Journal of Natural Science,
Pai Chai University, Korea
Vol. 9, No. 1: 1~7, 1996

Cell Cycle-Dependent Activity Change of Calcium/Calmodulin-Dependent Protein Kinase II

Kyong Hoon Suh
Department of Biochemistry, Pai Chai University

칼슘/calmodulin-의존적 단백질 인산화 효소 II의 동물세포 주기에 따른 활성도 변화에 관한 연구

서 경 훈
배재대학교 화학 및 생명과학부 생화학전공

Calcium/calmodulin-dependent protein kinase II (CaMK-II) is responsible for the phosphorylation of proteins involved in various cellular functions. Since the level of intracellular calcium (Ca^{2+}) oscillate during the cell cycle, it is expected that the activity of CaMK-II is also dependent on the cell cycle. The kinase activity in NIH3T3 cells which were arrested at or released from certain phase of the cell cycle was measured and compared to that in the normally growing asynchronous control cells to investigate whether the activity of this kinase is cell cycle-dependent. Cells were arrested at G0, G1, G1/S, G2/M and M phase, respectively by use of various drugs which do not have any effect on the kinase activity. Total activity of CaMK-II at G0, G1, G1/S and G2/M phase was similar to that of the control cells, whereas lower at M. Calcium-independent activity of CaMK-II by autophosphorylation was higher at M and, thus, higher autonomy at M, which represented the physiologically relevant activity of CaMK-II. A similar pattern of activity change of the kinase was demonstrated during the cell cycle of synchronized cells which were released from G1 arrest. These results indicate that the activity of CaMK-II is cell cycle-dependent and is activated during the mitosis.

칼슘/calmodulin-의존적 단백질 인산화 효소 II (CaMK-II)는 세포의 여러 기능을 조절하는 다양한 단백질들을 인산화시키는 효소이다. 세포 내부의 칼슘의 농도는 세포의 주기에 따라 변하므로 CaMK-II의 활성도 역시 세포주기에 따라 변하는 지를 조사함으로 세포주기에서의 CaMK-II의 역할을 알아보려 하였다. NIH3T3 세포를 CaMK-II의 활성도에는 전혀 영향을 주지 않는 여러 가지 약제로 처리하여 세포주기상의 특정한 시점에 동일하게 정지시킨 후, 세포내의 CaMK-II 활성도를 합성 펩타이드 기질을 이용하여 측정하였다. 또한 일정 시점으로부터 동조화된 세포내의 CaMK-II의 활성도의 변화를 측정하여 한 세포주기 동안 효소의 활성도 변화의 양상을 조사하였다. 세포주기상 각각 G0, G1, G1/S, G2/M기에 정지된 세포내의 CaMK-II 총활성도는 대조군과 차이가 없었으나 M기에서는 낮았다. 그러나 자가인산화에 의한 CaMK-II의 칼슘-비의존성 활성도는 M기에서 가장 높았다. 이러한 양상은 G1기에서부터 동조화된 세포내 CaMK-II의 칼슘-비의존성 활성도 변화 양상과도 일치하였다. CaMK-II의 생리학적 의미를 지닌 활성도는 인산화에 의한 calcium-비의존성 활성도임을 비추어 볼 때 M기에서 CaMK-II가 세포분열의 과정에서 중요한 기능을 하고 있음을 보여주고 있다.

Key words : Calcium, Calmodulin, Protein kinase, Phosphorylation, Cell cycle

I. Introduction

CaMK-II is an ubiquitous multifunctional enzyme. It requires Ca^{2+}-bound CaM for its activation and its abilities to phosphorylate and alter the function of a variety of substrates involved in the performance of many cellular functions (for a review, see Braun and Schulman, 1995). The kinase is in a large multimeric form of its subunits *in vivo*. Each subunit con tains an autoregulatory region, whose displace ment by autophosphorylation is critical in maintaining autonomous catalytic activity and CaM trapping (Mukherji and Soderling, 1994). Phosphorylation of autoregulatory site is not essential for the kinase activity, but it does have important consequences. Autophosphorylation increases the affinity of the kinase for CaM several hundred-fold by reducing the dissociation rate of kinase-CaM complex (Meyer *et al.*, 1992). Even when Ca^{2+} is reduced to physiological levels, CaM is trapped on the kinase for several seconds. Therefore, the kinase retains 100% activity as long as CaM is trapped, regardless of the Ca^{2+} level. In addition, the presence of phosphate on the autonomy site is itself sufficient to disrupt the autoinhibitory domain, and the kinase retains partial activity (20-80%) even after CaM dissociates in this autonomous site (Meyer *et al.*, 1992). The assay of autonomous CaMK-II activity in a cell homogenate from control and stimulated cultures gives a direct measure of the fraction of the kinase that has been activated by stimulation of the cells (Bennett-Jefferson *et al.*, 1991; Fukunaga *et al.*, 1989).

In order to reproduce and multiply, every cell must execute an orderly series of event, generally called the cell cycle, at some time during its life span (for a review, see Inoue, 1981; Pardee *et al.*, 1978). The cell cycle consists of the S phase (a period of DNA synthesis), the G2 phase (a gap), the M phase (a period of cell division), and the G1 phase (a gap). Physiologically G1 phase is the decision phase in which either commit to undergo another round of DNA synthesis and continue to cycle or to exit the cell cycle to enter a quiescent state referred to as G0 (a phase of terminal differentiation or resuming proliferation upon addition of an appropriate mitogen). These sequential events of the cell cycle are precisely controlled by the changes of external or internal signals such as growth factors, cell size, nutrients, the completion of genome replication, and the activity or content of various cell cycle specific proteins such as cyclins. It is very well known that the levels of Ca^{2+}, an intracellular second messenger, and CaM, a high affinity intracellular receptor of Ca^{2+}, are changing during the cell cycle (for a review, see Lu and Means, 1993). Intracellular level of Ca^{2+} transiently increases at early G1/S and G2/M and during M, and that of CaM also changes in a similar pattern. These temporal changes of Ca^{2+}/CaM are essential for cells to execute each round of the cell cycle. If cells fail to increase Ca^{2+} and/or CaM level transiently at certain specific time in the cell cycle, they become arrested at a critical point of the cell cycle (Sasaki and Hidaka, 1982; Kao *et al.*, 1990). Since the level of essential components for the activation of CaMK-II is being changed during the cell cycle, it is reasonable to assume that the activity of CaMK-II is also changing. In this report, the activity of CaMK-II was measured from NIH3T3 cells which were arrested/released at/from different phases of the cell cycle by various drugs to investigate whether the kinase activity is cell cycle-dependent.

II. Experimental Procedures

1. Material

Dulbecco's modified eagle medium (DMEM), Eagle's minimum essential medium (EMEM),

bovine calf serum, Hank's balanced salt solution (HBSS), antibiotics, hydroxyurea, Hoechst 33342, nocodazole, and CaM (as a phosphodiesterase activator) were from Sigma. [γ-^{32}P]-ATP was from Amersham.

2. Cell Culture

NIH3T3 cells were grown as monolayer cultures in DMEM containing 10% (v/v) BCS and antibiotics (penicillin at 100 units/ml and streptomycin at 50 μg/ml) at 37℃ in an atmosphere of 5% CO_2. Cells in exponential growth phase were used for all experiments.

3. Synchronization of cells

When cells grow to 60% confluency, they were incubated in medium without BCS for 2 to 3 days to be arrested at G0 (Tobey *et al.*, 1988). Cultivation of cells in isoleucine-deficient EMEM supplemented with 5% BCS for 1 day arrested cells at G1 (Tobey, 1973). Incubation of exponentially growing cells in normal medium supplemented with 1 mM hydroxyurea for 14-16 hours arrested at G1/S (Tobey *et al.*, 1988). An inhibitor of DNA-topoisomerase II, Hoechst 33342 (bisbenzimide H33342 fluorochrome, 7.5 μg/ml), was applied to the medium of exponentially growing cells for 14-16 hours to arrest cells at G2/M (Tobey *et al.*, 1990). Mitotic cells were obtained by incubation with 1 mM nocodazole for 14 hours (Brizuela *et al.*, 1988). For a study of one round of cell cycle, cells were released from arrest simply by washing cells with HBSS and then incubated in fresh medium supplemented with 10% BCS. The proportion of arrested cells in total cell population was determined by flow cytometry after staining of nucleus with propidium iodide.

4. Cell cycle analysis

NIH3T3 cells were harvested at different phases of the cell cycle by detachment with trypsin treatment and centrifuged at low speed (1,000 x g) for 5 min. Washing cells with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) was followed. The cells were suspended with small volume of PBS and added 5 ml of PBS-ethanol (3:7) while mixing gently. After 60 min at room temperature, cells were collected and washed twice with PBS and were resuspended with 500 ml of 5 μg/ml RNase A and incubated for 30 min at 37℃. Ice-cold PBS (2 ml) containing propidium iodide (50 μg/ml) was applied to washed cells for 30 min to stain nuclei (Minami *et al.*, 1994). Flow cytometric analysis was carried out to assess the proportion of cells arrested at a specific phase. Ten thousand cells were counted per analysis.

5. Protein extraction

Cells from different phase of the cell cycle were washed with ice-cold Tris-buffered saline (TBS) two times and harvested by scrapping. After collecting cells by low speed centrifugation, cells were resuspended in 5 volumes of extraction buffer (25 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 20 μM leupeptin) and were sonicated for 15 s. This homogenate was spun at 100K x g for 30 min to obtain cytosolic protein fraction. Protein content was determined by Bradford method and aliquots of protein was used as a source of the kinase or stored at -70 ℃ until use.

6. Kinase assay

CaMK-II activity was measured in a reaction mixture (final volume 100 μl) containing of 30 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 20 μg/ml CaM, either 1 mM EGTA (minus Ca^{2+}), or 1 mM EGTA, 2 mM $CaCl_2$ (plus Ca^{2+}), 5 μg of cytosolic protein of soluble fraction, and 2 μM of synthetic substrate, Syntide, at 30℃ (Kuret and Schulman, 1984). The mixture was incubated for 1 min and the reaction was started by addition of 20 mM [γ-^{32}P]-ATP (1-3 x 10^2 cpm/pmol). The phos-

phorylation reaction was terminated after 1 min by stop solution containing 100 mM EDTA, 25 mM Tris-HCl, pH 7.4, 33 mM EGTA, 10 mM pyrophosphate, and 10 mM β-glycerophosphate. Each reaction mixture (60 μl) was spotted on a P-81 phosphocellulose paper was washed in 75 mM phosphoric acid and in water. The radioactivity incorporated into the Syntide as the kinase activity was quantified by scintillation counting. Each sample was assayed in duplicate.

III. Results and Discussion

1. Synchronization of cells

In order to assess the activity of CaMK-II at specific phases of the cell cycle, cells should be synchronized. Among many ways of synchronization, treatment of cells with drugs which have been known to arrest exponentially growing cells at specific phases was adopted in this study. Three kinds of drugs (hydroxyurea, Hoechst 33342 and nocodazole) were selected from many candidates after examining their inability to exert any effect on the catalytic activity of the kinase by in vitro assay using purified enzyme and synthetic substrates (data not shown). Hydroxyurea, Hoechst 33342, and nocodazole treatments arrested the cells at G1/S, G2/M, and M phase, respectively (Table 1). For the better resolution of the kinase activity change along the cell cycle, the cell cycle was stopped at different time points as frequently as possible. Therefore, the cells were additionally arrested at G0 and G1 by cultivating cells in low serum (0.5%) and isoleucine-deficient medium, respectively. When the cells were growing in low serum medium for 2 days, almost of cells were arrested at G0, which was determined by fluorescent dye staining of chromosome, measurement of lag time to start DNA synthesis after release from the arrest, and cell morphology. The proportion of cells arrested at other phase was determined by flow cytometry. As shown in Table 1, more than 80% of total cells were arrested at specific phase by corresponding drug. These results indicated that each drug worked efficiently to arrest cells at specific time point of the cell cycle and that the activity of CaMK-II could be measured specifically at each time point. This method has another advantage to study cell cycle, since effects of the drugs and the specific media were easily reversible. The cells were reentering or restarting the cell cycle simply by washing off the drugs or changing media. Except reentering G1 from G0 arrest which took 2-3 additional hours to reach S, releasing from each arrest point drived cells to start very next phase of the cell cycle without a considerable lag time.

Table 1. Proportion of arrested cells in total cell population

Arresting point	Treatment	Frequency (%)
G0	0.5% BCS	> 98
G1	EMEM minus isoleucine	91
G1/S	Hydroxyurea	79
G2/M	Hoechst 33342	81
M	Nocodazole	86

NIH3T3 cells were harvested, fixed, and their nuclei were stained by propidium iodide after the cells were arrested at different time points of the cell cycle. Fluorescent intensity of stained nuclei were measured by flow cytometry to quantitate the content of DNA except G0 phase arrested cells.

2. Activity of CaMK-II in arrested cells

The activity of CaMK-II was measured in the cells arrested at different phases. Total activity (plus Ca^{2+} activity) of CaMK-II at G0, G1, G1/S and G2/M was similar to that of control (exponentially growing asynchronous) cells (Fig. 1).

However, the activity of cells at M phase showed relatively lower activity compared to that

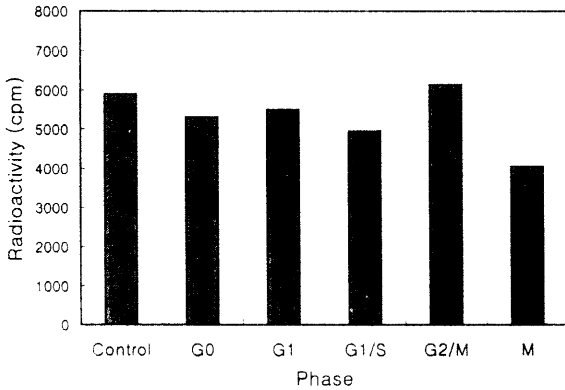

Fig. 1. Total activity (plus Ca2+ activity) of CaMK-II at different phases of the cell cycle. The kinase activity was measured in protein extract from the cells arrested at different phases of cell cycle by addition of 2 mM Ca2+ to the enzyme assay mixture. The enzyme activity at each phase is an average value from the five separate experiments.

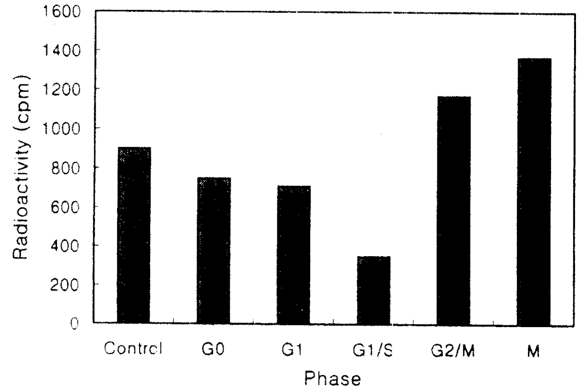

Fig. 2. Calcium-independent activity of CaMK-II at different phases of the cell cycle. The enzyme activity was measured in the absence of Ca^{2+}. In order to rule out any possible contamination of Ca^{2+} during the enzyme assay, 1 mM EGTA was added to the assay mixture. The enzyme activity at each phase is an average value from the five separate experiments.

activity of CaMK-II activated by 2 mM Ca^{2+}, which includes Ca^{2+}-dependent and -independent activity. As reported already, physiologically relevant activity is Ca^{2+}-independent activity which is known to be achieved by autophosphorylation after Ca^{2+}/CaM binding (Mukherji and Soderling, 1994; Braun and Schulman, 1995). This kind of activity can last relatively longer time even after Ca^{2+} level is lowered to the basal level. The Ca^{2+}-independent activity (minus Ca^{2+} activity) was measured directly from the assay mixture without Ca^{2+} and with EGTA to chelate any possible Ca^{2+} contamination. This Ca^{2+}-independent activity reflects *in vivo* level of active CaMK-II at a specific phase of the cell cycle. When cells were arrested at different phases of the cell cycle, Ca^{2+}-independent activity was not changed at G0 and G1 phases (Fig. 2). Ca^{2+}-independent activity at G1/S was lower (63% of control) than that of control whereas those at G2/M and M phases were higher than that of control (30 and 56% higher than control, respectively).

That is, total activity and Ca^{2+}-independent activity were not changed at G0 and G1, and total activity was not changed but Ca^{2+}-independent activity was changed at G1/S and G2/M, and both activities were changed at M. In order to understand better the activity change of CaMK-II, the proportion of Ca^{2+}-independent activity to the total activity (as a term of autonomy) at each phase of the cell cycle was calculated and plotted (Fig. 3).

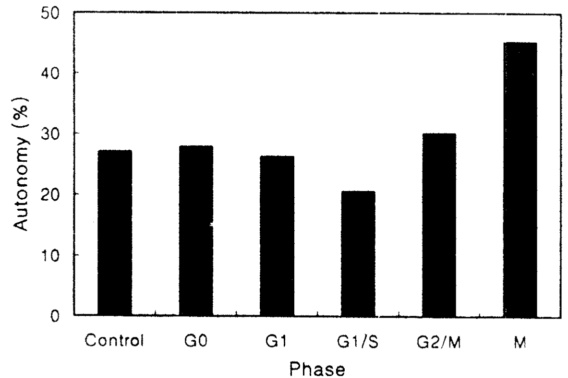

Fig. 3. Autonomy of CaMK-II activity at different phases of the cell cycle. The proportion of Ca^{2+}-independent activity to the total activity represents autonomy of the enzyme, which reflects the fraction of physiologically activated CaMK-II at each phase of the cell cycle.

Autonomy is the direct expression of the physiologically effective activity of CaMK-II. The cells arrested at G0, G1, and G2/M showed similar autonomy to that at control (27-30%), which means no meaningful net activation of the kinase at those phases. However, autonomy at G1/S was lower (37%) and at M was higher (209%) than that in control. Therefore, net physiological activation of CaMK-II was carried out at mitosis whereas low degree of inhibition was exerted at G1/S phase. These results indicates that CaMK-II activity is regulated during the cell cycle and that the kinase plays an important role in the transition of G1/S and the progression of M phases.

3. Change of CaMK-II activity during the cell cycle

Even though the Ca^{2+}-independent activity of CaMK-II was higher at M and lower at G1/S phase, it was not representing activity change of CaMK-II in normally proliferating native cells. Since arresting cells at different time points of the cell cycle by drug treatments was not physiological event, the changes in autonomy at G1/S and M shown above might result from unknown side effect of drugs rather than from the modification of enzyme activity itself. Therefore, it was necessary to examine how CaMK-II activity was changing during normal cell cycle. In order to examine whether the similar pattern of activity change was carried out during the one turn of cell cycle, a release experiment was performed. For one turn of the cell cycle, the cells arrested at G1 was released by changing medium to normal DMEM. The cells at designated time points from release was harvested and assayed for CaMK-II activity (Fig. 4).

Total activity showed slowly increasing pattern during the cell cycle and two distinctactivity peaks at 16 h and 22 h time points. A fall of total activity during the early phase of the cell cycle was obvious, which was believed to be during G1/S transition. The pattern of Ca^{2+}-independent activity was similar to that of total activity. When the change of autonomy during the cell cycle was analyzed

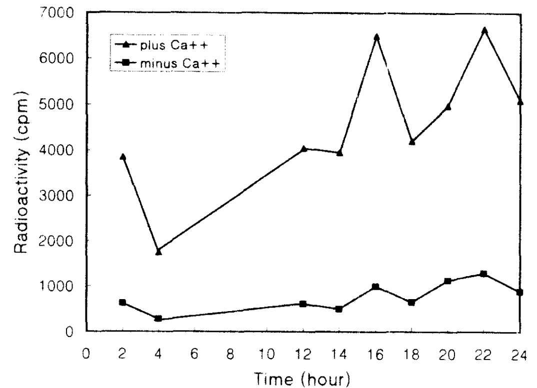

Fig. 4. Change of CaMK-II activity during the cell cycle. NIH3T3 cells arrested at G1 phase were released and the kinase activity was measured at designated time points while the cell proceeded for one turn of the cell cycle. The enzyme activity at each phase is an average value from the three separate experiments.

(Fig. 5), the highest autonomy (at 20 h time point) was almost 2 times higher than the lowest one (at 14 h time point), which was agree with the result from arrested cells.

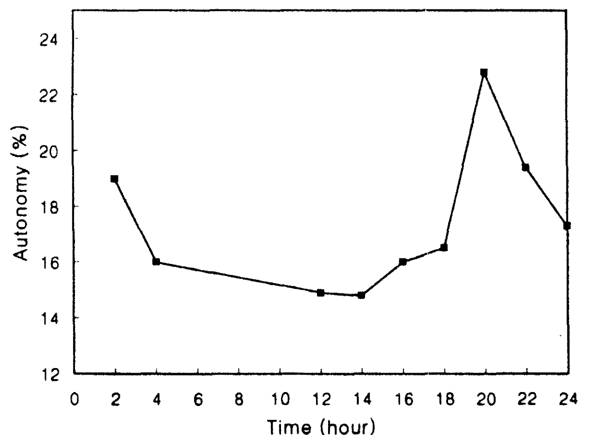

Fig. 5. Change of CaMK-II autonomy during the cell cycle. The proportion of Ca^{2+}-independent activity to the total activity of CaMK-II

was calculated and plotted to show how physiologically relevant activity of CaMK-II was changed during the cell cycle.

Although the exact duration of one cycle of NIH3T3 cell division was not measured in this study, it has been known that the cell completed one cycle in 22-23 hours. Therefore, the 20 h time point of the highest autonomy might indicate mid M phase. These results suggest that CaMK-II activity is changed along with the progression of cell cycle of synchronized NIH3T3 cells.

IV. Acknowledgment

This project was financially supported by Central Research Fund from Pai Chai University for the year of 1996.

V. References

1. Bennett-Jefferson, A., Travis, S.M., and Schulman, H. (1991) Activation of multifunctional Ca^{2+}/CaM-dependent protein kinase type II in GH3 cells. *J. Biol. Chem.* 266: 1484-1490.
2. Braun, A.P., and Schulman, H. (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu. Rev. Physiol.* 57: 417-445.
3. Brizuela, L., Draetta, G., and Beach, D. (1988) Association of human CDC2 protein as a histone H1 kinase is associated with complex formation with the p62 subunit. *Proc. Natl. Acad. Sci., USA* 86: 4362-4366.
4. Fukunaga, K., Rich, D.P., and Soderling, T. R. (1989) Generation of the Ca^{2+}/CaM-independent form of Ca^{2+}/CaM-dependent protein kinase II in cerebella granule cells. *J. Biol. Chem.* 264: 21830-21836.
5. Inoue, S. (1981) Cell division and the mitotic spindle. *J. Cell Biol.* 91: 132s-147s.
6. Kao, J.P.Y., Alderton, J.M., Tsien, R.Y., and Steinhardt, R.A. (1990) Active involvement of Ca^{2+} in mitotic progression of Swiss 3T3 fibroblasts. *J. Cell Biol.* 111: 183-196.
7. Kuret, J. and Schulman, H. (1984) Purification and characterization of Ca^{2+}/CaM-dependent protein kinase from rat brain. *Biochemistry* 23: 5495-5504.
8. Lu, K.P., and Means, A.R. (1993) Regulation of the cell cycle by calcium and calmodulin. *Endocrine Rev.* 14: 40-58.
9. Meyer, T., Hanson, P.I., Stryer, L., and Schulman, H. (1992) Calmodulin trapping by calcium-calmodulin-dependent protein kinase. *Science* 256: 1199-1202.
10. Minami, H., Inoue, S., and Hidaka, H. (1994) The effect of KN-62, Ca^{2+}/CaM-dependent protein kinase II inhibitor on cell cycle. *Biochem. Biophys. Res. Comm.* 199: 241-248.
11. Mukherji, S., and Soderling, T.R. (1994) Regulation of Ca^{2+}/CaM-dependent protein kinase II by inter- and intrasubunit-catalyzed autophosphorylation. *J. Biol. Chem.* 269: 137 44-13747.
12. Pardee, A.B., Dubarow, R., and Hamlin, J.L. (1978) Animal cell cycle. *Annu. Rev. Biochem.* 47: 715-750.
13. Sasaki, Y., and Hidaka, H. (1982) Calmodulin and cell proliferation. *Biochem. Biophys. Res. Comm.* 104: 451-456.
14. Tobey, R.A. (1973) Production and characterization of mammalian cells reversibly arrested in G1 by growth in isoleucine-deficient medium. in Methods in Cell Biol., ed. Prescott, D.M., Vol. 6, pp. 67-112, Academic Press, New York.
15. Tobey, R.A., Oishi, N., and Crissman, H.A. (1990) Cell cycle synchronization: reversible induction of G2 synchrony in cultured rodent and human fibroblasts. *Proc. Natl. Acad. Sci., USA* 87: 5104-5108.
16. Tobey, R.A., Valdez, J.G., and Crissman, H. A. (1988) Synchronization of human diploid fibroblasts at multiple stages of the cell cycle. *Exp. Cell Res.* 179: 400-416.