NUCLEOLAR AND NUCLEAR RNA SYNTHESIS DURING THE CELL LIFE CYCLE IN MONKEY AND PIG KIDNEY CELLS IN VITRO

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ABSTRACT

The incorporation of 5-^{3}H-uridine and 5-^{3}H-cytidine into nucleolar and nonnucleolar RNA in the nucleus of monkey and pig kidney cells was measured in vitro during the cell life cycle. Time-lapse cinematographic records were made of cells during asynchronous exponential proliferation, in order to identify the temporal position of individual cells in relation to the preceding mitosis. Immediately following cinematography, cells were labeled with uridine-^{3}H and cytidine-^{3}H for a short period, fixed, and analyzed by radioautography. Since the data permit correlation of the rate of RNA labeling with the position of a cell within the cycle, curves could be constructed describing the rate of RNA synthesis over the average cell cycle. RNA synthesis was absent in early telophase, and rose very abruptly in rate in late telophase and in very early G_1 in both the nucleus and the reconstituting nucleolus. Thereafter, through the G_1 and S periods the rate of nuclear RNA synthesis rose gradually. When we used a 10-min pulse, there was no detectable change in the rate for nucleolar RNA labeling in monkey kidney cells during G_1 or S. When we used a 30-min labeling time, the rate of nucleolar RNA labeling rose gradually in pig kidney cells. With increasing time after mitosis, the data became more variable, which may, in part, be related to the variation in generation times for individual cells.

INTRODUCTION

Both nucleolar and nonnucleolar RNA syntheses are apparently continuous throughout interphase, but not much is known about the rate of such syntheses during progress of a cell through G_1, S, and G_2. In mammalian cells, the rate of total nuclear RNA synthesis has been reported to rise linearly through the cycle or to rise abruptly at the G_1 to S transition (3, 26). We have studied the rate of synthesis in the nucleolus and the nonnucleolar regions of the nucleus during the cell cycle, measuring uridine-^{3}H and cytidine-^{3}H incorporation into individual cells. The temporal position of individual cells in the life cycle was determined from a time-lapse cinematographic record taken in the hours prior to RNA labeling. Isotope incorporation was measured by radioautography. The cinematographic method of staging of cells is at least as precise as any of the methods of synchronization currently in use. As in almost all cell cycle analyses, the precision of the data is limited by the variation in generation times for individual cells.

METHODS

Cell Types and Growth Conditions

MONKEY KIDNEY: Cells with prominent nucleoli derived from the kidneys of green monkeys

(10) were used at subcultivation levels 53–72.[1] The cells were epitheloid in appearance; as the number of subcultivations increased, intracellular granules became more frequent and the growth potential declined. In the older cultures, irregularly shaped nuclei occurred and some cell death was observed. These passage levels (53–72) appeared to correspond to the late phase II and phase III described by Hayflick and Moorhead (9). The limited migration of these cells during growth was an advantage in the analysis of the time-lapse cinematographic records.

The individual generation times of the monkey kidney cells determined by time-lapse cinematography varied from 8 to 37 hr with a mean value of 16.2 hr and a standard deviation of 3.6 hr. The distribution of individual generation times was skewed toward the longer times and resembled those obtained with a number of established cell lines (4, 7, 12, 15, 18, 27). The average G_1 period, determined by thymidine-^{3}H pulse labeling of cells staged by cinematographic recording, was 6 hr. Cells were detected in S as early as 3 hr after mitosis and as late as 24 hr, indicating considerable variation in G_1 times for different cells. Similar variations in the length of the G_1 interval have been reported in a cell line by Sisken and Morasca (28). The average DNA synthetic period was 6 hr, the G_2 period approximately 4.5 hr, and division 0.8 hr.

The monkey kidney cells were grown at 37°C in medium 199 with 20% fetal calf serum, 0.1% yeastolate, penicillin (20 units/ml), and streptomycin (50 mcg/ml). For subcultivation, cell sheets were trypsinized as described by Hopps et al. (10). Cells were plated initially at 25,000 cells/ml and at later passage levels at 50,000 cells/ml. Stock cultures were maintained in bottles, and cells were diluted 1:1 for transfer at intervals of approximately 1 wk. The medium was renewed every 3 days.

PIG KIDNEY: A limited number of experiments was performed with pig kidney cells established in continuous culture in 1955 by Dr. R. Stice and obtained under the designation PK-14 from Dr. Morgan Harris in 1963. The cells were epitheloid with generally one or two prominent nucleoli. During growth, compact colonies formed, and the cells showed only limited migration. Occasional cells were observed to die; this is apparently characteristic of the line under the growth conditions employed. The individual generation times of these PK-14 cells varied from 9 to 34 hr with a mean individual generation time of 16.0 hr determined by time-lapse cinematographic methods. The G_2 period was approximately 3.5 hr, the S period 6 hr, and the average mitotic period (D) 1 hr. The G_1 period, estimated as the difference between G_2, S, and D and the mean generation time, was 5.5 hr.

The PK-14 cells were grown in medium F10 with 7% fetal calf serum. Cells were freed from the substrate by exposure to 0.1% trypsin-Versene. Cell suspensions were diluted to contain 15,000 cells per ml for plating.

Labeling and Radioautographic Procedures

RNA synthesis was studied by pulse-labeling cells of known ages. The age of the individual cells, measured as time elapsed since mitosis, was determined by analysis of time-lapse cinematographic film records taken prior to addition of the isotope. Following cinematography, monkey kidney cells were pulsed for 10 min with 5-^{3}H-uridine (sp. act. 14.3 c/mmole; Nuclear-Chicago) at a concentration of 5 μc/ml plus 5-^{3}H-cytidine (sp. act. 23.8 c/mmole; Nuclear-Chicago) at a concentration of 5 μc/ml. Pig kidney PK-14 cells were labeled for 30 min with 5-^{3}H-uridine (sp. act. 11.3 c/mmole; Schwartz) at a concentration of 12.6 μc/ml.

After isotope labeling, the coverslips with attached growing cells were rinsed with balanced salt solution at pH 6.8, fixed for 40 min in 100% ethyl alcohol:glacial acetic acid (3:1), and air dried. Unincorporated isotope still remaining was removed by extraction for 5 min with cold 5% trichloracetic acid. Following exposure to cold 0.1 M sodium pyrophosphate for 5 min, the preparations were washed in tap water for 1 hr, rinsed through an alcohol series, and dried. The cells were coated with Kodak NTB 3 liquid emulsion. Preparations of the monkey kidney cells were exposed for 17 days, while those of the pig kidney cells were exposed for 4 days. Radioautographs were developed for 4 min in D-11, rinsed in water, fixed for 1 min in Kodak Rapid Fixer at 68°C, and washed with tap water for 10 min. The radioautographs were then stained for 30 sec in 0.25% toluidine blue adjusted to pH 6.8 with phosphate buffer. Incorporation of the isotopes was measured by counting grains over nucleoli and the nonnucleolar portions of the nuclei of cells that had been staged by cinematography. The background count was negligible.

Ribonuclease (0.2 mg/ml at pH 6.8 in balanced salt solution) treatment of fixed cell preparations for 30 min at 37°C effectively prevented grain development in subsequent radioautographs, indicating that the RNA precursors supplied were incorporated only into RNA under the conditions employed.

Procedures for Time-Lapse Cinematography

Cells were established on marked 24- × 34-mm coverslips in 60-mm plastic petri dishes. At the time

[1] Obtained from frozen stocks through the courtesy of Mrs. Hope Hopps, Division of Biological Standards, National Institutes of Health, Bethesda, Maryland.

of plating, a companion dish was seeded with cells around the inner bottom periphery close to the wall. 3 or 4 hr prior to the start of a time-lapse analysis, the marked coverslip with the attached cells was transferred to fresh medium in the companion dish. After the cells had been conditioned in the CO_2 incubator, the regular petri dish cover was replaced with an inverted plastic top which permitted low-power microscopic observation of the cells (2). Silicone along the sealing surface retarded loss of CO_2 from the medium while filming was in progress and allowed an air space above the medium. After being sealed, the experimental dish was transferred to the time-lapse cinematographic apparatus. A field containing a number of cells in proximity to one of the marked areas on the coverslip was located and filming begun. The temperature of the microscope stage was maintained at 37°C.

Time-lapse cinematographs were taken at the rate of one frame per minute with phase microscopy using a 10 × phase objective. The light passing through a green filter was adjusted for a 1-sec exposure on 16-mm Plus X reversal film. Photography was continued until the field had almost filled with cells, but was not too crowded to prevent history tracing. Photography extended over periods of 16–66 hr. A movie projector equipped with a frame counter and clutch control was used to edit records of generation times, cell ages, and division times.

RESULTS

Rates of RNA Synthesis

MONKEY KIDNEY CELLS: Considerable variation in the average total grain count occurred from one experiment to another following incorporation of labeled precursors into RNA. For the purpose of permitting grouping of the data from different experiments, the grain counts over the nucleolus and total nucleus of individual cells from the different experiments have been normalized and plotted together in Fig. 1. Normalization was carried out as follows: a mean for the total grain count for all cells of all ages was determined for each experiment. From these, an intermediate set of experiments with the same mean was chosen as equal to 100%. Data from the other experiments were then expressed on the same percentage basis by multiplication with appropriate factors. The grain counts over individual nuclei and nucleoli are widely dispersed, and the variability becomes greater with increasing cell age.

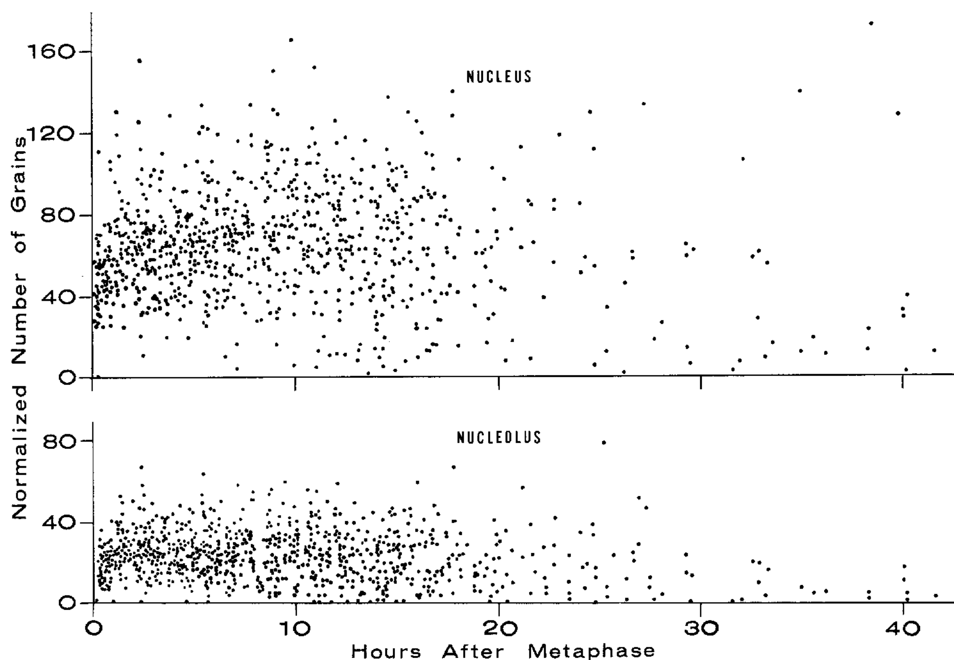

FIGURE 1 Distribution of normalized grain counts over the nucleolus and total nucleus of individual monkey kidney cells with increasing cycle age. The cells were labeled with tritiated uridine and cytidine for 10 min. The data are taken from 14 separate experiments.

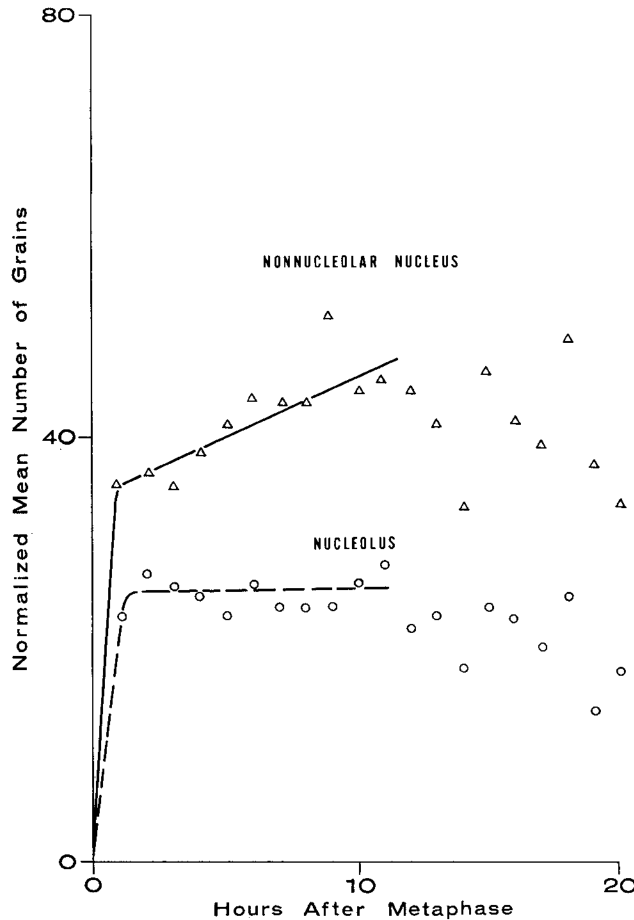

FIGURE 2 Rates of RNA labeling in nucleolus and nonnucleolar nucleus of monkey kidney cells with increasing cycle age. Cell ages were recorded by time-lapse cinematography prior to a 10-min pulse with a mixture of tritiated uridine and cytidine. The points represent mean grain count values collected over hourly intervals (derived from the normalized grain counts for individual cells presented in Fig. 1).

Part of the dispersion is probably due to biological variation, part to the randomness of tritium decay, and part to varying amounts of overlying cytoplasm. A portion of the low RNA incorporation values shown by nuclei of greater cycle age may represent cells in late G_2 that are about to divide, while others may be associated with cells that will fail to reach division and ultimately die. Without correction (see 17 and 20) for differential efficiency in the detection of tritium in nucleolus versus nonnucleolar nucleus, the rate of RNA labeling in the nucleolus is about one-third of the total nuclear rate. In these raw data there are no apparent discontinuities in either nucleolar or nuclear RNA synthesis.

Average rates of RNA synthesis in the nonnucleolar and nucleolar portions of the nucleus were derived by averaging the grain counts obtained for successive hourly intervals. These averaged values are shown in Fig. 2. The period illustrated extends from 0 to 20 hr and is equivalent in length to one mean generation time plus one standard deviation. The mean values of the normalized mean grain counts over the nucleolus with increasing age are shown by the lower curve. RNA synthesis began shortly after mitosis and rose sharply to the maximum rate at the beginning of G_1. The rate did not change significantly during the next 10 hr and then fell slightly between 10 and 20 hr.

The rate of incorporation of RNA precursors into the nonnucleolar portion of the nucleus with increasing age is shown by the upper curve in Fig. 2. At metaphase (zero time), no RNA labeling was detectable. This observation agrees with other reports that RNA synthesis does not occur in the nucleus of a dividing mammalian cell (for example, references 5, 22). Very shortly after mitosis, RNA

synthesis recommenced and the rate of labeling rose rapidly over the next 30–40 min. A much more gradual increase in the rate of incorporation into the nonnucleolar region extended from about 60 min after metaphase to about 10 hr into interphase (latter part of S). After 10 hr, the average rate of incorporation of isotope into nonnucleolar RNA of the nucleus appeared to decline, but the data in this interval became irregular, and there is no clearly discernible trend. The values in this interval are less meaningful because of the variation in individual generation times. Little can be said about the rate of RNA synthesis during G_2

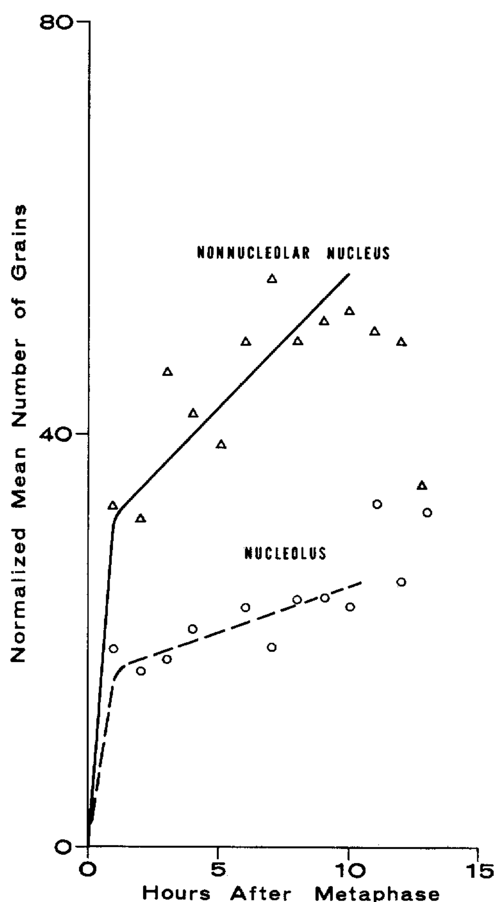

FIGURE 3 Rates of RNA labeling in nucleolus and nonnucleolar nucleus of pig kidney line PK-14 cells with increasing cycle age. Cell ages were recorded by time-lapse cinemicrography and the cultures were pulsed for 30 min with uridine-^{3}H. Points represent mean grain count values from the normalized means of grains over 217 cells.

except that marked changes in rate from S are hardly likely in view of the data in Figs. 1 and 2.

When the grains for the nucleolus and the nonnucleolar nucleus are added to give total nuclear values, the slope for the rate increases up to 10 hr and is similar to that defined by the data of Terasima and Tolmach (29). In the latter work, measurements were made on cells synchronized by the metaphase "shake-off" method. Other studies with partially synchronized HeLa cells have also shown a gradual increase in the rate of RNA synthesis in the nucleus for up to 14–20 hr (16, 25).

PIG KIDNEY CELLS: In the experiments with pig kidney PK-14 cells, only uridine-^{3}H was used to label RNA, and the duration of the pulse was increased to 30 min. A limited number of cells (217) was examined over a period of 13 hr. The data are shown in Fig. 3.

The rate of nucleolar labeling increased sharply in the hour immediately after mitosis and, in contrast to that of monkey kidney cells, continued to increase slowly during the next 10 hr of interphase. Nucleolar labeling accounts for roughly one-third of the total nuclear labeling. As in the monkey kidney cells, there was a sharp rise in incorporation into RNA in the nonnucleolar regions of the nucleus during the 1st hr after mitosis. Over the next 10 hr, the rate of RNA labeling increased steadily and then declined during the final 2 hr of measurements. Again, it is difficult to know what significance to attach to the decline because of the loss of "synchrony" incurred by this time in the cycle.

DISCUSSION

The cinematographic method of determining the stage of a cell in the life cycle is limited in accuracy only by the variation in individual cell generation times. Since metaphase is used as the marker for determining cell age, the correlation between rate of RNA synthesis and interphase stage is most precise in early G_1 and gradually becomes less precise as the cycle proceeds. By the end of the average S period, a significant number of cells has reached mitosis, and we are disinclined to attach much significance to the data beyond this point (beyond about 10 hr in Figs. 2 and 3). To examine RNA synthesis in G_2, it would be preferable to use the beginning of S as the marker for synchrony, using FUdR (19, 23) or thymidine (14) block methods for imposition of synchrony. Un-

fortunately, one cannot assume with complete safety that such inhibitors are free of effects other than the one on DNA synthesis.

The very rapid rise in the rate of RNA synthesis for the nucleus and the nucleolus is expected since nuclear RNA synthesis stops completely during mitosis. Since tRNA synthesis probably contributes only in a minor way to nuclear RNA labeling, we are tempted to assume that all nonnucleolar labeling represents synthesis of mRNA. With such an assumption, the simplest interpretation of the data in Figs. 2 and 3 is that the rate of mRNA synthesis increases steadily throughout G_1 and S, and we should expect to find an increasing rate of cytoplasmic protein synthesis over the corresponding interval. Accurate data on this latter point are not yet available. In experiments on both monkey and pig kidney cells the labeling times were short enough to exclude migration of significant amounts of labeled RNA to the cytoplasm, and the rate curves are not complicated by this factor. The interpretation that the nonnucleolar RNA labeling reflects the rate of mRNA synthesis is complicated by at least two other considerations. First, changes in the size of RNA precursor pools could conceivably change with a regular pattern over the cycle, and the rate of RNA labeling would not remain in the same proportional relation to RNA synthesis. Second, a substantial part of the rapidly labeled RNA in the nonnucleolar regions appears to have a very high molecular weight (greater than 60S) (11), and its function is unknown. Houssais and Attardi (11) and Nemer[2] have presented some suggestive evidence that such RNA is not a form of mRNA.

Nucleolar RNA labeling is presumed to represent primarily rRNA synthesis (1, 6, 8, 13, 21, 24, 30). The difference in rates for nucleoli in the two-cell types may stem from differences in the experimental conditions (a 10-min pulse versus a 30-min pulse). The longer pulse time might allow for RNA migration to or from the nucleolus and not give so true a picture of nucleolar RNA synthesis as a 10-min pulse. An increased incorporation such as observed in the pig kidney nucleoli during G_1 and S could also be due, for example, to a decrease in pool size during the longer labeling period.

The data reported here agree in general with those of Terasima and Tolmach (29), Scharff and Robbins (25), and Kim and Perez (16) on the rate of total nuclear RNA synthesis. Our results differ somewhat from those of Seed (26) and Crippa (3). Seed reported that the rates of labeling on nucleolar and chromatin RNA in monkey kidney cells followed a course of increase parallel to DNA synthesis, i.e. low RNA synthesis in G_1 with a rise in S. For nucleolar and chromatin RNA labeling in HeLa cells, however, the rates rose steadily after mitosis in a manner similar to that for the pig kidney cells in Fig. 3.

Crippa identified the position of hamster cells within the life cycle by spectrophotometric methods, and concluded that the rate of RNA synthesis increased during S, but the methodology does not permit any meaningful conclusions about G_1 or G_2. The interpretation of these data is additionally complicated by the uncertainties introduced by differences in ploidy in the cell line employed.

The finer points (and probably the most important ones) about RNA synthesis over the cell life cycle will probably remain concealed until precursor pool questions have been settled and methods are found for very accurate staging of well synchronized cells in sufficient quality to permit detailed biochemical information on the rates of synthesis of various RNA species over the cycle.

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[2] M. Nemer. In press.

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