

Mitosis of Contact-Inhibited 3T3 Preadipocytes Precedes Chemically Induced Differentiation into Adipocytes (41672)

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Abstract. We studied the conversion of 3T3 cells into adipocytes *in vitro* after pulsed exposure of confluent, nongrowing cultures to various combinations of the "triggering" agents, methylisobutylxanthine, dexamethasone, and fetal calf serum. Conversion of nongrowing 3T3 preadipocytes into adipocytes takes place after the cells have been stimulated to undergo one round of cell division. Maximal cell division and cytodifferentiation occur only when all three triggering agents are present.

The role played by a specific round of cell division in the process of cytodifferentiation is unclear although a requirement for critical mitoses in the sequence of events leading to the differentiated state has long been postulated (1). We report here that confluent, nongrowing 3T3 preadipocytes biochemically induced to differentiate into adipocytes undergo one round of cell division prior to expression of the adipose phenotype. Furthermore, there is a direct correlation between the incidence of cell division and the extent of cytodifferentiation when cells are exposed to the various combinations of agents used to induce preadipocyte into adipocyte conversion.

During logarithmic growth the preadipocyte 3T3 cells are indistinguishable from other nonadipogenic 3T3 cells (2). A number of compounds including methylisobutylxanthine (MIX) (3), dexamethasone (DEX) (4), and an "adipogenic factor" (5) present in fetal calf serum (FCS) which appears to be growth hormone (6) play a role in effecting the differentiation of preadipocytes into adipocytes. Preadipocytes maintained during growth and confluence in medium supplemented with newborn calf serum (NCS) do not undergo the adipose conversion. When cultures grown to confluence with NCS are given a 2-day "pulsed" exposure to medium containing MIX, DEX, and FCS (MIX-DEX-FCS) (mi-

nus NCS), then refed with the original medium containing NCS, adipocyte clusters develop over the ensuing 3-5 days (7). Thus, a combination of three agents is required to instigate the cytodifferentiation process. These agents need only be present for 2 days to activate the process.

Rubin *et al.* (4) reported that MIX-DEX treatment of preadipocytes leads to an increase in cell number. Once we established that a 2-day treatment with MIX-DEX-FCS is sufficient to promote differentiation, we began studies to evaluate the role of cell division in the cytodifferentiation.

Methods. *Cell line and cell culture.* The 3T3 preadipocytes were isolated from the original stock of Swiss 3T3 fibroblasts (2). Cells were grown in Dulbecco modified Eagle's medium supplemented with 10% newborn calf serum (NCS). Petri-dish cultures were maintained in an atmosphere of 5% CO₂ in air at 37°C. Under these conditions the cells grow to confluence but do not become adipocytes. To induce conversion to adipocytes, medium was removed from confluent cultures and replaced with medium supplemented with 10% FCS, 0.25 μM DEX, and 0.5 mM MIX. Various combinations of serum, MIX, and DEX were tested to determine what combination of these agents is necessary to induce the round of cell division and conversion into adipocytes. Supplemented medium was left on the cells for 2 days, then removed, and replaced with a fresh lot of the original medium (without MIX, DEX, or FCS). At 5, 10, 17, 20.5, and 31 hr thereafter, cultures were analyzed to evaluate cell proliferation and adipocyte conversion.

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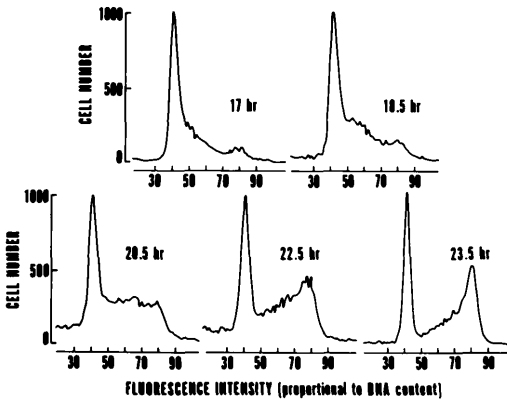


FIG. 1. Effect of MIX-DEX-FCS on DNA content in confluent 3T3 preadipocytes. Cultures were grown to confluence in medium supplemented with NCS. At $t = 0$, confluent, nongrowing cultures were fed with MIX-DEX-FCS (0.5 mM, 0.25 μ M, and 10%, respectively). At the indicated times, cells were harvested, stained with propidium iodide, and analyzed on an EPICS IV cell sorter. The DNA content in channels 40–41 corresponds to a DNA content of $2n$ (G_1) and the peak channels in 80–82 corresponds to a DNA content of $4n$ ($G_2 + M$).

Duplicate cultures were fixed in the petri dishes and stained to demonstrate fat. For flow microfluorometry, the cells were removed from the culture plates by trypsinization, centrifuged, supernatant was removed, and cells were resuspended by pipetting in phosphate-buffered saline (PBS).

Staining. To stain cells *in situ* in the petri dish, the supernatant culture medium was removed and the cell layer fixed by covering it with buffered 2.5% glutaraldehyde for 30 min. The fixing solution was then removed and the cell layer was covered with a 60% solution of oil red O in a 6:4 (v:v) mixture of isopropanol and water for 30 min. The stained, fixed preparation was examined using an inverted microscope. These preparations are semipermanent.

Propidium iodide, a DNA intercalating stain, was used for flow microfluorometry (FMF). The cell suspension in PBS was adjusted to contain $1-2 \times 10^6$ cell/ml. An equal volume of Vindelov's solution, which contains RNase, NP-40, and 7.5×10^{-5} M propidium iodide (8), was added and the suspension was allowed to stand at room temperature for 5 min or at refrigerator temperature for 10 min or longer before analysis by FMF.

TABLE I. EFFECT OF MIX-DEX-FCS ON CELL NUMBER^a

MIX-DEX-FCS	Time (hr)	Cell number $\times 10^{-6}$
—	48	1.3 ± 0.1
—	72	1.1 ± 0.1
—	96	1.3 ± 0.1
+	48	1.92 ± 0.12
+	72	2.12 ± 0.08
+	96	2.2 ± 0.1

^a Cells were grown to confluence in medium containing NCS. At $t = 0$, dishes were fed either with medium plus NCS or medium plus MIX-DEX-FCS. At the times indicated, cell number was determined using a Coulter counter. The numbers represent the average of duplicate determinations \pm the range.

Flow microfluorometry. Flow microfluorometry was used to develop frequency distribution histograms of DNA contents of large numbers of individual cells at various times after the addition of MIX-DEX-FCS to the confluent cultures. Cells were analyzed using an EPICS IV cell sorter (Coulter Electronics, Inc., Hialeah, Fla.) interfaced to a PDP 11/34A computer (Digital Equipment Corp., Maynard, Mass.) and a Tektronics 4025 graphics display terminal (Tektronics, Inc., Beaverton, Oreg.). The Epics was equipped with a Loxel 95 5-W argon-ion laser which

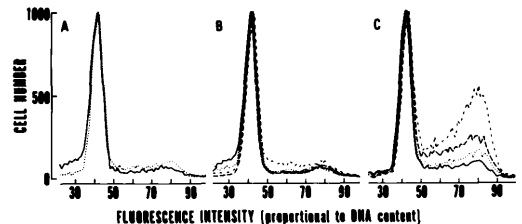


FIG. 2. Effect of combinations of MIX, DEX, FCS, and NCS on DNA content of 3T3 preadipocytes. All cultures were grown to confluence in medium supplemented with NCS. At $t = 0$ confluent, nongrowing cultures were fed with A: NCS (—) and FCS (---); B: NCS (—), NCS + MIX (---), NCS + DEX (— — —), and NCS + MIX + DEX (---); C: FCS (—), FCS + MIX (---), FCS + DEX (— — —), and FCS + MIX + DEX (---). At $t = 23$ hr cells were harvested, stained with propidium iodide, and analyzed on an EPICS IV cell sorter. The DNA peak in channels 40–41 corresponds to a DNA content of $2n$ (G_1) and the peak in channels 80–82 corresponds to a DNA content of $4n$ ($G_2 + M$).

TABLE II. EFFECT OF INDUCING AGENTS ON CELL CYCLE^a

Additive	Percentage in G ₁	Percentage in S + (G ₂ + M)
FCS	67.3	32.7
FCS + MIX	59.4	40.6
FCS + DEX	44.5	55.5
FCS + MIX + DEX	31.7	68.3

^a Computed from data illustrated in Fig. 2C by Dr. A. Brunsting, Coulter Electronics, Inc.

was tuned to 488 nm. A 510-nm blocking filter and a 630-nm-long pass filter were used in measuring the red fluorescence from pro-

pidium iodide bound to DNA. Flow rate was typically a few hundred cells per second.

Results. DNA histograms showed that by 17 hr after the addition of MIX-DEX-FCS to confluent cultures, preadipocyte cells have entered S phase, signaling the onset of a round of cell division (Fig. 1). By 48 hr the cell number nearly doubles in cultures with MIX-DEX-FCS (Table I). The increase in cell number invariably precedes the appearance of adipocyte clusters which develop between 24 and 72 hr after removal of MIX-DEX-FCS (7). When cultures are fed with medium plus NCS or FCS, relatively few cells enter the cell cycle (Fig. 2A). MIX and DEX singly

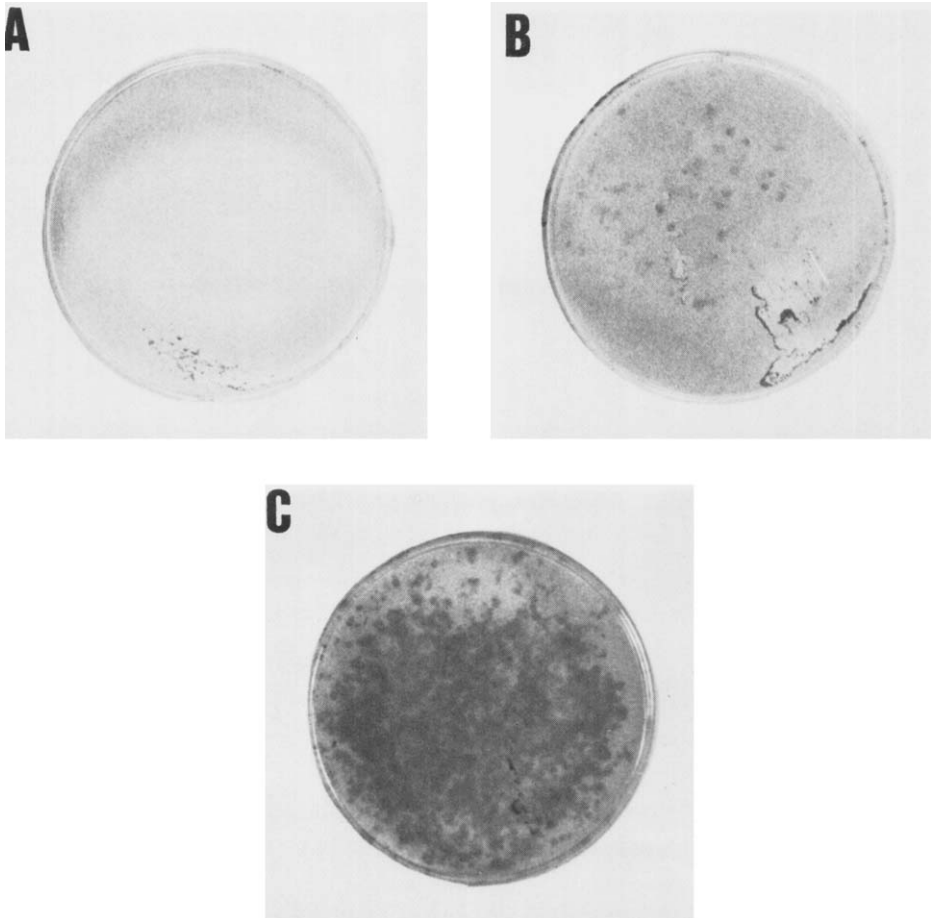


FIG. 3. Effect of NCS, NCS + MIX + DEX, and FCS + MIX + DEX on cytodifferentiation. Cultures were grown to confluence in medium containing NCS. At $t = 0$, cultures were pulsed for 48 hr with NCS (A), NCS + MIX + DEX (B), and FCS + MIX + DEX (C). At $t = 48$ hr, cultures were rinsed free of triggering agents and all cultures were fed with medium plus NCS. Five days later cultures were fixed, stained with oil red O, and photographed.

in the presence of NCS have negligible effect on cell cycle parameters the DNA histograms resembling those of confluent, untreated cells in which cell division is limited to that required to replace dead cells (Fig. 2B). The addition of MIX plus DEX in the presence of NCS promotes a slight increase in cycling cells (Fig. 2B). In the presence of FCS results are quite different: MIX and DEX, singly, promote an increase in cycling cells greater than that seen with FCS alone, in contrast to the ineffectiveness of these agents when present singly in medium containing NCS. In the experiment represented in Fig. 2C and Table II, the combination of MIX plus DEX in medium supplemented with FCS results in the cycling of 68% of the cells as determined by computer analysis of the data. As illustrated in Fig. 3A-C, the degree of preadipocyte to adipocyte conversion in cultures presented with the various combinations of additives is: MIX-DEX-FCS > MIX-DEX-NCS > NCS. MIX-DEX-FCS, which produces the highest degree of adipose conversion, also results in the greatest increase in cell number. In 3T3 C2 cells, a clone that does not undergo the adipose conversion, MIX-DEX-FCS does not promote an increase in cell number.

Though a majority of the cells undergo a single round of cell division in response to MIX-DEX-FCS (68% in Fig. 2C), there remains a question about the cells that do not enter S phase. Are they incapable of responding to the triggering stimulus? To answer this question we plan to utilize the EPICS IV cell sorter and study separately the resting cells that do not enter S phase after exposure to MIX-DEX-FCS and the cells that do enter S phase and prepare for cell division (S + G₂ and M).

Discussion. In studying the events that occur during the 2-day exposure of 3T3 cells to agents that promote their conversion into adipocytes, we have found that MIX-DEX-FCS promotes a single, synchronous round of cell division that is completed prior to the expres-

sion of the adipose phenotype. Experiments in which various combinations of MIX, DEX, NCS, and FCS were used showed that the entry of cells into cell cycle correlates directly with the subsequent accumulation of stainable fat. The small number of cycling cells and adipocyte clusters seen with MIX-DEX-NCS could be due to the presence of small amounts of adipogenic factor (growth hormone) (6) in NCS. Since the preadipocytes are contact-inhibited, hence are not growing prior to adding the triggering agents, there appears to be a correlation between the round of cell division and the ensuing cytodifferentiation.

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