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Cell cycle regulation in mammalian cells: hormones and commitment to DNA synthesis

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Regulación del ciclo celular en células de mamíferos: hormonas y compromiso con la síntesis de DNA

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The growth regulation of cultured mouse fibroblasts and functional adrenal cells was studied. Variants or mutants from these cell lines were obtained. The effects of classical hormones (insulin, hydrocortisone and adrenocorticotropin) and of growth factors (EGF and PF) were analysed. These hormones stimulate or inhibit the entry of cells into S phase. However G_1 cells become irreversibly committed to DNA synthesis 5 hours before entering S phase.

FIBROBLASTS/ADRENAL CELLS/HORMONES/GROWTH FACTORS

Hormones and hormone-like growth factors are the basic extracellular regulators of mammalian cell proliferation. Cell cultures are useful experimental models to study the mechanisms of action of these regulators (Armelin, 1973 and 1975; Armelin and Armelin, 1975). Lines of mouse fibroblasts and mouse functional adrenal cells were used as model systems. Hormones and growth factors which are active on these cells were studied.

The results recently obtained indicate that these regulators control cell proliferation by stimulating or inhibiting the entry of cells into S phase (or DNA synthesis), a step which we have been refering to as the $G_0 \rightarrow G_1$ transition.

MATERIALS AND METHODS

a) *Cells:* Swiss mouse embryo 3T3 fibroblasts (Todaro and Green, 1963) and Y-1 mouse adrenal cells (Yasumura *et al.*, 1966) were originally obtained from the American Type Culture Collection. ST1 mouse fibroblasts were derived from the 3T3 line (Armelin and Armelin, 1977). Culture procedures are described elsewhere (Armelin *et al.*, 1977; Armelin and Armelin, 1978).

b) *Cell countings, ^{3}H-thymidine uptake into DNA and autoradiography:* acoording to methods described before (Armelin 1973; Armelin *et al.*, 1977).

c) *Serum starved cells* were obtained using the following protocol: cells were plated in 3% fetal calf serum medium, at $1.5—2.0 \times 10^4$ cells/cm^2 onto plates orwashed glass coverslips. After 24 hours cultures were eashed with phosphate buffered saline and fresh 0.2% serum medium was added. Upon a 24 hours incubation period the cultures were considered "serum starved" and were then used for the experiments.

d) *Hormones and growth factors:* crystalline insulin and hydrocortisone were obtained from Sigma Chem. Co. and Nutritional Biochemical Corp. respectively. ACTH was the Acthar from Armour Pharmaceutical Co. EGF is a purified preparation of mouse epidermal growth factor (Cohen, 1962). PF is a preparation of pituitary growth factors obtained from frozen bovine pituitary gland by a process described elsewhere (Armelin *et al.*, 1977). Prostaglandin $F_{2\alpha}$-tromethamine salt ($PGF_{2\alpha}$) was from The Upjohn Co. Partially purified somatomedin B and highly pure somatomedin C were kindly provided by Dr. J Van Wyck.

RESULTS AND DISCUSSION

a) *Cell cycle regulation in fibroblasts*

Serum starvation causes fibroblasts to accumulate in the G_0 state. Serum step up stimulates fibroblasts to leave G_0 towards S phase (Fig. 1). This cell response is characterized by two

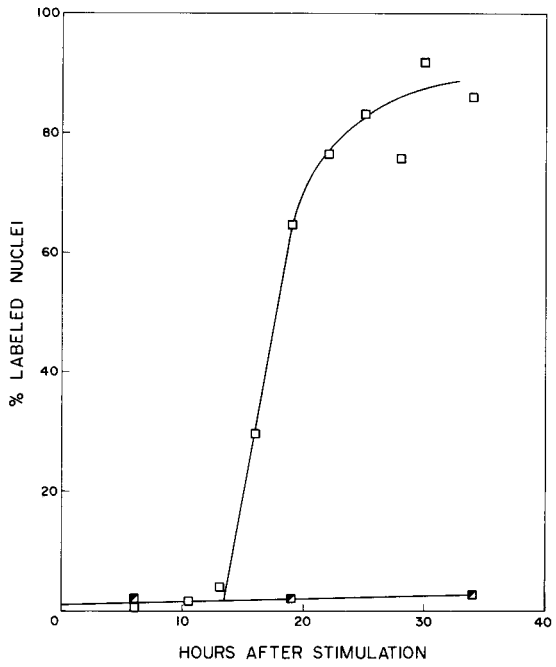

Fig. 1. Restimulation of serum starved fibroblasts, kinetics of the response. Serum starved hydrocortisone ST1 treated cells. Five % fetal calf serum was added at zero time; ^{3}H-thymidine added at the same time. Labeled nuclei estimated by autoradiography.

parameters: the lag before the onset of DNA synthesis and the rate at which cells enter S phase. The lag before DNA synthesis is constant and independent of the concentration of serum used for restimulation. On the other hand cells leave G_0 following a first order kinetics (Figure 2) and the rate constant increases with increasing serum concentrations.

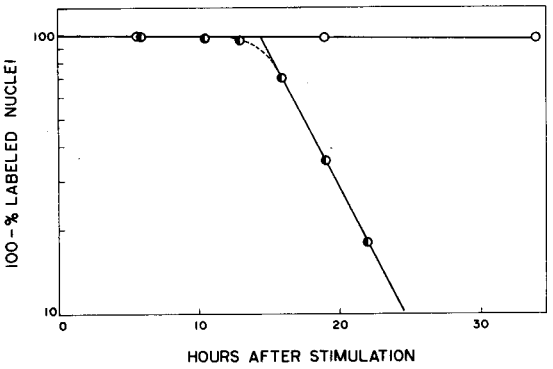

Fig. 2. Data of Figure 1 replotted in a semi-log scale.

The serum action described above is due to hormones and hormone like factors (Armelin, 1973 and 1975). A number of factors active with the 3T3 line of mouse fibroblasts have been identified (Table I). All of these factors stimulate 3T3 cells in the G_0 state to enter S phase.

TABLE I

Hormones and factors active on 3T3 fibroblasts and on the mutant line ST1

Hormones or Factor	*Cells* 3T3	ST1
EGF	S	—
PF	S	—
Insulin	S	S[b]
Somatomedin B and C	S	—
Hydrocortisone	S	I[c]
$FGF_{2\alpha}$	S	—
BSA[a]	S	S

S stimulatory; *I* inhibitory; -inactive; *a* bovine serum albumin: activity due to a contaminant; *b* only at high doses; *c* at physiological doses. For further informations see Armelin and Armelin, 1978.

The kinetics of this cell response (Figure 3) shows the same pattern presented by serum stimulation, namely: a) the lag before DNA synthesis is constant and independent of the concentration or the type of stimulator, and b) the rate of cell entry into S phase increases with increasing concentrations of the factor or with com-

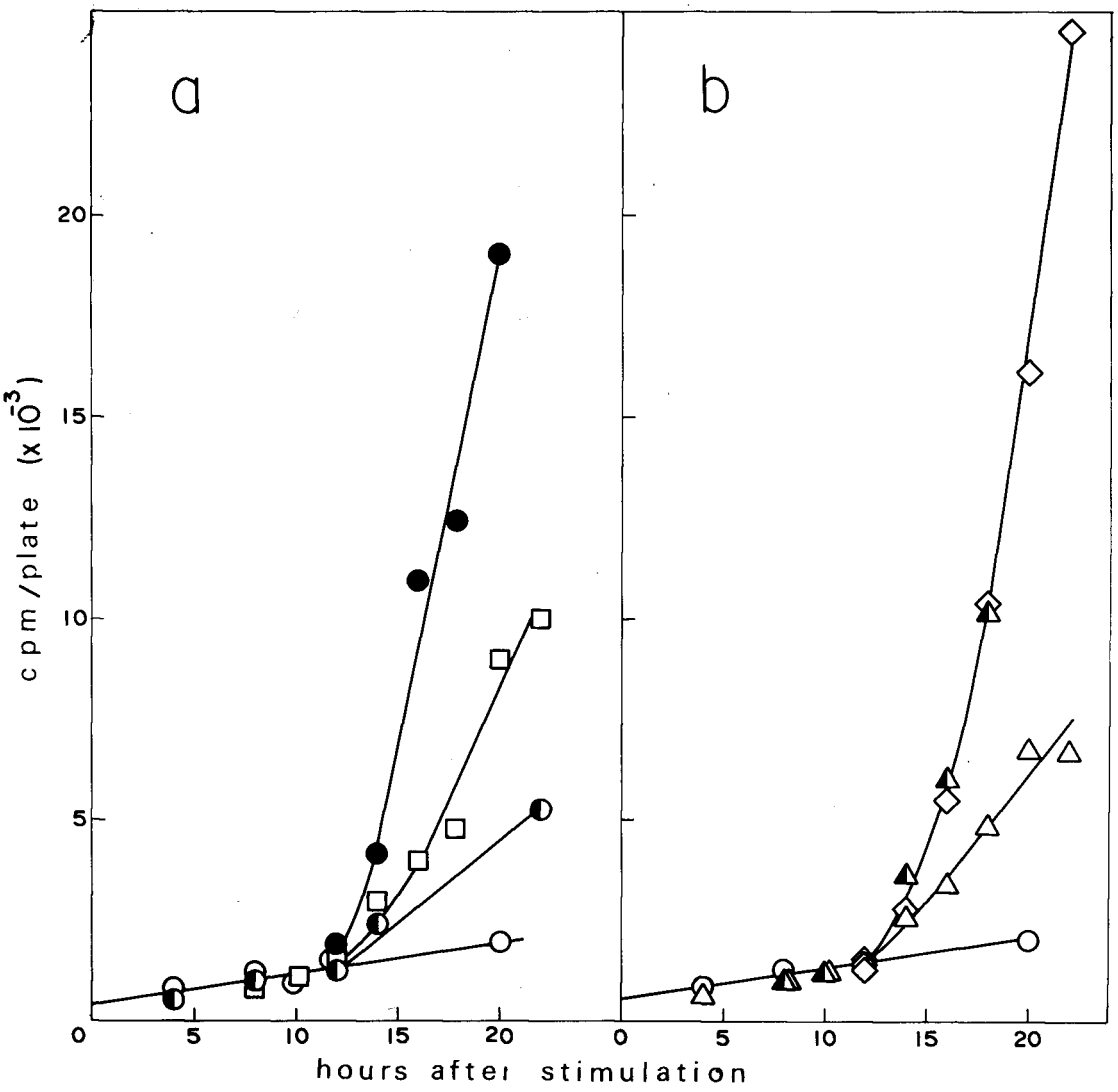

Fig. 3. Restimulation of serum starved 3T3 cells by several factors and serum: kinetics of the response (^{3}H-thymidine incorporation into DNA). At time zero. o——o Control; o——o EGF (0.1 μg/ml); □—□ insulin (2.5 μg/ml); △_△ PF (1 μg/ml; ●——● EGF + PF + insulin; ▲-▲ PF + hydrocortisone, ◇—◇ 10% fetal calf serum. Lag before onset of DNA synthesis: 13 hours.

binations of different factors. These results suggest that stimulating factors increase the probability of cells leaving G_0 without interfering with the time required to reach S phase. This interpretation is further supported by the data of Fig. 4. In this case resting 3T3 cells were stimulated with PF at zero time and, 5 hours later, with hydrocortisone plus insulin. Fig. 4 shows that the curve for cells' entry into S phase is biphasic presenting, initially, a slow rate and, 5 hours later, the rate characteristic for the combination of PF, hydrocortisone and insulin.

The results presented above suggest a rather complex picture for growth regulation of 3T3 cells by hormones and growth factors. The recog-

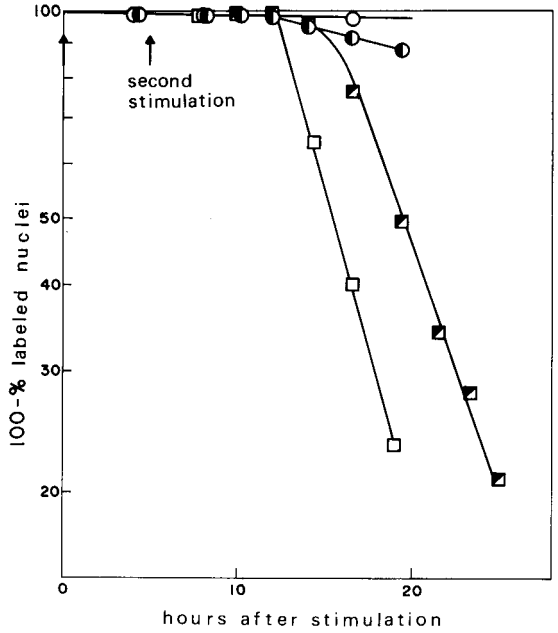

Fig. 4. Kinetics of the restimulation of serum starved 3T3 cells by PF, insulin and hydrocortisone. ○——○ Control; ◐——◐ PF (1 μg/ml) added at time zero; □——□ PF + hidro-cortisone (0.3 μg/ml) + insulin (1 μg/ml) combined and added at time zero; ◪——◪ PF added at time zero and hydrocortisone + insulin at t = 5 hours. ^{3}H-thymidine added at time zero to all cultures. Lag before onset of DNA synthesis: 12 hours.

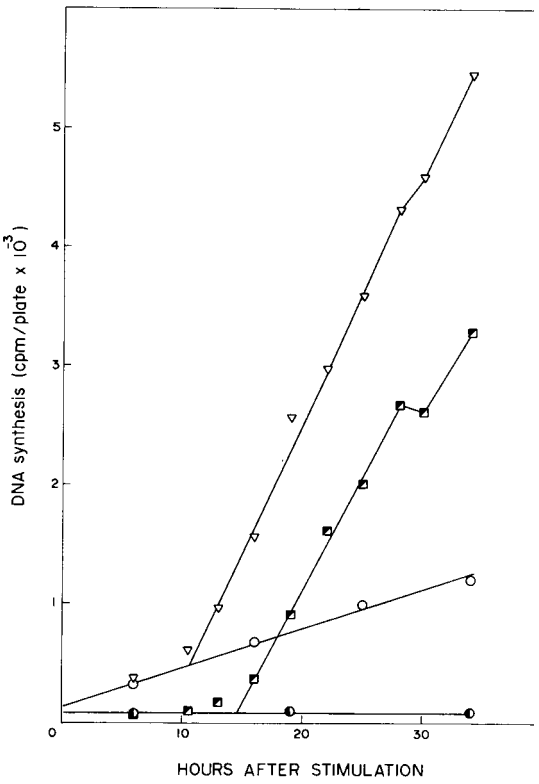

Fig. 5. Restimulation of serum starved STl cells treate and untreated with hydrocortisone. Serum step up (0.2% to 5%) and ^{3}H-thymidine addition at time zero. ○——○ 0.2% serum (control, untreated); △——△ 5% serum; ◐——◐ 0.2% serum + hydrocortisone (control, treated); ◪——◪ 5% serum + hydrocortisone.

nition of this fact led us to search for variants or mutants of 3T3 with different responses towards these hormones or factors. With this approach in mind we isolated the line STl (Armelin and Armelin, 1977), a transformant derived from our 3T3 strain, showing response to just a few hormones (Table 1). Hydrocortisone, particularly, has a striking effect on STl cells. Upon serum step down these cells keep synthesizing DNA at a significant level. But in the presence of hydrocortisone serum step down leads to negligible levels of DNA synthesis and cells enter a strict resting state. Moreover, when serum starved cells are restimulated by serum step up, the outcome is different depending on the presence or absence of hydrocortisone: glucocorticoid treated cells present a lag of 14.5 hours before DNA synthesis whereas untreated cells show a lag of 10.5 hours. These results suggested to us that the steroird hormone was resetting the controls of the $G_0 \rightarrow G_1$ transition in STl cells.

Actually, further studies indicated that hydrocortisone induces a reversible transitior between a transformed and a "normal" phenotype in STl cells (Armelin and Armelin 1977b 1978). An important point to emphasize here is that hydrocortisone only interferes with cells which are post-mitotic and pre-replicative STl fibroblasts growing exponentially in 5% serum, present a mean doubling time (T_D) equal to 15 hours and estimates of T_{G1}, T_S T_{G_2} and T_M equal to 6.0, 6.1, 2.4 and 0.(hours, respectively. On the other hand, for cells growing in 5% serum medium plus hydrocortison T_D and T_{G1} are equal to 20 and 11 hours respectively, whereas T_S, $T_{G_2}$and T_M are the same. When hydrocortisone was addec to exponential cultures in 5% serum medium, the labeling index (after ^{3}H-thymidine pulses) did not change for the first 5 hours. These results indicate that cells which are 5 hours before S phase are not affected by the hormone action.

Measurements of intracellular levels of cAMP in the absence or presence of hydrocortisone gave identical results showing that this hormone does not lead to changes in the levels of this cyclic nucleotide. Therefore the effects of hydrocortisone on STl cells are not secondary effects due to changes in cAMP levels.

In the context of the results presented above it is worth considering the effect of serum step down on the labeling index of 3T3 and STl cells. Pulses of ^{3}H-thymidine (30 minutes) after serum step down (5% to 0.2%) showed that the labeling index does not change during the first 5 hours. This observation indicates that 3T3 and STl cells which are 5 hours before S phase are already committed to DNA synthesis.

TABLE II

Estimates of the lag before DNA synthesis stimulation in serum starved Y-1 cells

Experiment	*Stimulator*	*Lag (in hours) before DNA synthesis stimulation*
1	10% serum	11.2
	1 μg/ml PF	12.0
2	5% serum	11.2
	1% serum	11.2
	1 μg/ml PF	11.0
3	5% serum	10.5
	0.75% serum	10.0
	1 μg/ml PF	10.5

Figures on the table were obtained from kinetic experiments like those described in Figures 1 through 5 for fibroblasts.

b) *Cell cycle regulation in adrenal cells*

The pattern of regulation we observed for fibroblasts seems to repeat for adrenal cells. In the case of the line Y-1 of functional adrenal cells, the only factors we found to be active are the classical hormone adrenocorticotropin (ACTH) and a novel pituitary protein factor (Armelin *et al.*, 1977). ACTH inhibits DNA synthesis and growth in Y-l cells whereas PF stimulates them.

DNA synthesis of serum starved Y-1 cells can be restimulated with serum or PF. The onset of DNA synthesis occurs after a lag of 11 hours irrespective of the kind or concentration of the stimulator: serum or PF (Table III). ACTH completely blocks the restimulation given by serum or PF if added to medium up to 6 hours after stimulation; later additions of ACTH allows the stimulation to be expressed with increasing magnitudes (Armelin *et al.*, 1977). Therefore cells become resistant to ACTH 5 hours before entering S phase.

ACTH has also been added to exponentially growing cultures of Y-1 cells in 10% serum medium. ^{3}H-thymidine pulses showed that the labeling index did not change during the first 5 hours but then fell continuously to essentially zero at approximately 12 hours after ACTH addition. During the first 15 hours of ACTH treatment the mitotic index did not change. These results demonstrate that ACTH blocks exclusively post-mitotic cells which are 5 hours or more apart from S phase.

cAMP (10^{-3} M) mimics the inhibitory activity of ACTH. However, differently from ACTH, cAMP blocks at two points of the cell cycle: G_1 and G_2.

We have been selecting for ACTH resistant Y-1 cells and recently obtained the line AR-1. DNA synthesis of serum starved AR-1 cells can be restimulated with either serum or PF. The entry of AR-1 cells into S phase is not blocked by ACTH or cAMP. However cAMP still arrests AR-1 cells at the G_2 phase. This new cell type is of obvious interest for studies of the mechanisms of action of ACTH and cAMP.

Concluding remarks

We propose to represent the cell cycle by the schemme of Figure 6. G_0 is assumed as an

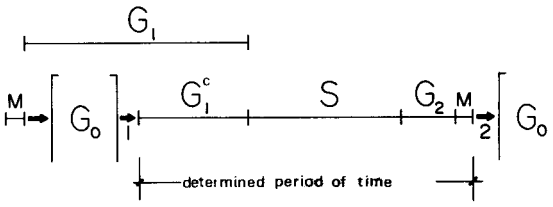

Fig. 6. Schematic model for the cell cycle. G_o is an obligatory step after mitosis (M). Cells remain in G_o for an undefined period of time. Upon stimulation cells leave G_o randomly. Cells in G_1^c are irreversibly committed to DNA synthesis and cell division. Therefore the critical step in the control of the cell cycle is the transition G_o to G_1^c (transition 1). Transition 2 (M → G_o) occurs obligatorily after cell division.

obligatory step after mitosis. This as already been proposed by Burns and Tannock (1970). In our view, when cells enter G_1^c they are irreversibly committed to DNA synthesis and cell division. Regulation of growth is at the level of G_0 cells. Hormones or growth factors control proliferation by stimulating or inhibiting the transition $|G_1^c| \rightarrow G_1^c$. From the results described above we estimate that G_1^c is equal to 5 hours.

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REFERENCES

ARMELIN, H.A. Proc. Nat. Acad. Sci. USA. *70:*2702, 1973.

ARMELIN, H.A. *in* Biochemical Actions of Hormones *3:*1 (Ed. G. Litwack), Academic Press, 1975.

ARMELIN, H.A. and ARMELIN, M.C.S. Biochem. Biophys. Res. Comm. *62:*260, 1975.

ARMELIN, M.C.S. and ARMELIN, H.A. Nature *265:*148, 1977.

ARMELIN, M.C.S. and ARMELIN, H.A. Proc. Nat. Acad. Sci. USA *75:* 2805, 1978.

ARMELIN, M.C.S., GAMBARINI, A.G. and ARMELIN, H.A.J. Cell Physiol. *48:*1, 1977.

BURNS, F.J. and TANNOCK, I.F. Cell Tissue Kinet. *3:*321, 1970.

COHEN, S. J. Biol. Chem. *237:*1555, 1962.

TODARO, G. and GREEN, H. J. Cell. Biol. *17,* 299, 1963.

YASUMURA, Y., BUONASSISSI, V. and SATO, G. Cancer Res. *26:*529, 1966.