1984) *FEBS Lett.* **167,** 193–198.
20. Woodford, T. A. & Pardee, A. B. (1986) *J. Biol. Chem.* **261,** 4669–4676.

21. Russell, P. & Nurse, P. (1987) *Cell* **49,** 559–567.
22. Russell, P. & Nurse, P. (1987) *Cell* **49,** 569–576.
23. Simanis, V. & Nurse, P. (1986) *Cell* **45,** 261–268.
24. Wasserman, W. J. & Masui, Y. (1975) *Exp. Cell Res.* **91,** 381–388.
25. Gerhart, J., Wu, W. & Kirschner, M. (1984) *J. Cell Biol.* **98,** 1247–1255.
26. Adlakha, R. C., Davis, F. M. & Rao, P. N. (1985) in *Control of Animal Cell Proliferation*, eds. Boynton, A. L. & Leffert, H. L. (Academic, New York), pp. 485–513.
27. Swenson, K. I., Farrell, K. M. & Ruderman, J. V. (1986) *Cell* **47,** 861–870.
28. Evans, T., Rosenthal, E. T., Youngbloom, J., Distel, D. & Hunt, T. (1983) *Cell* **33,** 389–396.
29. Sunkara, P. S., Wright, D. A. & Rao, P. N. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2799–2802.
30. Adlakha, R. C., Sahasrabuddhe, C. G., Wright, D. A. & Rao, P. N. (1983) *J. Cell Biol.* **97,** 1707–1713.