CYCLOHEXIMIDE STIMULATES RIBOSOMAL RNA TRANSCRIPTION IN AMINO ACID-STARVED ASCITES TUMOR CELLS

A. O. POGO and Valerie J. ZBRZEZNA

The Lindsley F. Kimball Research Institute of The New York Blood Center, New York, NY 10021, USA

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1. Introduction

The restriction of rRNA transcription that amino acid starvation induces in nucleated cells has features similar to the stringent phenomenon described in prokaryotes [1–5]. Since amino acid deprivation also inhibits protein synthesis and since inhibition of protein synthesis per se produces template restriction, it is generally accepted that this effect is mediated through a high turnover polypeptide different from any of the subunits of RNA polymerase [2,4,6,7]. However, the fact that de-repression of rRNA synthesis can be achieved by addition of cycloheximide to amino acid-starved yeast cells strongly suggests that in addition to the high turnover polypeptide another (factor(s) plays an essential role in limiting the availability of yeast RNA polymerase to the rRNA cistrons [2–4].

Nevertheless stimulation of rRNA synthesis by cycloheximide is rarely observed in other eukaryotes. It has been postulated that one reason why this phenomenon is difficult to detect is that entrance of uridine into the UTP and CTP pools is feedback-inhibited by these ribonucleotides [3]. Since amino acid starvation produces an expansion of these pools, it prevents the conversion of uridine into UTP and CTP and may mask the real stimulatory effect of cycloheximide on rRNA transcription [3]. To avoid this complication rRNA synthesis has been studied in isolated nuclei and in this communication we present the first evidence that the addition of cycloheximide to amino acid-starved cells produces a bona fide de-repression of rRNA transcription.

This is the fourth paper in a series on control of RNA synthesis in eukaryotes; the first was [2]

2. Materials and methods

2.1. *Culturing conditions of ascites tumor cells*

The cells were grown to a density of 0.5×10^6 cells/ml in Joklik medium (Spinner), supplemented with 10% heat-inactivated and dialyzed fetal calf serum as in [8]. For the experiments the cells were concentrated to a density of 2.0×10^6/ml in Eagle's minimum essential medium supplemented with 10% dialyzed fetal calf serum and maintained in this medium for 1 h (recovery time). For starvation the cells were harvested after recovery and resuspended in a medium containing one-tenth the concentration of essential amino acids (amino acid-deficient medium).

2.2. *Nuclear isolation and assay of RNA polymerase 1.*

At the end of each experiment the cells were rapidly poured onto 4 vol. semi-frozen, crushed 0.9% NaCl solution, collected by centrifugation at $800 \times g$ for 7 min, washed once with a buffer containing 0.35 M sucrose, 10 mM piperazine,*N,N'*-bis (2-ethane sulfonic acid) (Pipes), pH 7.9, 1.5 mM $MgCl_2$ and 1 mM dithioerythritol. The pellet was resuspended in 20 vol. same buffer, and Triton X-100 and saponin were added to the cell suspension to make final conc. 0.5% for each. The cells were disrupted with a Dounce homogenizer (12 strokes), centrifuged for 10 min at $1000 \times g$ and washed again with this buffer minus the detergents. The pellet, consisting of nuclei with attached cytoplasmic tags, was resuspended in 10 vol. buffer

containing 0.35 M sucrose, 4 mM $MgCl_2$, 20% glycerol, 25 mM Pipes, pH 7.9 and 1 mM dithioerythritol. Electron microscopic examinations show that these nuclei are devoid of the nuclear envelope but the cytoskeletal network with aggregate ribosomes remains attached to them [8]. The crude nuclear fraction was frozen in liquid nitrogen and stored at −70°C. The standard incubation mixture for assaying RNA polymerase contains in vol. 75 μl: 25 mM Pipes, pH 7.9, 30 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.36 mM $MnCl_2$, 15 mM KCl, 1 mM dithioerythritol, 0.1 mM sodium phospho(enol)pyruvate, 6 μg pyruvate kinase (Sigma), 0.65 mM each of ATP, GTP, CTP, 0.18 mM [5-^{3}H]UTP (spec. act. 200 cpm/pmol) and ~0.5–0.7 × 10^6 nuclei. This mixture was incubated at 30°C for 20 min and the reaction was stopped by rapid cooling to 0°C followed by the addition of 0.2 mg non-radioactive UTP, 1 ml cold 10% trichloroacetic acid containing 0.04 M sodium pyrophosphate and 200 μg albumin. The precipitate was collected on Whatman GF/B filters and washed 5 times with cold 10% trichloroacetic acid containing 0.04 M sodium pyrophosphate. It was then washed once with ethanol, dried and placed in vials. Triton–toluene scintillation mixture was added and radioactivity was counted. The reaction was done in triplicate in the absence and presence of 400 μg/ml α-amanitin. The SD is between ±0.31–±0.88 pmol [^{3}H]UMP/10^6 nuclei per condition. A separate reaction was done in duplicate in the presence of 15 μg actinomycin D. Polymerase 1 activity was computed by substracting the cpm of the actinomycin-treated samples (40–60 cpm or 0.34–0.51 pmol [^{3}H]UMP/10^6 nuclei) from the α-amanitin-treated samples. The reaction was linear from 0.15–1.5 × 10^6 nuclei in the presence and absence of α-amanitin.

3. Results and discussion

As indicated in fig.1, small doses of cycloheximide stimulate nuclear RNA polymerase 1 activiy when added to amino acid-starved cells. Puromycin, on the other hand, has no effect at this or higher doses, e.g., 3.6 × 10^{-6} M. However, at 18.0 × 10^{-6} M a 20% inhibition was detected after 2 h treatment (not shown).

When cells are transferred from complete to amino acid deficient medium (shift down), there is a slight increase in RNA polymerase 1 activity in the first 2 h;

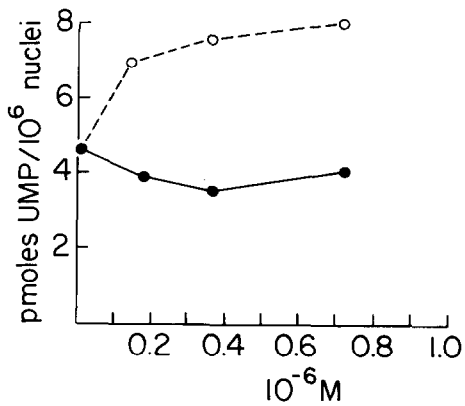

Fig.1. Effect of different concentrations of cycloheximide and puromycin on the activity of nuclear RNA polymerase 1. At the time of starvation the culture was divided into 3 aliquots: control and cycloheximide- and puromycin-treated and kept for 2 h. Then nuclear RNA polymerase 1 activity was determined. (○——○) cycloheximide-treated sample; (●——●) puromycin-treated sample.

later on the activity reaches a plateau and in some experiments there is a significant diminution after 4 h. Although the degree of the early increase as well as the subsequent diminution varies between experiments, the pattern shown in fig.2A is consistently observed. As shown fig.2, addition of a small dose of cycloheximide cancels the inhibitory effect of starvation. The effect of the drug is observed after a lag period of 1 h and is maintained for another hour. In general, rRNA synthesis in nuclei isolated from cells treated with cycloheximide for 2 h is approx. 40–60% higher than in non-treated cells. This difference increases to 100%, primarily in those experiments where there is a significant diminution of RNA polymerase 1 activity in non-treated cells.

When cells are transferred to a complete medium (shift up) there is an initial slight stimulation in nuclear RNA polymerase activity followed by an abrupt increase after 1 h incubation and a slower rise in the next two hours (fig.2B). This pattern varies and in some experiments the rapid increase in observed during 1 h incubation. However, the addition of a small dose of cycloheximide does not alter this pattern as it does in starved cells (fig.2B).

Since stimulation or inhibition of transcription in isolated nuclei is not due to changes in 'pool size' and

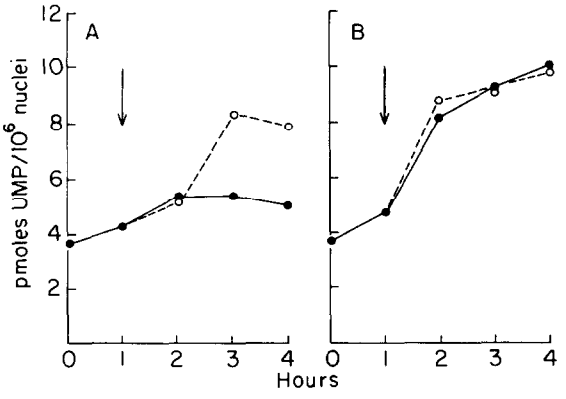

Fig.2. Effect of a small dose of cycloheximide on rRNA transcription in cells transferred to (A) amino acid-deficient medium (shift down) and to (B) complete medium (shift up). Cycloheximide, 0.1 μg/ml (0.36×10^{-6} M) was added to half of the cultures and nuclear RNA polymerase 1 activity was determined. The values are averages of 3 different experiments and were normalized by the amount of activity obtained after the recovery period (0 time). The arrow indicates the time at which the drug was added. (○——○) Cycloheximide-treated samples; (●——●) control.

Table 1
Effect of small doses of cycloheximide and puromycin on protein synthesis of ascites tumor cells cultured in amino acid starved and non-starved conditions

Treatment	Incorporation of [^{3}H]Lysine (cpm × 10^3/2 × 10^6 cells)	
	Starved	Non-starved
–	166.9	33.9
Cycloheximide		
0.36×10^{-6} M	127.9	12.0
Puromycin		
0.72×10^{-6} M	158.0	35.5
18.0×10^{-6} M	139.0	18.6

The cells were resuspended in starved and unstarved media and 90 min after treatments with the antibiotics they were labelled for 30 min with 150 μCi [^{3}H]lysine (spec. act. 7Ci/mmol; Schwarz/Mann). The incorporation was stopped with 10% cold trichloroacetic acid and the precipitate was washed once with the same solution and with alcohol–ether (2:1, v/v). The insoluble material was heated to 90°C for 15 min in 10% trichloroacetic acid and the acid-insoluble material was dissolved in concentrated formic acid. Scintillation mixture was then added to determine radioactivities. Note that the low [^{3}H]lysine incorporation in non-starved cells is due to radioisotope dilution

does not reflect alterations of nuclear envelope permeabilities to ribonucleoside triphosphates or inhibitors and activators of polymerases, this stimulation must reflect, to a large extent, a real augmentation in the number of enzyme molecules engaged in rRNA transcription [2–4]. It has been reported that antibiotic release of restricted RNA synthesis in amino acid-starved *Escherichia coli rel*$^+$ can be detected at the level of nucleoids, i.e., an in vitro system comparable to isolated nuclei in eukaryotes [9].

The concentration of cycloheximide that de-represses rRNA synthesis inhibits by 25% and 65% the activity of the protein synthetic apparatus in starved and non-starved cells, respectively (table 1). However, puromycin at twice this concentration has no inhibitory effect on starved or non-starved cells. In order to produce a similar inhibition it is necessary to add as much as 50-times the concentration of cycloheximide (table 1). It is obvious that the specific release of restricted RNA synthesis by a low dose of cycloheximide, known as phenotypic relaxation, is independent of the rate of protein synthesis. In another communication it will be shown that this low dose produces an increase in the accumulation of 45 S rRNA precursor; the detection of this accumulation depends upon specific culture conditions of the cells (in preparation). Since high doses of cycloheximide, like those of other protein synthesis inhibitors, inhibit nuclear RNA polymerase 1 activity, it is evident that the effect on transcription is dose-dependent.

De-repression of rRNA transcription in amino acid-starved cells is identical to that elicited in prokaryotes by chloroamphenicol, a drug which has effects similar to cycloheximide [5]. This is the main feature of the stringent phenomenon in which RNA synthesis can continue in the absence of, or with little, protein synthesis and can be obtained in prokaryotes by mutation (relaxed mutants) [5]. Relaxed mutants have yet to be obtained in eukaryotes, however, the cycloheximide response in amino acid-starved ascites tumor or yeast cells strongly suggests a basic mechanism common to prokaryotes, yeast, and ascites cells in the regulation of rRNA metabolism [2–4,10,11,14]. Similar observations have been made for the synthesis of 4 S RNA in confluent monolayers of chick fibroblasts, 3T6

mouse fibroblasts during transition from resting to growing state, and Chan's liver cells [12–15].

It has been a general belief that guanosine 3,5'-bipyrophosphate (ppGpp), which accumulates in amino acid-starved *E. coli rel*$^+$ strains, is the stringent signal for repression of stable RNA synthesis [16]. However, it has been shown in some bacterial strains that the close relationship between inhibition of rRNA synthesis and accumulation of ppGpp does not exist [17–18]. These observations and the well known fact that stringency does not produce accumulation of ppGpp in high and low eukaryotes, indicate that ppGpp may not be directly involved in controlling rRNA synthesis [19–21]. In any case, our observations in ascites tumor cells indicate that cycloheximide has the ability to prevent the synthesis or the functioning of a compound which restricts the amount of promoter sequence available for interaction with the RNA polymerase or limits the number of molecules which are able to interact with the promoter. It is obvious that this compound, which remains to be identified, is not produced by the idling reaction of protein synthesis.

The observation reported here does not support the assumption that the stimulatory effect of cycloheximide on RNA synthesis is due to changes in ribonucleoside triphosphate pools [22]. There is little doubt that cycloheximide stimulation of nuclear RNA synthesis in starved ascites tumor cells is a bona fide gene de-repression process. It is concluded that the effect of the drug in starved eukaryotes is, at the phenomenological level, comparable to phenotypic relaxation in prokaryotes.

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