CONTROL OF DNA SYNTHESIS AND MITOSIS IN 3T3 CELLS BY CYCLIC AMP

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SUMMARY: DNA synthesis and mitosis in synchronized Swiss 3T3-4 and Balb 3T3 cells is prevented by the addition of $(but)_2$cAMP during the early G_1 phase of the cell cycle. Removal of $(but)_2$cAMP at up to eleven hours, or addition of $(but)_2$cAMP as early as three hours after planting fails to block DNA synthesis. However, in Swiss 3T3-4 cells, but not Balb 3T3 C1A31 cells, the addition or removal of $(but)_2$cAMP at three or six hours after planting causes DNA synthesis to reach a peak at an earlier time. Adding $(but)_2$cAMP in early S-phase does not inhibit DNA synthesis, but prevents subsequent mitosis. This indicates the presence of a cyclic AMP-dependent block in G_2. Our results suggest that cyclic AMP has at least three regulatory functions during the cell cycle, two negative and one positive.

INTRODUCTION

Previous studies have shown that 3T3 cells at confluency demonstrate contact inhibition of DNA synthesis and mitotic activity and are arrested in the early G_1 phase of the cell cycle (8,4). Three lines of evidence suggest that adenosine-3',5'-monophosphate (cyclic AMP) regulates this phenomenon. Treatment of 3T3 cells with $N^6,O^{2'}$ - dibutyryl cyclic AMP [$(but)_2$cAMP] decreases their saturation density (3), intracellular levels of cyclic AMP rise sharply at confluency (5), and finally stimulation of confluent 3T3 cells by serum addition (8) or trypsinization with replanting (4) produces a synchonous wave of DNA synthesis and mitotic activity which is preceeded by a fall in the intracellular levels of cyclic AMP (6).

The purpose of this study was to investigate the effects of $(but)_2$cAMP on DNA synthesis and mitosis in 3T3 cells synchronized by trypsinization and replanting. Since $(but)_2$cAMP presumably acts by increasing intracellular cyclic AMP levels (1), it should prevent the lowering of cyclic AMP levels and thus

prevent synchronous DNA synthesis and mitosis. This would support the hypothesis that intracellular cyclic-AMP levels in the early G_1 phase are an important factor in the growth control of confluent 3T3 cells.

MATERIALS AND METHODS

Swiss 3T3-4 mouse embryo fibroblasts were supplied by Dr. Howard Green. Balb 3T3 ClA31 fibroblasts were supplied by Dr. Allan Rein. They were propagated in Dulbecco-Vogt's modified Eagle's medium supplemented with penicillin - streptomycin (50 units/ml), and 10% calf serum (Flow Laboratories) at 37° under an atmosphere of 95% air, 5% CO_2. Trypsin (Microbiological Associates) in T-D buffer at 0.25% was used in all experiments. Methyl-^{3}H-thymidine (6C/mM) was obtained from Schwarz/Mann. $(But)_2$cAMP was purified as previously described (2).

Confluent 3T3-4 cells were incubated without media change for 3-4 days and then trypsinized. The cells were resuspended in media after centrifugation and, if required by the experiment, were divided in two aliquots, one containing $(but)_2$cAMP (1-1.5mM). Equal numbers of cells (the number varying with the experiment) were pipetted into 20 cm^2 dishes (Falcon Plastics) and incubated at 37°. At designated times (3, 6, 11, 12 hr) the medium on every dish was changed. Each experiment consisted of a control group and an experimental group treated with $(but)_2$cAMP, both coming from the same pool of trypsinized cells.

After appropriate incubation, one dish from each group was removed and its medium replaced with labelled medium. If $(but)_2$cAMP was to be present at the time of labelling, it was included in the labelled media of the treated group. After one hr incubation at 37°, the medium was removed and each dish was rinsed four times with 5-10 ml phosphate buffered saline (PBS).

Cells were trypsinized with 0.25% trypsin, and, after centrifugation, were resuspended in PBS. An aliquot was removed for cell counting (Particle Data model #112TA particle counter), and the remainder was precipitated with 5% trichloracetic acid (TCA) on ice and washed four times with 5 ml of 5% TCA.

The precipitate was collected on glass fiber filters or recovered by centrifugation. Both methods yielded identical results. The precipitated DNA was mixed with 0.5 ml NCS (Amersham/Searle) and heated to 60° for 10 - 15 min. A mixture of the solubilized pellet and 10 ml of toluene-Liquifluor (New England Nuclear) solution was counted in a Packard model #3375 liquid scintillation spectrometer. In some experiments, 5% TCA was added directly to the dish without trypsinization and the cells were removed by scraping. After centrifugation the supernatant was added to a counting vial with 10 ml of Aquasol (New England Nuclear) as an index of the TCA-soluble fraction.

DNA synthesis during the first S-phase period after replanting was routinely measured, and in most experiments cell numbers and/or (^{3}H)-thymidine incorporation was also measured during a portion of the second cycle.

RESULTS

Cell number: The addition of $(but)_2$cAMP to the cells at the time of planting or up to 12 hr following planting prevented the cells from entering mitosis at the end of the first cycle (Table 1). However, a short three hr treatment with $(but)_2$cAMP in early G_1 seemed to have no effect on mitosis (Table 2). A longer eleven hr treatment did inhibit mitosis partially, perhaps due to the related decrease in DNA synthesis prior to mitosis in the treated cells (discussed below).

The number of cells remained constant during the first cycle in all experiments, and no difference was noted between control and $(but)_2$cAMP treated dishes in any experiment. This indicated that $(but)_2$cAMP did not alter plating efficiency.

DNA synthesis: The (^{3}H)-thymidine incorporation is shown in Fig. 1. $(But)_2$cAMP (1mM) added at the time of planting markedly diminished the synchronous DNA synthesis peak that occurred about 28 hours after planting (Fig. 1A). When the addition of $(but)_2$cAMP was delayed until 12 hours, no inhibition was observed (Fig. 1D). This indicates that $(but)_2$cAMP acts at a step prior to the beginning of DNA synthesis. If $(but)_2$cAMP is added at the time of planting and removed at

Table I. Percentage of Cells Subsequently Dividing at the End of the First Cell Cycle. The number of cells present during S-phase of the second cell cycle were compared to the number present during the first cell cycle S-phase, the difference being expressed as the percentage increase in total cell number. The separate experiments represent differences in the time of addition of $(but)_2cAMP$ to the treated group.

Table I

Experiment Number (from Fig. I)	Time of $(But)_2cAMP$ Addition (hrs after planting)	% of Cells Subsequently Dividing $(But)_2cAMP$	
		-	+
C	+ 6	88%	0%
D	+12	45%	5%

Table II. The experiment was performed as in Table I except that $(but)_2cAMP$ was added at planting and removed at 3 and 11 hours.

Table II

Experiment Number (from Fig. I)	Time of $(But)_2cAMP$ Removal (hrs after planting)	% of Cells Subsequently Dividing $(But)_2cAMP$	
		-	+
F	+ 3	44%	44%
H	+11	117%	14%

eleven hours, just before S-phase (Fig. I, H) DNA synthesis occurs at the usual time, although the amount is diminished.

In contract to this inhibitory effect, if $(but)_2cAMP$ was added at 3 or 6 hr after planting, it failed to block DNA synthesis (Fig. I, B, C), and DNA synthesis actually peaked earlier than in the control. Similarly, if $(but)_2cAMP$ was added at planting, and then removed at +3 or +6 hr (Fig. I, F, G), the treated group showed an earlier peak of synthesis. This advanced wave of DNA synthesis observed with $(but)_2cAMP$ addition at 3 hours after planting was observed with Swiss 3T3-4 cells but not Balb 3T3 cells (results not shown).

At 28 hours after planting, the uptake of ^3H-thymidine into the TCA soluble fraction was the same in treated and control cells, even though the

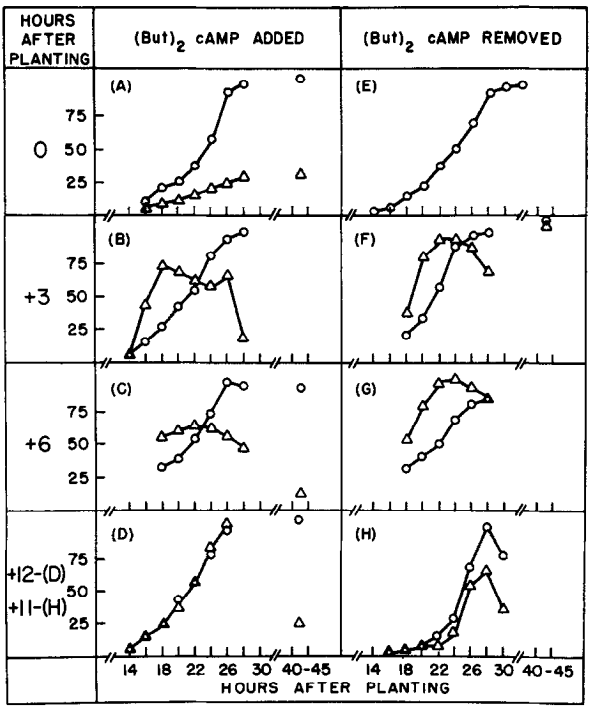

Figure I. ^{3}H-thymidine incorporation into DNA after one hour incubation at the times indicated after trypsinization and replanting. Each experiment is shown separately with control (●) and (but)$_2$cAMP-treated (▲) values after addition (A, B, C, D) or removal (E, F, G, H) of 1-1.5 mM (but)$_2$cAMP at the times indicated after planting (0, +3, +6, +11, or +12 hr).

incorporation of ^{3}H-thymidine into DNA was markedly decreased in the treated group.

DISCUSSION

Confluent 3T3 cells are thought to be arrested in G_1 (4), since trypsinization and replanting leads to synchronous DNA synthesis followed by mitosis. Confluent 3T3-4 cells have higher cyclic AMP levels than logarithmically growing cells (5), and the addition of serum or trypsin to these cells, which stimulates cell growth, results in a fall in cAMP levels (6). These facts plus the inhibition of DNA synthesis and mitosis by addition of (but)$_2$cAMP to trypsinized 3T3 cells observed in this study argue strongly that elevated intra-

cellular levels of cAMP are important in the inhibition of cell growth at confluency. With 3T3-4 cells, this sensitivity to $(but)_2$cAMP repression passes after an interval of less than three hours.

In addition to this blockage of cell growth in early G_1, cyclic AMP appears to have at least two additional regulatory functions in the cell cycle. The second cyclic AMP-sensitive mechanism appears in the three to six-hour period. When $(but)_2$cAMP is added at this time, the cells show an earlier peak of DNA synthesis.

Cyclic AMP also appears to have an inhibitory function in G_2 phase when $(but)_2$cAMP is added at the beginning of DNA synthesis. The nucleotide fails to affect S-phase itself (12 hours), but does inhibit mitosis at the end of the first cycle. Thus the nucleotide may be useful in obtaining cells arrested in G_2.

The results of this study should be useful in elucidating the biochemical events in the cell cycle.

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