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ONLY ONE OF THE TWO SIGNALS REQUIRED FOR INITIATION OF THE CELL CYCLE IS ASSOCIATED WITH CELLULAR ACCUMULATION OF RIBOSOMAL RNA

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SUMMARY: Two intracellular functions are elicited by the serum component in culture media in order to initiate the cell cycle: "competence" and "progression". Although both functions have to be present simultaneously for start of cell division, it is shown here for Swiss 3T3 cells that only one of them, the progression signal, is associated with reaccumulation of ribosomal RNA. This result points to the cellular complement of ribosomes as one of the limiting parameters for cell division.

The growth-stimulating activity of the serum component in the culture medium has been found to provide for two necessary functions termed induction of "competence" and of "progression", which only in conjunction are sufficient for initiation of the cell cycle (1). For 3T3 cells, these different functions may largely be induced separately by different growth factors (2,3) and are characterized by different kinetics (1,4). Since the level of ribosomal RNA (rRNA) appears to be of considerable importance for regulation of the cell cycle, as reemphasized recently (5,6), it has been of interest to resolve whether competence or progression or both are associated with regulation of rRNA metabolism. In previous work, it was shown clearly that the induction of progression by platelet-poor blood plasma is associated with elevation of cellular content of rRNA (7). A

ABBREVIATIONS
EFG, Epidermal Growth Factor; FGF, Fibroblast Growth Factor; PDGF, Platelet Derived Growth Factor; AO, Acridine Orange; NBCS, Newborn Calf Serum; DMEM, Dulbecco's Modification of Eagle's Medium; EDTA, Ethylenediaminetetraacetic Acid; rRNA, ribosomal RNA; RNase, Ribonuclease A.

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second result from this work was stimulation of rRNA metabolism by the competence-inducer platelet-derived growth factor (PDGF), albeit in the presence of depleted medium with 2% serum. However, in the light of more recent results of a sensitization of cells to progression factors by the action of competence factors (3), this second result does not appear to hold for a clear-cut interpretation. Therefore, we have tested several conditions of pure competence induction with respect to the cellular content of rRNA. The result is that progression, but not competence is associated with reaccumulation of rRNA in 3T3 cells stimulated from quiescence. This result appears of considerable interest in the context of modern models of the cell cycle requiring a continuous growth cycle in addition to the chromosome cycle (8,9); in the light of the present evidence, the growth cycle might be defined by the ribosome complement of the cells.

MATERIALS AND METHODS

Cell culture: Stock cultures of Swiss 3T3 cells were obtained from Flow Laboratories (Bonn) and propagated as described previously (10). In all experiments, newborn-calf serum (NBCS) from Gibco-Biocult (Karlsruhe) was used. Fibroblast Growth Factor from pituitary (FGF) and Epidermal Growth Factor (EGF) were from KOR Biochemicals, Cambridge, MA (obtained through Renner, Dannstadt). Insulin (from bovine pancreas) was obtained from Sigma, München. Details regarding experimental protocols are given in the legends to the figure and tables.

RNA-staining procedure: Cells to be assayed for their RNA content were released from the plate in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (buffer A: 138 mM NaCl, 27. mM KCl, 6.5 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing 60 µg/ml trypsin of bovine pancreas (EC 3.4.21.4) from Boehringer, Mannheim. For staining of the cells with acridine orange (AO), the following modified version of the two-step procedure developed by Darzynkiewicz et al. (11) was used. First step: 1 ml of the cell suspension was mixed at 37 °C with 0.5 ml of a citrate/phosphate buffer (buffer B: 0.15 M NaCl, 10^{-3} M EDTA, 0.1% (v/v) Triton X-100, $4.3 \cdot 10^{-2}$ M citric acid, and Na_2HPO_4 added until pH 3.0) and maintained under these conditions for 1 min. Second step: The cell suspension resulting from the first step was transferred onto ice and stained by addition of 1 ml of an ice-cold citrate buffer (buffer C: 0.15 M NaCl, 0.015 M citric acid, and Na_2HPO_4 added until pH 4.3) containing $8.7 \cdot 10^{-5}$ M AO (Fluka, Buchs). Thus, the cells in the staining solution are exposed to a final AO concentration of $3.2 \cdot 10^{-5}$ M and held on ice under this condition until fluorescence measurement (usually 5 to 20 min after staining).

Tests for specificity of RNA staining: After performing the first step of the staining procedure, cells were washed once at 4 °C in buffer A, resuspended in 1 ml of buffer A, and after addition of 50 µl of buffer A containing 250 µg ribonuclease A (RNase: EC 2.7.7.16) from bovine pancreas (Boehringer, Mannheim) incubated at 37 °C for 15 min. Controls were treated without enzyme. Thereafter, 0.5 ml of buffer B (without Triton X-100) and 1 ml of buffer C containing AO were added at 0 °C, and this suspension used for fluorescence measurement. By this procedure it was shown that according to our staining method more than 90% of cellular red fluorescence is specific for RNA.

Fluorescence measurements: Fluorescence of single stained cells was measured at two wavelengths using an impulse cytophotometer ICP22 (Biophysics Systems, Bensheim) interfaced to a desk computer (Commodore CBM 3032) via an analog/digital converter. Excitation light (λ < 450 nm) was provided by a high-pressure mercury lamp; red fluorescence was recorded at λ > 630 nm. Red fluorescence per cell was determined as the weighted average from histograms extending over 64 channels.

RESULTS

Kinetics of rRNA content during growth regulation. Fig.1 (upper) shows the population dynamics of 3T3 cells during cell-density dependent proliferation at 5% NBCS (3 x weekly medium renewal) and after stimulation from about 12 days of quiescence by a serum step-up to 20% NBCS without any further medium renewal. After cells have reached confluence, cellular RNA content decreases within 5 days to about half the figure characteristic for exponentially growing cells (Fig.1, middle). Since 80 to 90% of total cellular RNA is rRNA (5), this result supplements earlier findings on different rRNA contents of sparse and confluent 3T3 cells (12). Stimulation after about 11 days of confluence leads to an increase of cellular rRNA after a delay of > 8 h (Fig.1, lower). At 24 h after stimulation, cellular RNA content is increased by about 50% as compared to the unstimulated cells. This result agrees with numerous studies demonstrating substantial rises of cellular rRNA content after stimulation from quiescence (7,13).

Effects of defined growth factors on RNA content. From the kinetic results shown in Fig.1 (lower), an assay for changes of rRNA content at 24 h after growth stimulation appeared appro-

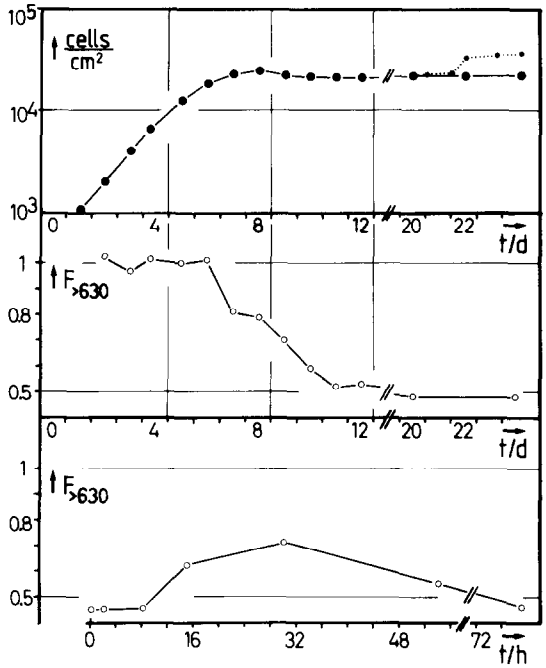

Fig.1. Upper: Growth curve of 3T3 cells at 5% newborn-calf serum (3 x weekly medium renewal). On day 20 of growth, the culture was stimulated by once applying fresh medium containing 20% newborn-calf serum. Middle: Cellular RNA content versus time of growth as measured flow-cytometrically by red fluorescence of cells stained with acridine orange. Fluorescence of exponentially growing cells was taken as unity. Lower: Time course of cellular RNA content after 20% serum stimulation as indicated in the upper part. Note different time scale of lower part of the figure.

priate. Furthermore, RNA content of non-stimulated cells at t > 11 days was independent of time at the lower level (Fig.1, middle). Following these precepts, we have stimulated 3T3 cells after 17 days of growth with fibroblast growth factor (FGF) or the combination of epidermal growth factor (EGF) and insulin, or with the combination of all three growth factors and, after incubation for 24 h, determined the changes of cellular RNA content relative to the non-stimulated control. The results are shown in Table 1. As rRNA comprises 80 to 90% of total cellular RNA (5), our results indicate that treatment of cells with EGF/insulin stimulates accumulation of cellular rRNA content (Table 1) to a similar extent as treatment with increased serum concentration (Fig.1,.lower, and Table 2). Thus, our results on EGF/insulin

Table 1
RNA Content of 3T3 Cells 24 h after Stimulation with Defined Growth Factors

Treatment	RNA content per cell (percent of control)
Control (DMEM)	100
20 ng/ml EGF + 10^{-5} M insulin	159
125 ng/ml FGF + 20 ng/ml EGF + 10^{-5} M insulin	142
125 ng/ml FGF	105
250 ng/ml FGF	102

Cells were seeded at 10^3 cells/cm^2 in 6 cm plastic petri plates and grown for 17 days in Dulbecco's modified Eagle medium (DMEM) with 2.5% newborn-calf serum (3 x weekly medium renewal). Before treatment with growth factors (or control treatment), cells were washed once in DMEM at 37 °C. After 24-h incubation with 2.5 ml of DMEM containing the given concentrations of growth factors, cells were processed for fluorescence measurement as described in Materials and Methods. Figures given are averages from duplicate measurements, with less than 8% deviation from the mean in all cases.

Table 2
RNA Content of 3T3 Cells 24 h after Stimulation with Different Regimes of Serum Application

Treatment (3 h/21 h)	RNA content (percent of control)	Cell density (percent of control)
Control (DMEM/DMEM)	100	100
10% NBCS/10% NBCS	148	116
10% NBCS/10 ng/ml EGF +10^{-5} M insulin	151	113
10% NBCS/DMEM	91	102

Cells were seeded at 10^3 cells/cm^2 in 6 cm plastic petri plates and grown for 14 days in Dulbecco's modified Eagle medium (DMEM) with 2.5% newborn-calf serum (3 x weekly medium renewal). Treatment was in all cases a pretreatment of 3 h (condition A) followed by a prolonged treatment for 21 h (condition B), which in the first column of the table is indicated as "A/B". After a total incubation period of 24 h, cells were processed for measurement as described in Materials and Methods. Figures given are averages from duplicate measurements, with less than 6% deviation from the mean in all cases.

are consistent with and supplement earlier work on stimulation of rRNA content in BALB/c-3T3 cells by treatment with platelet-poor plasma, which induces progression in competence-induced cells (7). In contrast, treatment of 3T3 cells with FGF from the same source and at the same concentrations shown to induce competence (2), does not stimulate cellular rRNA content (Table 1). The same result was obtained in another experiment, where the cells had not been washed with DMEM prior to treatment with 250 ng/ml FGF in DMEM. These results do not necessarily contradict earlier results on an increase of rRNA content upon stimulation with PDGF, albeit in the presence of depleted medium with 2% serum (7). It was shown recently that PDGF treatment may effect a sensitization of cells to low levels of EGF (3). Thus, there is the real possibility in the results (7) of a sensitization of the cells by PDGF to residual progression activities in the depleted medium.

We have exploited the kinetics of competence induction for another independent test on the effect of competence on rRNA content. It has been demonstrated previously (1) that the relatively long-lived (4) competence signal may be induced by a comparably short ($\approx$ 3 h) treatment by a competence inducer, but that the progression inducer is required to be present continuously ($\approx$ 12 h). Therefore, we have tested the effect on RNA content and cell density of a brief (3 h) serum treatment for competence induction with and without a subsequent longer (21 h) treatment with serum or EGF/insulin for induction of progression. As shown in Table 2, the competence treatment alone does not stimulate an increase of rRNA content or cell division, but competence and progression treatments together do. The increase in cell densities upon competence and progression treatments are still relatively small, because the mitotic wave is substantial only at

about 30 to 40 h following stimulation (Fig.1, upper); the rise in cell density in Table 2, however, is significant.

Thus, the data from Tables 1 and 2 consistently yield the result that induction of progression, but not of competence, stimulates an increase of cellular rRNA content.

DISCUSSION

The results presented above point to a salient role of re-accumulation of cellular content of ribosomes in preparation for cell division. Specifically, the "progression" requirement for initiation of the cell cycle might be in essence the need for rising the ribosome number or concentration to some threshold value, before the competence signal can become effective. Although this notion certainly is not yet proven, there are numerous lines of evidence for its support. Duration of the cell cycle was shown to be inversely related to cellular rRNA content (14). SV40-virus was shown to contain coding sequences to activate ribosomal RNA genes and different sequences necessary for induction of cellular DNA replication (15). We have demonstrated a detailed correlation between cellular rRNA content and the proliferative capacity during cell-density and serum dependent population dynamics of 3T3 cells ([16], Adam et al., to be published elsewhere). In the light of this and other evidence, our present results appear to offer a relevant specification to modern models of the cell cycle requiring a continuous growth cycle in addition to the chromosome cycle (8,9). In fact, cellular growth might be identical with the progression function and defined for the cell by its number or concentration of ribosomes.

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