# HORMONE-TREATED CHO CELLS EXIT THE CELL CYCLE IN THE G2 PHASE

J.A. Remington and R.R. Klevecz

Division of Biology
City of Hope National Medical Center
Duarte, California 91010

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SUMMARY: Determinations of the rate of DNA synthesis, quantity of DNA and mitotic index in synchronized and unsynchronized Chinese hamster ovary cells (CHO) indicate that these cells can exit the cell cycle at several points. CHO cells in stationary phase are arrested in G1 whereas CHO cells at confluency in the presence of dibutyryl cyclic AMP and testosterone are arrested in the G2 phase. The hormone-treated cells can also be arrested, or delayed, at two points in the S phase, and in the G1 phase. The periodicity of DNA synthesis and the length of the components of the cell cycle do not change in CHO cells in the presence of dibutyryl cyclic AMP and testosterone. The findings are discussed in relation to the change in the differentiation state of CHO cells induced by these hormones.

Cyclic AMP appears to be involved in the regulation of the growth rate and the process of contact inhibition in cultured fibroblasts (1-3). Dibutyryl cyclic AMP [$(but)_2$cAMP] also regulates the appearance of differentiated functions in neuroblastoma (4,5), melanoma (6) and 3T3 cells (7). Chinese hamster ovary (CHO) cells induced by $(but)_2$cAMP and testosterone show an increased doubling time, altered morphology and a change in the differentiation state (8,9).

The purpose of this study was to determine the cause of the increased doubling time and possible stages of the cell cycle from which differentiation of CHO cells induced by $(but)_2$cAMP and testosterone occurs.

## MATERIALS AND METHODS

A recently cloned derivative of the CHO line originally described by Tjio and Puck (10) was grown free of PPLO contamination as a monolayer in McCoy's 5a medium with 20% fetal calf serum. $1 \times 10^{-4}$ M or $2 \times 10^{-4}$ M $(but)_2$cAMP (Sigma) plus $1.5 \times 10^{-5}$ M testosterone propionate (Sigma) were added as indicated in legends.

Cells were synchronized by colcemid (2.5-3.5 hrs; 0.06 µg/ml), and incorporation of $H^3$-methyl-thymidine ($H^3$-TdR; Schwarz/Mann, 6.0 Ci/mmole) into DNA of cells in scintillation vials was determined as previously described (11,12).

The amount of DNA per culture was determined by its fluorescence following the reaction of diaminobenzoic acid with the 2-deoxy sugar group according to Hinegardner's modification (13) of the technique of Kissane and Robins (14). Cells were counted in a Coulter counter (Model A).

Autoradiography was performed according to standard procedures (15). From 50-150 metaphases were scored for the percent labelled metaphases and 500-2500 cells were counted in each sample to determine the mitotic index.

## RESULTS

If $(but)_2cAMP$ and testosterone are added at the beginning of the cell cycle, the timing and extent of DNA synthesis is not significantly altered (Fig. 1). The mitotic index of the hormone-

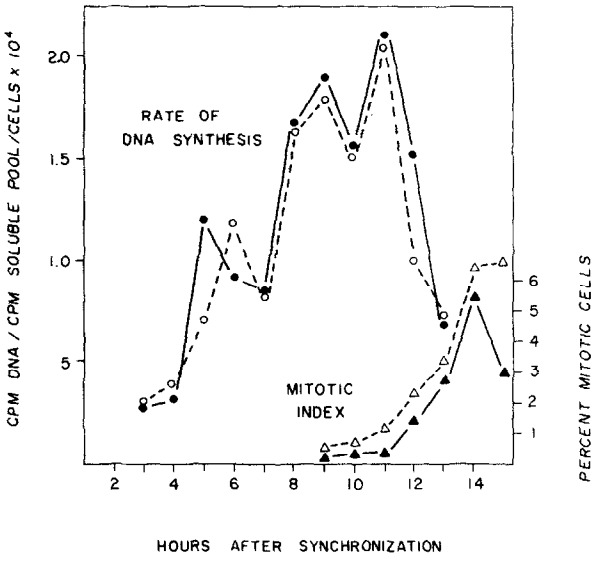


Figure 1. Incorporation of $H^3$-TdR into DNA and mitotic index during the cell cycle in control cells (--o,--Δ) and in cells administered $2 \times 10^{-4}$ M $(but)_2cAMP$ + $1.5 \times 10^{-5}$ M testosterone (—o,—Δ) at hour 0. $6 \times 10^5$ cells/vial were used. Points indicate average of duplicate samples.

treated cells (Fig. 1) shows a small depression in relation to the non-treated cells, suggesting the arrest of some cells late in the cell cycle.

The arrival of labelled cells at mitosis in hormone-treated cells is similar to that of the non-treated cells (Fig. 2A) for two waves of labelled mitoses (second wave not shown). It is evident therefore that the length of the cell cycle and each of its components is the same although in this experiment the mitotic index for the treated cells is much lower (unpublished data). These experiments indicate that the increased doubling time of hormone-treated cells is not due to an increase in the cell cycle length, but must be due to a decrease in the number of cells traversing the cycle.

In order to ascertain where cells exit the cycle, hormone-treated cells and cells in stationary phase were released with fresh medium (Fig. 2B). In this way the arrest of cells at any point in the cell cycle should be revealed by an increase in the mitotic index at times thereafter. The greatest increase in mitotic index for released stationary cells (II) is attributable to cells blocked in the G1 phase, whereas the greatest increase for treated cells (I) occurs within one hour after addition of fresh medium, indicating arrest in G2. Arrest, or delay, in the treated cells also occurs at two points in the S phase and in the G1 phase. The G1 peak in the hormone-treated cells is artificially depressed by averaging separate experiments in which there was a 2-3 hr difference in the time of a G1 increase in the mitotic index.

Determinations of DNA quantity (Table 1) on stationary phase, untreated cells (medium depleted) show values identical with those of G1 cells. Confluent, treated cells have almost double the G1 value, indicating an arrest of most, if not all, cells in the G2 phase. Arrest, or delay, in the cell cycle in the presence of these hormones is dependent upon, or to some extent mediated by, cell contact or interaction. This is indicated by an increase in the DNA value as cell density increases.

## DISCUSSION

Willingham *et al.* (16) have shown that $(but)_2$cAMP can inhibit the progression of cell cycle traverse in the G2 and early G1 phase, and Burger *et al.* (17) demonstrated a large decrease in

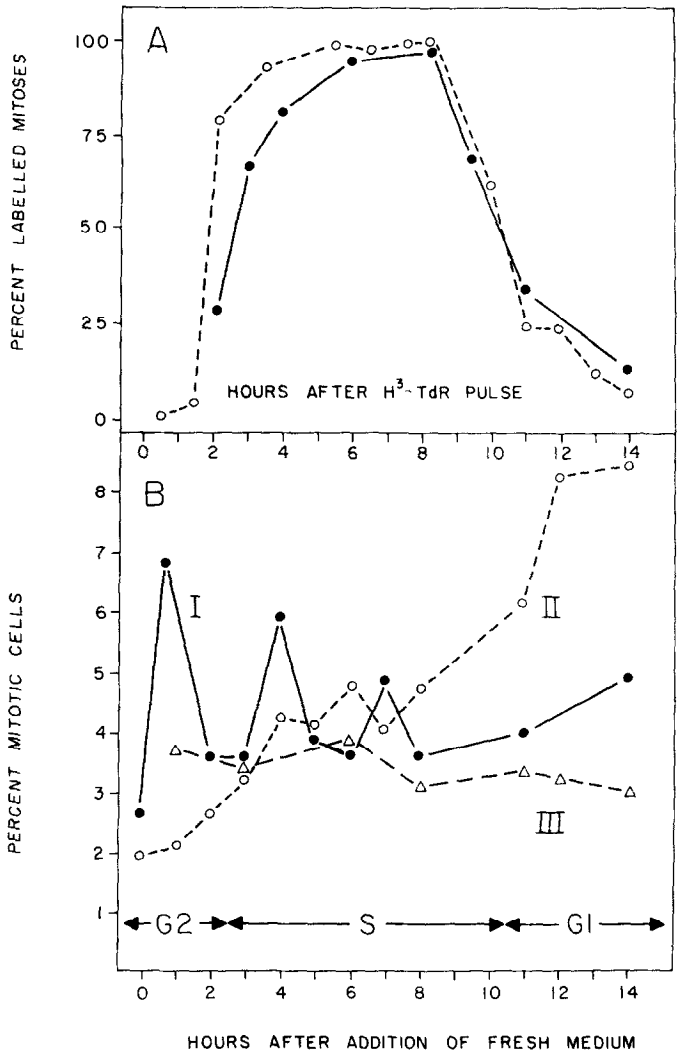


Figure 2A. Percent labelled mitoses in hormone-treated [1 X $10^{-4}$ M $(but)_2cAMP$ + 1.5 X $10^{-5}$ M testosterone; —o] and untreated (--o) cells. 6 X $10^5$ log-phase cells were subcultured into 25 $cm^2$ Falcon flasks and half were administered hormone. 15 hrs later cells were pulse-labelled for 15 min with 5 µCi/ml $H^3$-TdR and were washed and chased in medium supplemented with 5 µg/ml unlabelled thymidine. $(But)_2cAMP$ and testosterone were re-added to half the cultures at the end of the pulse, and samples were collected for autoradiography at intervals thereafter.

B. Mitotic index of cells released from hormone-treatment [1 X $10^{-4}$ M $(but)_2cAMP$ + 1.5 X $10^{-5}$ M testosterone] or stationary phase by the addition of fresh medium. Three days prior to the release by fresh medium cells were subcultured into 25 $cm^2$ flasks. Non-treated cells (II,--o) grew to confluency in non-replenished medium. Cells treated (—o, I) with hormone 24 hrs prior were approaching confluency at the time of release. Log-phase cells (III,-Δ). Colcemid was added 1 hr prior to collection. Average of two similar experiments.

Table 1. Quantity of DNA (units fluorescence/$10^3$ cells) for G1 cells, hormone-treated cells [$10^{-4}$ M $(but)_2$cAMP + 1.5 X $10^{-5}$ M testosterone, three days] and untreated cells at different cell densities. Standard error indicated.

TABLE 1

| Growth Condition | Cell Density ($10^4$ cells/$cm^2$) | Hormone | DNA Value (Fluor./$10^3$ cells |
|---|---|---|---|
| 1) Exponential | Light (0.6-2.0) | - | 14.0 ± 2.5 |
| 2) Exponential | Light (2.6-3.4) | + | 12.8 ± 0.3 |
| 3) Exponential | Medium (4.7-9.6) | - | 14.8 ± 0.5 |
| 4) Exponential | Medium (5.6-6.0) | + | 17.0 ± 0.4 |
| 5) Stationary (confluent) | Heavy (15.9-16.5) | - | 10.6 ± 0.5 |
| 6) Stationary (confluent) | Heavy (8.0-9.7) | + | 19.4 ± 0.1 |
| 7) G1 Cells (>88%) | ___ | - | 10.9 ± 0.8 |

cellular cAMP levels around the time of mitosis (G2-M-early G1) in 3T3 cells. Our study indicates that the progression of the cell cycle can be regulated by $(but)_2$cAMP and testosterone in the G1 and G2 phases in CHO cells. Confluent, hormone-treated cells ultimately come to rest in the G2 phase. The decrease in $H^3$-thymidine incorporation into DNA that we and others (18) have observed in dividing cultures treated with $(but)_2$cAMP can be attributed to the exit of increasing numbers of cells from the cell cycle. It is significant that cell density is a factor in this process. The importance of the G2 phase for growth regulation in other cell types has been discussed (19).

It is pertinent to the induced change in CHO cell morphology (8) that microtubule protein, which functions in the maintenance of fibroblastic morphology (19), is synthesized predominately in

the late S and G2 phases (20). The change in the differentiation state of hormone-treated CHO cells (collagen synthesis) under the conditions described by Hsie *et al.* (9) evidently must occur from the G2 phase of the cell cycle. In this respect the differentiation of *Dictyostelium* from either the G1 or G2 phases of the cell cycle depending upon nutritional conditions (22) is of interest.

It may be significant that the times of S delay (Fig. 2B) occur at 4 and 7 hrs from the end of the cell cycle when troughs in the rate of DNA synthesis are observed. It appears likely that the DNA of CHO cells is synthesized in three large families of synchronously replicating units (23,15) and that arrest, or delay, can occur before the initiation of synthesis of each round of replication. The increase in the mitotic index attributable to cells delayed at two points in the S phase (Fig. 2B) may indicate the existence of transient fluctuations in endogenous cAMP levels at these times. It is also possible that fine growth control during the S phase is lost in established cell lines.

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