CIRCAHORALIAN RHYTHM OF *t*RNA AMINOACYLATION IN HEPATOCYTE CULTURE

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UDC 612.352.3.015.36.085.23"414"

Key words: liver; biological rhythms; protein synthesis.

Fluctuations of incorporation of labeled amino acids into proteins with periods of 20 to 100 min have been found in various types of cells both in vivo and in vitro [3]. Changes in the amino-acid pool as the cause of these fluctuations were eliminated by research in several laboratories, in which attention was paid to changes in the labeled and unlabeled amino-acid pool, the kinetics of accumulation of labeled amino acids in the pool, and their adsorption on cell structures. Secretion of labeled proteins likewise cannot be the cause of fluctuations of the specific radioactivity of proteins [2]. Moreover, changes in the amino-acid pool and protein transport as the cause of instability of incorporation were eliminated by the discovery of a similar rhythm in a cell-free system [1]. On the basis of these observations, fluctuations of incorporation of labeled amino acids into proteins can be interpreted as a rhythm of protein synthesis. It was accordingly essential to discover the causes of the fluctuating kinetics of protein synthesis: which stage of polypeptide formation is most subject to fluctuations in time?

The aim of the present investigation was to study the first stage, namely aminoacylation of *t*RNA, and it brought to light a rhythm similar to the rhythm of protein synthesis in a monolayer of a primary hepatocyte culture.

EXPERIMENTAL METHOD

Hepatocytes were isolated from the liver of Wistar rats weighing 130-150 g. A suspension containing more than 90% of intact cells (about 10^6 hepatocytes/ml) was introduced into a Petri dish and cultured in enriched Eagle's medium on slides [4]. A monolayer of resting cells, formed on the 2nd day of culture, was incubated two or three slides at a time, in successive tests, each for 10 min every 10 min, on medium with ^{3}H-leucine (30 μCi/ml, specific radioactivity 89 μCi/mmole). The total duration of the experiment was 1.5-2.5 h. In each experiment one culture was studied. After incubation with labeled leucine the cells were washed with cold medium containing an excess of unlabeled leucine and sodium pyrophosphate (10^{-2} M), an inhibitor of *t*RNA aminoacylation, quickly treated with cold (4°C) 0.5 M $HClO_4$, and frozen at −70°C. The content of radioactive leucine in aminoacyl-*t*RNA (aa-*t*RNA$_{Leu}$) and the specific radioactivity of the proteins were then determined. For this purpose the cells were washed 3 times with 5% TCA, cooled in an ice bath, and centrifuged at 1000*g* for 10 min (4°C). The washed cell residue was treated with 5% TCA, heated to 90°C, and incubated at this temperature for 15 min to hydrolyze the aa-*t*RNA. After centrifugation the supernatant was collected for counting radioactivity of ^{3}H-leucine from the aa-*t*RNA. The residue was washed with 5% cold TCA and dissolved in 1 N NaOH, diluted with water to 0.1 N NaOH, and the concentration and radioactivity of the proteins were then determined by the usual method. A cell-free system for protein synthesis was prepared from rat liver cells by the method described in [7]. The system consisted of postmitochondrial supernatant and could maintain a relatively long period of high-intensity protein synthesis.

Laboratory of Cytology, N. K. Kol'tsov Institute of Developmental Biology, Academy of Sciences of the USSR. Laboratory of Molecular Biology, Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR I. P. Ashmarin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 109, No. 4, pp. 393-395, April, 1990. Original article submitted April 5, 1989.

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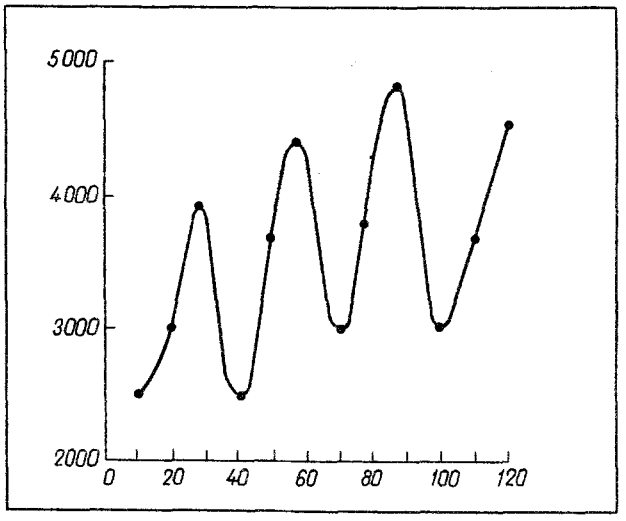

Fig. 1. Time course of ^{3}H-leucine incorporation into proteins of hepatocyte monolayer in vitro. Abscissa, time (in min); ordinate, incorporation of ^{3}H-leucine into proteins, during pulse (10 min) labeling (in cpm/mg protein). Error of individual measurements did not exceed 10%.

EXPERIMENTAL RESULTS

The characteristic dynamics of ^{3}H-leucine incorporation into protein of the hepatocyte monomer, just as in previous investigations of the same object [3], is illustrated in Figs. 1 and 2. In the course of 2 h of observations at least three periods of fluctuations of specific radioactivity of proteins could be observed. Comparison of changes in the radioactivity of leucine in the proteins and in the aa-tRNA$_{Leu}$ fraction of the same monolayer showed quite close agreement between the fluctuations (Fig. 2). In the curves studied in 11 cases the maxima coincided with maxima or minima with minima, and in five cases the radioactivity was opposite in phase.

One possible cause of these changes in the intensity of aminoacylation could be the recently discovered circahoralian fluctuations in most cases were opposite in phase to changes in the intensity of protein synthesis. Protein synthesis is known to be an energy-intensive process, and among the phases of protein synthesis, aminoacylation is particularly demanding for ATP. It is therefore natural to explain the time course of tRNA aminoacylation by energy-dependence on the fluctuations of ATP in the cell, and to explain fluctuations in the rate of protein synthesis by substrate dependence on the aa-RNA concentration. Comparison of the time course of labeling of tRNA and proteins in fact indicates their mutual dependence. However, this dependence is strictly observed at a low level of intensity of tRNA aminoacylation. A low level of aminoacylation remains constant throughout the experiment. It evidently assigns the minimal velocity of total protein synthesis in the cell, which also is relatively stable (Fig. 2). The maximal velocity of protein synthesis, however, does not always correspond to the increased aa-tRNA$_{Leu}$ concentration. This may be due to changes in the ratio of the velocities of tRNA aminoacylation and the resultant rate of amino-acid assembly in the cell, i.e., the life span of aa-tRNA, which evidently reflects the degree of coordination of the protein-synthesizing conveyor. Besides its energy-dependence on ATP, aminoacylation of tRNA is also controlled independently of the other stages of protein synthesis by pyrophosphatase [5], pyrophosphorylase [9], and protease, which have specific signaling sequences in a multienzyme complex consisting of eight aminoacyl-tRNA-synthetases, and which includes leucine tRNA [8]. High rates of protein synthesis do not always correspond, therefore, to an increased concentration of labeled aa-tRNA (Fig. 2). Interference with unlabeled aa-tRNA likewise cannot be ruled out.

It is thus clear that fluctuations of the rate of protein synthesis are connected with fluctuations of tRNA aminoacylation, but are not rigidly linked. The degree of coordination of these processes in the cell varies in the course of the same experiment and from one experiment to another (Fig. 2).

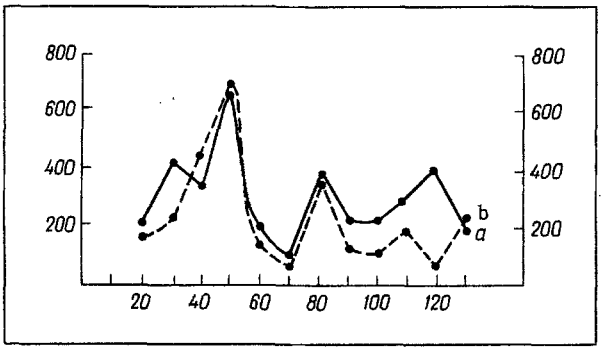

Fig. 2. Example of ^{3}H-leucine incorporation into proteins (a) and into aa-tRNA$_{Leu}$ (b) of hepatocytes from the same monolayer culture (pulse labeling, 10 min). Abscissa, time (in min); ordinate: on left — incorporation of ^{3}H-leucine into proteins (in cpm/mg protein), on right — incorporation of ^{3}H-leucine into aa-tRNA$_{Leu}$ (in cpm/mg protein).

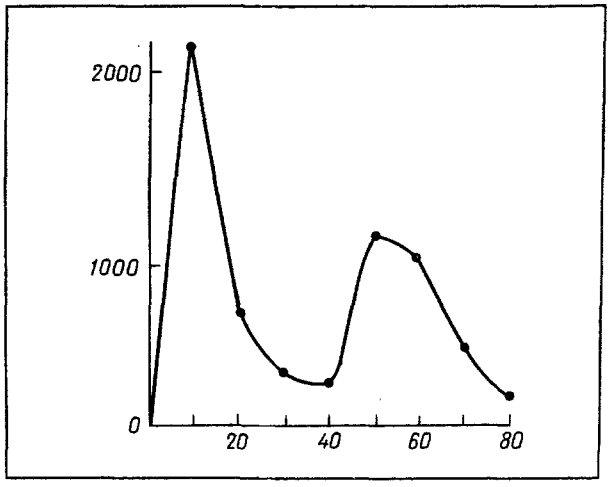

Fig. 3. Changes in rate of incorporation of ^{3}H-leucine into TCA-insoluble polypeptides. Abscissa, time (in min); ordinate: incorporation of ^{3}H-leucine into TCA-insoluble polypeptides (in cpm). Cell-free system for protein synthesis composed of rat liver cells. Pulse labeling, 10 min at 30°C.

The question therefore arises: what is the time course of protein synthesis under conditions of ATP and aa-tRNA saturation? If it is linear in character, the pacemakers of protein synthesis must be exclusively the rhythms of change in ATP and aa-tRNA concentrations. This situation with an excess of ATP and of aa-tRNA can be created in a cell-free system of protein synthesis. The system of rat liver cells [7] guarantees a high rate and long duration of protein synthesis, and several rounds of translation of endogenous and exogenous mRNA. By virtue of these properties, and also the complete homology of the cells, we chose this system for analysis of the dynamics of protein synthesis. Fluctuations of intensity of incorporation of ^{3}H-leucine into polypeptides also are observed in a cell-free system (Fig. 3) in the presence of a high concentration of ATP and aa-tRNA. Consequently, the fluctuating kinetics of the velocity of protein synthesis also is generated in the subsequent stages of protein synthesis, independently of fluctuations in ATP and aa-tRNA concentrations.

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TRANSFORMATION OF HUMAN CORNEAL ENDOTHELIAL CELLS BY MICROINJECTION OF ONCOGENES

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UDC 617.713-008.97:578.826.2]-018.15-092.4

Key words: corneal endothelium; oncogenes; microinjection; immortalization.

Human corneal endothelial cells (HCEC) normally form a monolayer covering the posterior surface of the cornea. HCEC are highly specialized cells with as yet unexplained mechanisms of differentiation and regulation of their functional activity and also an unexplained origin. The main function of HCEC is to maintain the water and electrolyte balance of the corneal stroma. Disturbance of the function of HCEC usually leads to edema and opacity of the cornea, resulting in loss of sight.

The properties of the corneal endothelium have been studied mainly on cultures of animal (rabbit, bovine) cells, and in vitro studies of HCEC have been limited to the search for methods of obtaining cultures of these cells, for they possess low proliferative activity and are unable to grow in culture in the absence of stimulating factors [12, 13]. By a combination of methods already developed it is possible to isolate and cultivate HCEC for a long time (up to 50-fold doubling of the population), while preserving their basic morphological characteristics [8]. Cells in culture can be used to compare preparations for use in ophthalmology, and also to reconstruct the injured corneal endothelium. Since the cells to be studied may change their properties while in culture, the need arises to obtain primary cell lines with a stable phenotype.

Combinations of oncogenes of adenovirus (regions E1a and E1b) and cellular origin, differing in their neoplastic transforming efficiency, have been used as transforming agents [6, 10]. It has been shown that microinjection of the E1 region of the strongly oncogenic type 7 simian adenovirus (SA 7) leads to the immortalization and neoplastic transformation of mammalian embryonic kidney cells [5]. The same region of the genome of the nononcogenic human type 5 adenovirus (Ad 5), while preserving the ability to transform and immortalize primary cells, does not induce tumor formation in the experimental animals [3]. The Ha-ras oncogene, in combination with the E1a region of the adenovirus, can induce effective immortalization and transformation of primary cells [9].

Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. All-Union Research Institute of Agricultural Biotechnology. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 109, No. 4, pp. 395-398, April, 1990. Original article submitted November 5, 1988.