PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 456–460 (1974)

Initiation of DNA Synthesis in Primary Fetal Rat Hepatocytes in Culture (37830)

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(Introduced by J. Salk)

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The regulation of growth of mammalian cells in tissue culture is effected by a complex balance of signals which reach the cell membrane, e.g., factors in serum which control the biochemical events in the cell cycle (1–5). One of the most interesting aspects is how DNA synthesis is controlled. Quiescent 3T3 mouse cells can be induced to synthesize DNA and undergo mitosis after addition of serum to a culture (6). DNA synthesis is detected following a lag period of approximately 14–16 hr after the initial stimulus has reached the cells. Similarly, after excision of 90% of the rat liver, DNA synthesis is induced in the liver remnant approximately 16 hr after partial hepatectomy (7, 8). Numerous experiments have provided suggestive evidence that one or more humoral agents regulate this response *in vivo* (9–12). Recently, evidence has been presented that DNA synthesis can be initiated in the liver of the intact animal after injection of a mixture of amino acids and hormones with a time course similar to the one observed after partial hepatectomy in the rat (13). In the present communication, evidence is presented that quiescent primary fetal rat liver (FRL) cells in culture can be induced to synthesize DNA and to divide by serum, ornithine, or arginine, suggesting that low-molecular-weight nutrients might be involved in the control of DNA synthesis.

Materials and Methods. Fetal rat liver cells were plated in 2 ml arginine-free Dulbecco and Vogt's modification of Eagle's medium in the presence of 1650 U penicillin and 33.3 μg streptomycin/ml, supplemented with 1.75% dialyzed fetal calf serum (dialyzed extensively vs isotonic NaCl solution) in 30-mm NUNC plastic dishes, and incubated at 37° in a humidified 10% CO_2 environment according to the plating procedure described elsewhere (14, 15). Arginine-free culture medium was used for all experiments. The rate of DNA synthesis was measured by determining the incorporation of [^{3}H]-thymidine (New England Nuclear) into trichloroacetic acid (TCA)-precipitable material. After cultures were incubated with [^{3}H]-thymidine (sp ac 20 Ci/mmole; final concentration 5 μM), the medium was removed, cultures were washed with Tris-buffered saline (pH 7.4), and incubated at 37° for 30 min in 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free Tris-buffered saline (pH 7.4). The resulting cell suspension was added into 1 vol of 10% TCA, and radioactivity retained on 2.4-cm Whatman GF/C glass fiber filters was determined in a Beckman scintillation counter. Autoradiograms were performed with Kodak AR-10 stripping film.

Results and Discussion. It has been shown that FRL cells grow in arginine-deficient medium in the presence of dialyzed fetal calf serum (14). When FRL cells are plated in 1.75% dialyzed fetal calf serum in arginine-deficient medium, DNA synthesizing activity gradually decreases until the cells are quiescent 10–13 days after plating. After addition of 10% dialyzed fetal calf serum to the cultures, DNA synthesis is initiated as shown by the appearance of labeled nuclei in autoradiograms or increase of TCA-precipitable radioactivity after pulse labeling with [^{3}H]-thymidine (Table I). It can be seen that DNA synthesis starts 14–16 hr after serum

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TABLE I. Initiation of DNA Synthesis in Quiescent Fetal Rat Liver Cells by Serum.[a]

Hours after serum addition	cpm per culture	% Nuclei labeled
0–3	105	≤1
3–6	115	≤1
6–9	120	≤1
9–12	90	≤1
12–15	135	4
15–18	215	14
18–21	360	15
21–24	680	15
24–27	590	15
36–39	130	6

[a] At Day 13 after 4×10^5 fetal rat liver cells were plated, 10% dialyzed fetal calf serum was added to the cultures. [^{3}H]-Thymidine was added to the dishes for 3-hr periods at the times indicated.

was added to the cultures. Cells divide approximately 34–38 hr after serum addition, indicating that quiescent FRL cells are arrested in the G_1 (G_0) phase of the cell cycle (data not shown; see also Ref. 14). Therefore, the situation in tissue culture is similar to the one *in vivo* after partial hepatectomy in the rat.

FRL cells require a "conditioning activity" in the medium for growth (14). It is not clear whether this activity is released by dying cells or secreted by surviving cells. Studies were made as to whether the serum factor(s) required for the initiation of DNA synthesis in FRL cells acts synergistically with the "conditioning activity" or whether the events which ultimately lead to DNA synthesis can be initiated by serum factor(s) in the absence of "conditioning activity." Fresh medium containing 10% serum (nonconditioned medium) was added to quiescent cultures. At either 3 or 5 hr after the start of the experiment, the medium was removed and replaced with conditioned medium (collected from untreated sister dishes at Day 13 after plating) plus 10% fresh serum. In control cultures, 10% serum was added to conditioned medium; the medium was removed at either 3 or 5 hr and replaced with conditioned medium plus 10% fresh serum. The onset of DNA synthesis was recorded by determining TCA-insoluble radioactivity after 3 hr [^{3}H]-thymidine pulses. The results are shown in Table IIa. They indicate that the onset of DNA synthesis occurs not earlier than 16 hr after cells were incubated in 10% serum *and* conditioned medium regardless of the duration of the previous exposure to 10% serum in fresh (nonconditioned) medium. The data suggest that serum factor(s) *and* "conditioning activity" are required simultaneously in order to initiate the events that lead to the induction of DNA synthesis. The number of cells which synthesize DNA after induction by serum is proportional to the amount of "conditioning activity" present in the culture medium (Table IIb).

In order to determine the minimal exposure time of quiescent FRL cells to serum which is required to program the cells to synthesize DNA, the following experiment was performed. Cells were exposed to 10% fresh serum in conditioned medium for different periods, after which the medium was

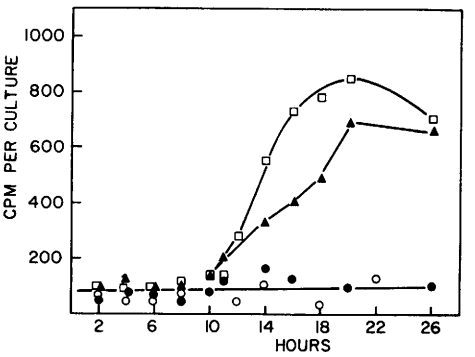

FIG. 1. Initiation of DNA synthesis in quiescent fetal rat liver cells after different periods of exposure to serum. At Day 13 after 4×10^5 fetal rat liver cells were plated, dialyzed fetal calf serum (10%) was added to the cultures. At the times indicated, the medium was removed and replaced with either conditioned medium obtained from untreated sister dishes at Day 13 after plating (▲—▲) or with fresh serum free (nonconditioned) medium (□—□). Control cultures received no serum and were incubated either in conditioned medium (●—●) or in fresh medium (○—○) for the duration of the experiment. At 24–26 hr, all cultures were pulsed with [^{3}H]-thymidine, and the TCA-precipitable radioactivity was determined as described in *Materials and Methods*. See also text for details. Abscissa: exposure time of the cultures to dialyzed fetal calf serum. Ordinate: cpm per culture.

TABLE II. Initiation of DNA Synthesis in Quiescent Fetal Rat Liver Cells by "Conditioning Activity" and Serum.[a]

A

Culture conditions	Hours after start of experiment			
	13–16	16–19	19–22	22–25
	(cpm per culture)			
Control 1				
No addition, no change	82	82	90	88
Control 2				
Fresh medium (no serum)	42	42	54	88
Control 3				
Fresh medium + 10% serum	45	53	46	53
Control 4				
Conditioned medium + 10% serum (0–3 hr) followed by conditioned medium + 10% serum (3–26 hr)	63	125	255	425
Experiment 1				
Fresh medium + 10% serum (0–3 hr) followed by conditioned medium + 10% serum (3–26 hr)	58	50	95	210
Experiment 2				
Fresh medium + 10% serum (0–5 hr) followed by conditioned medium + 10% serum (5–26 hr)	58	56	80	122

B

Culture conditions		cpm per culture	% Nuclei labeled
Conditioned medium		111	≦3
Conditioned medium plus 10% dialyzed fetal calf serum		393	21
Fresh medium, no serum		47	≦1
Fresh medium plus 10% dialyzed fetal calf serum		51	1
Fresh medium (1.5 ml) plus conditioned medium (0.5 ml)	plus 10% dialyzed fetal calf serum	168	6
Fresh medium (1 ml) plus conditioned medium (1 ml)	plus 10% dialyzed fetal calf serum	192	8
Fresh medium (0.5 ml) plus conditioned medium (1.5 ml)	plus 10% dialyzed fetal calf serum	241	11

[a] At Day 13 after 3 × 10^5 fetal rat liver cells were plated, culture media of different composition were added to the cultures. They were pulsed for 3 hr with [^{3}H]-thymidine (a) at different times and (b) at 23–26 hr after the start of the experiments. Dialyzed fetal calf serum was used in all experiments. Conditioned medium was collected from untreated sister dishes at Day 13 after plating.

removed and replaced with conditioned medium without fresh serum, and DNA synthesis was recorded by determination of TCA-precipitable radioactivity with [^{3}H]-thymidine pulses (24–26 hr after start of the experiment). The results, summarized in Fig. 1, show that the cells have to be exposed to serum *and* "conditioning activity" for at least 12 hr in order to become committed to synthesize DNA. Control experiments showed that under these conditions DNA synthesis starts 14–16 hr after start of the experiment (data not shown). Cultures which had been exposed to fresh serum in conditioned medium for ⩾12 hr and then incubated in serum-free fresh (nonconditioned) medium showed an increased incorporation of [^{3}H]-thymidine into TCA-

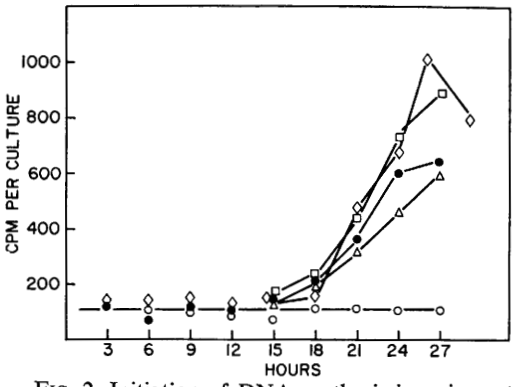

FIG. 2. Initiation of DNA synthesis in quiescent fetal rat liver cells by serum, ornithine, or arginine. At Day 13 after 4×10^5 fetal rat liver cells were plated, dialyzed fetal calf serum (10%) and/or ornithine or arginine (final concentration: $4 \times 10^{-4} M$) were added to the cultures in conditioned medium and DNA synthesis recorded by determination of TCA-precipitable radioactivity retained on glassfiber filters after 3-hr pulses with [^{3}H]-thymidine at different times after start of the experiment. Abscissa: time (hr). (○—○) Control; (△—△) ornithine; (●—●) fetal calf serum; (□—□) fetal calf serum plus ornithine; (◇—◇) arginine. Ordinate: cpm per culture.

precipitable material as compared with cultures which were incubated in conditioned medium after the 12th hr (Fig. 1).

It was shown in a previous communication that when ornithine was present in the arginine-deficient culture medium, the growth rate of FRL cells as well as the final cell density were enhanced (14). This suggested that the production of arginine (from ornithine) (15) in FRL cells which can be made available for protein synthesis might be limiting the growth rate of the cells. At higher serum concentrations in the absence of both ornithine and arginine in the medium, the cells grow and the growth rate as well as the final cell density increase with increasing serum concentrations (14), which suggests that more arginine is made available for protein synthesis in growing cells as compared with quiescent cells (14). Therefore, only an increase of the biosynthetic capacity for ornithine (or inhibition of its breakdown) in response to serum growth factors could account for an increased availability of arginine in the cells which can be utilized for protein synthesis without impairing the urea cycle activity. This suggested that DNA synthesis might be regulated as a result of increased intracellular levels of ornithine or/and arginine after serum addition. As shown in Fig. 2, DNA synthesis in quiescent FRL cells was initiated 14–16 hr after addition of ornithine ($4 \times 10^{-4} M$) or arginine ($4 \times 10^{-4} M$) to the cultures in conditioned medium. Similarly, DNA synthesis was also initiated when arginine ($4 \times 10^{-4} M$) was present in fresh (unconditioned) medium (data not shown). When both dialyzed fetal calf serum *and* ornithine were added to the cultures in conditioned medium, a larger number of cells (33%) was initiated to synthesize DNA, as in cultures to which only serum (20%) or ornithine (17%) was added (see also Fig. 2). These results suggested that the rate of DNA synthesis in a slowly growing culture could be accelerated by ornithine. When serum was added to a slowly growing culture, DNA synthesis was accelerated after a lag period of 8–10 hr (Fig. 3). Similarly, when ornithine was added to such a culture, an identical time course in the acceleration of DNA synthesis was observed as after addition of serum to the cultures.

In the experiments described above, ornithine or arginine seemed to be limiting for the biochemical events which led to the ini-

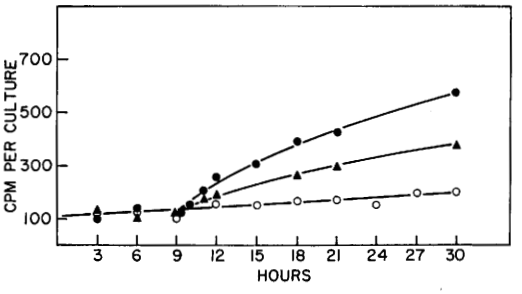

FIG. 3. Acceleration of DNA synthesis in fetal rat liver cells by serum or ornithine. At Day 4 after 3×10^5 fetal rat liver cells were plated, dialyzed fetal calf serum (10%) or ornithine (final concentration: $4 \times 10^{-4} M$) were added to the cultures. DNA synthesis was recorded by determination of TCA-precipitable radioactivity retained on glassfiber filters after 3-hr pulses with [^{3}H]-thymidine at different times after start of the experiment. Abscissa: time after addition of dialyzed fetal calf serum (●—●); ornithine (▲—▲); or isotonic salt solution (control) (○—○). Ordinate: cpm per culture.

tiation of DNA synthesis (14). It seems that when the pool size of ornithine (or arginine) drops below a certain level, the cells become arrested in the G_1 (G_0) phase of the cell cycle. Similar, although not identical, observations were published by Ley and Tobey (16) and Enger and Tobey (17), who reported that hamster ovary tumor cells become arrested in late G_1 (before entry into S) when the concentrations of isoleucine or glutamine in the culture medium were reduced. Also, zinc (18, 19) and putrescine (20) were shown to be involved in the regulation of cell growth under special conditions, because deficiencies in these nutrients led to the arrest of cell growth in G_1. Recently, it was suggested that the control of cell growth could be effected by the availability inside the cell of one or more nutrients required for cell division (21) (see also Ref. 22). The results presented above strongly support this hypothesis, and we propose that in FRL cells, ornithine and/or arginine and/or their proximal metabolites have regulatory functions in the control of DNA synthesis, the latter being regulated by intracellular levels of these compounds.

Summary. DNA synthesis can be initiated in quiescent fetal rat hepatocytes in culture by the addition of dialyzed fetal calf serum, arginine, or ornithine with a time course similar to the one observed after partial hepatectomy of the rat. A lag period of 14–16 hr was observed between the addition of the stimulus to the cultures and the onset of DNA synthesis. Cells are committed to synthesize DNA after incubation with serum for 12 hr. Serum or ornithine is active only when incubated in conditioned medium. It is suggested that DNA synthesis in cultured fetal rat hepatocytes is regulated by intracellular levels of arginine and/or ornithine and/or their proximal metabolites.

We gratefully acknowledge the assistance of M. Henahan, B. Raspenti, J. Rippon, L. White, and M. Williams. The work was supported by research grants from the American Cancer Society, California Division, The Damon Runyon Memorial Fund for Cancer Research, Inc., and by the National Institutes of Health, U. S., Public Health Service.

Note added in proof: That arginine stimulates DNA synthesis in quiescent fetal rat liver cells in chemically defined medium has also been demonstrated by K. Koch and H. L. Leffert (submitted for publication), and H. L. Leffert and S. Sell (J. Cell Biol., in press).

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Received June 19, 1973. P.S.E.B.M., 1974, Vol. 145.