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DNA SYNTHESIS IN CULTURED HUMAN FIBROBLASTS: Regulation by 3′:5′-Cyclic Amp

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The addition of serum to density-inhibited human fibroblast cultures induced a wave of DNA synthesis, measured as [^{3}H] thymidine incorporation into acid-precipitable material, beginning after 8–12 hr and reaching maximum levels at 16–24 hr. Addition of dibutyryl-3′:5′-cyclic AMP (DBcAMP) together with serum inhibited [^{3}H] thymidine incorporation by 75–95%. When DBcAMP was added for the first 4 hr of serum stimulation and then removed, the wave of DNA synthesis was not delayed. This suggested that serum could induce DNA synthesis even though cyclic AMP concentrations were maintained at high levels by DBcAMP during this initial period. These results are inconsistent with the hypothesis that it is the immediate transient reduction in 3′:5′-cyclic AMP concentration following the addition of serum that triggers DNA synthesis. By contrast, DBcAMP added 8 hr after serum inhibited [^{3}H] thymidine incorporation to the same extent as DBcAMP added at the same time as serum. This indicated that a step essential for DNA synthesis and occurring late in G_1 was inhibited by high concentrations of 3′:5′-cyclic AMP.

INTRODUCTION

Density-inhibited human fibroblast cultures can be induced to synthesize DNA by growth-promoting agents such as fetal calf serum, insulin, and certain polypeptides with insulin-like activity (1). The stimulation by serum appeared to proceed by a mechanism different from that by insulin or insulin-like polypeptides (1). Addition of insulin or serum

to nontransformed cell lines promptly reduced intracellular cAMP[1] levels (2, 3). It has been proposed that this decrease in cAMP concentration might be the signal for a cell to initiate DNA synthesis (4). In this report, we present evidence against this "trigger" hypothesis: DNA synthesis in human fibroblasts was stimulated by serum even when the initial reduction in intracellular cAMP concentration was prevented by the addition of DBcAMP. In contrast, fibroblast DNA synthesis was profoundly inhibited by DBcAMP added 8–18 hr after serum, indicating that a DBcAMP-sensitive step occurred late in G_1.

METHODS

Human fibroblast cultures were established from biopsies of normal skin. The cells remain diploid and have a finite lifespan in culture. Techniques of cell propagation have been described previously (1). For most experiments, fibroblasts were maintained in serum-free medium (Eagle's minimum essential medium supplemented with a 0.25% bovine serum albumin) for 5–10 days prior to the addition of 10% (V/V) fetal calf serum. Cultures then were pulsed with [^{3}H] thymidine or [^{3}H] uridine as indicated, and the incorporation of radioactivity into acid-precipitable material was determined (1).

RESULTS

Increased Cyclic AMP Concentration at Confluency

Human fibroblast cultures growing logarithmically have low intracellular cAMP concentrations, e.g., 4 pmoles/mg protein (5). As cell density increased (> 0.7 mg cell protein/25 cm^2 flask), the growth rate slowed markedly and the cAMP concentration showed a coincident four- to fivefold increase (5).

Inhibition of [^{3}H] Thymidine Incorporation by Dibutyryl Cyclic AMP

Confluent fibroblast cultures which had been maintained in serum-free medium for 5–10 days had high cAMP concentrations and incorporated [^{3}H] thymidine into DNA at an extremely low basal rate (5). The addition of serum to such a quiescent culture induced a wave of DNA synthesis, beginning after 8–12 hr and reaching maximum levels after 16–24 hr (1).

Serum-stimulated DNA synthesis was inhibited by the simultaneous addition of DBcAMP (5), a potent cyclic nucleotide phosphodiesterase inhibitor which causes a rapid increase in intracellular cAMP levels (6). DBcAMP, at concentrations from 0.25 to 1.0 mM, inhibited [^{3}H] thymidine incorporation by 75–95% (5). The inhibition of serum-stimulated DNA synthesis by DBcAMP was not a nonspecific toxic effect: cells did not die during treatment, normal growth was resumed upon removal of DBcAMP, and ribosomal RNA was synthesized at approximately control rates in the presence of DBcAMP (5).

Effect of Dibutyryl Cyclic AMP During the First 4 Hours of Serum Stimulation

Although 8–12 hr were required before the increase in [^{3}H] thymidine incorporation induced by serum was observed, the metabolic preparations for DNA synthesis began

[1] Abbreviations: cAMP, 3′:5′-cyclic adenosine monophosphate; DBcAMP, N^6, $O^{2\prime}$-dibutyryl-3′:5′-cyclic adenosine monophosphate.

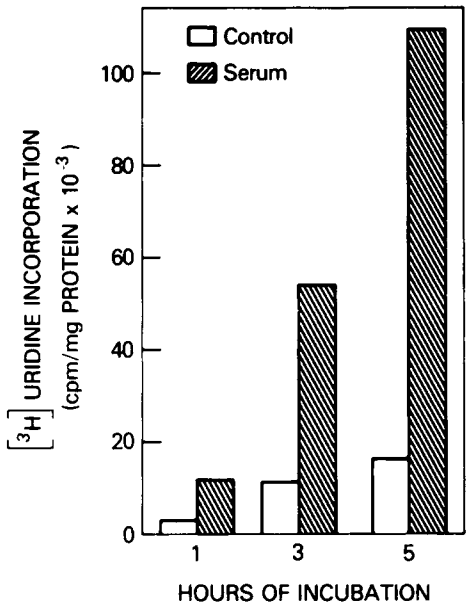

Fig. 1. Serum-stimulation of human fibroblast RNA synthesis. Confluent fibroblast cultures, maintained in serum-free medium for 4 days, were incubated with [^{3}H] uridine (1 μCi/ml, 10 μM) ± 10% fetal calf serum. The incorporation of ^{3}H into acid-precipitable material (RNA) was determined after incubations of different duration.

almost immediately. The incorporation of [^{3}H] uridine into RNA, for example, was increased fourfold within the first hr of serum treatment (Fig. 1). A parallel stimulation was observed for the uptake of [^{3}H] uridine from the incubation medium into intracellular acid-soluble material.

If a decrease in fibroblast cAMP levels were essential for serum to induce DNA synthesis (4), then maintaining elevated cAMP concentrations by the addition of DBcAMP during the first 4 hr of serum treatment should delay the wave of DNA synthesis by 4 hr. As seen in Fig. 2, this was not the case: the time course of [^{3}H] thymidine incorporation was identical for DBcAMP-treated and control cultures. These results conflict with those reported by Froehlich and Rachmeler (7). Thus, despite the presence of DBcAMP during the first 4 hr of serum treatment, serum was able to initiate the sequence of metabolic events which culminate in DNA synthesis.

Effect of Late Addition of Dibutyryl Cyclic AMP

DBcAMP was added to fibroblast cultures at different times after serum stimulation in order to determine when it exerted its inhibitory effects. DBcAMP added up to 8 hr after serum inhibited the incorporation of [^{3}H] thymidine during a pulse at 20.5 hr to the same extent ($\approx$ 95%) as DBcAMP added at the same time as serum (Fig. 3). The magnitude of the inhibition decreased progressively when DBcAMP was added at later times. DBcAMP still inhibited [^{3}H] thymidine incorporation by almost 40% when added

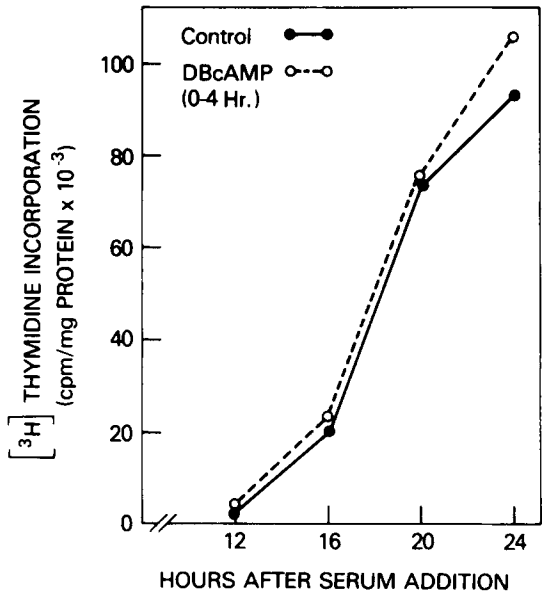

Fig. 2. Effect of DBcAMP during the first 4 hr of serum stimulation. Confluent fibroblast cultures, maintained in serum-free medium for 7 days, were transferred to regular (10% serum) medium (●——●) or regular medium supplemented with 0.5 mM DBcAMP (○---○). After 4 hr, the DBcAMP-treated cultures were changed to regular medium and the incubation continued. Cultures were pulsed for 30 min with [^{3}H] thymidine, 2 μCi/ml, at the indicated times after the initial media change. The incorporation of ^{3}H into acid-precipitable material (DNA) was determined.

18 hr after serum. We conclude that a DBcAMP-sensitive step occurred 8–18 hr after the addition of serum.

The DBcAMP-sensitive step could be in late G_1 or early S phase. To distinguish between these possibilities, the following experiment was performed. Fibroblast cultures were arrested at the G_1/S boundary with hydroxyurea, an inhibitor of DNA synthesis. After 16 hr the cultures were transferred to fresh media ± 0.5 mM DBcAMP and pulsed with [^{3}H] thymidine at different times thereafter. No direct effects of DBcAMP on S phase DNA synthesis were observed (5)*, indicating that the DBcAMP-sensitive step was in late G_1.

DISCUSSION

Increased intracellular cAMP concentrations were associated with reduced DNA synthesis in human fibroblast cultures. Cyclic AMP levels were low in growing cultures and increased at confluency. Serum-stimulated DNA synthesis was inhibited when cAMP was maintained at a high level with DBcAMP.

Although serum lowered the cAMP concentration and stimulated DNA synthesis in

*Note added in proof: In more recent experiments, DBcAMP inhibited S phase DNA synthesis 4–8 hr after the hydroxyurea-containing media was replaced, but not at earlier times.

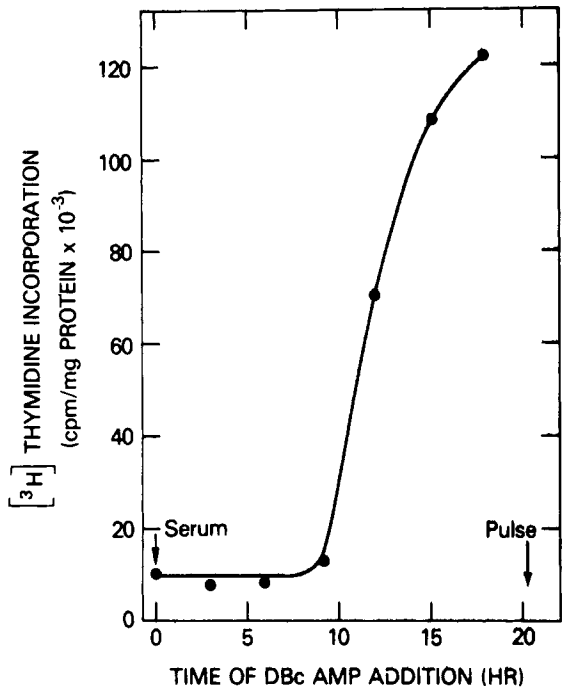

Fig. 3. Effect of adding DBcAMP at different times after serum stimulation. Confluent fibroblast cultures, maintained in serum-free medium for 7 days, were changed to medium containing 10% serum at t = 0. DBcAMP, 0.5 mM, was added at the time of the media change or at different times thereafter. All cultures were pulsed for 30 min with [^{3}H] thymidine, 2 μCi/ml, 20.5 hr after the media change. The incorporation of ^{3}H into acid-precipitable material was determined.

confluent fibroblasts, the transient reduction of cAMP concentration following serum administration was not the signal for a cell to initiate a new round of DNA synthesis. When DBcAMP was added for the first 4 hr of serum treatment so as to prevent a decrease in cAMP concentration, the wave of DNA synthesis induced by serum was not delayed. Thus, an initial reduction in cAMP concentration was not essential for serum to stimulate human fibroblast DNA synthesis. The effects of serum are undoubtedly more complex than a simple trigger mechanism, since serum must be present for 5–7 hr before DNA synthesis in human fibroblasts becomes serum independent (8, 9).

The intracellular cAMP concentration late in G_1, on the other hand, critically affected fibroblast DNA synthesis. Seifert and Rudland recently reported an abrupt decrease in cAMP levels in Balb c/3T3 fibroblasts occurring at this point in the cell cycle (10). In our experiments, DBcAMP added 8–18 hr after serum profoundly inhibited [^{3}H] thymidine incorporation into DNA. A similar late inhibiting effect of DBcAMP has been described by Froehlich and Rachmeler (7). Although the molecular event in late G_1 that is inhibited by high cAMP concentrations is unknown, its identification may help elucidate the sequence of biochemical events preparing a cell for DNA synthesis.

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