ABSENCE OF ppGpp PRODUCTION IN SYNCHRONISED BALB/C MOUSE 3T3 CELLS ON ISOLEUCINE STARVATION

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1. Introduction

Two unusual nucleotides guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp) accumulate when stringent or rel$^+$ strains of *E. coli* are starved for required amino acids [1]. Amino acid starvation of the relaxed or rel$^-$ strains does not result in a similar accumulation of ppGpp and pppGpp. Many lines of evidence indicate that accumulation of the nucleotide ppGpp may be related to the restriction of stable RNA synthesis in rel$^+$ strains of *E. coli* following amino acid starvation [see [2] and [3] for a review]. Attempts to demonstrate the production of ppGpp in intact mammalian cells following amino acid starvation have so far been unsuccessful [4–6].

It appeared to us that inability to observe an accumulation of ppGpp in mammalian cells may be related to the regulation of RNA synthesis as a function of the cell cycle [7–9]. Therefore, we starved synchronised mouse 3T3 cells for isoleucine, and then looked for the production of ppGpp. In addition, we have also utilised an isoleucine analogue, *O*-methyl threonine, to induce extreme amino acid deficiency. *O*-methyl threonine is a competitive inhibitor of eukaryotic isoleucyl-tRNA synthetase; treatment of HeLa cells with *O*-methyl threonine has been shown to result in an accumulation of uncharged isoleucyl tRNA [10,11]. Under these conditions of cell cycle time for maximal ribosomal RNA synthesis, combined with accumulation of uncharged isoleucyl tRNA in the cell, we did not observe magic spot formation.

2. Materials and methods

Balb/c 3T3 cells were grown in Falcon tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% calf serum in the presence of penicillin and streptomycin. For routine subculture, cells were treated with 0.25% trypsin (Gibco) and counted in haemocytometer. However, in experiments described in fig.1, cell counts were obtained after trypsin-EDTA treatment. Trypsin-EDTA solution was prepared by mixing one volume of 0.25% trypsin solution (Gibco) with 9 volumes of 0.2% EDTA in calcium- and magnesium-free phosphate buffered saline. Cells were treated with the trypsin-EDTA solution for 20 min and resuspended by pipetting back and forth several times.

In typical experiments about 5×10^4 cells were plated in 3.5 cm diameter Falcon tissue culture dishes in 2.5 ml medium and grown for nearly 24 h. Growing 3T3 cells were serum starved for 30–32 h by shifting to a medium containing 0.5% calf serum. Following serum starvation, cells were shifted back to the medium containing 10% calf serum for 9 h. Routinely, 9 h of serum stimulation was used as the zero time for amino acid starvation experiments. At the zero time, cells were shifted to a Control Dulbecco's medium or a complete medium lacking isoleucine as well as a medium lacking isoleucine but containing *O*-methyl threonine (Calbiochem) along with 10% calf serum

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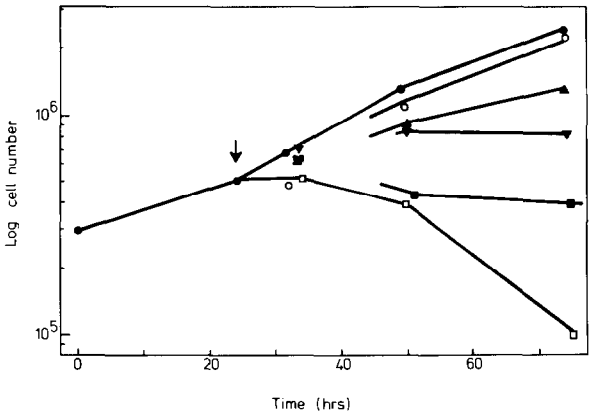

Fig.1. Effect of *O*-methyl threonine on the growth of 3T3 cells. 3 X 10^5 cells were plated per 6 cm dish and treated with the medium containing the analogue after 24 h in complete medium as described in Materials and methods. The arrow indicates the time of transfer from the complete medium to the medium indicated. The control plates (●—●) received complete medium containing 10% dialysed serum. Other plates received complete medium minus isoleucine, + 0 (○—○), (▲—▲), 4 (▼—▼), 10 (■—■), or 20 (□—□) mM *O*-methyl threonine. Cell counts were obtained as described in Materials and methods.

(dialysed against phosphate buffered saline). Labelling was done by using 300 to 400 μCi of [^{32}P]phosphate (carrier-free, sterile, obtained from Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland) in 2.5 ml of appropriate medium. At the end of a 3 h-labelling period, the radioactive medium was carefully removed, and cells were rinsed with appropriate pre-warmed medium. Washed cells were then treated with 1 ml of 2 M formic acid for 15 min at 4°C. Formic acid extracts were carefully removed and immediately frozen at −20°C.

^{32}P-Labelled nucleotides in formic acid extracts were separated by thin-layer chromatography on PEI-cellulose sheets (Machery-Nagel & Co.) developed with 1.5 M KH_2PO_4 (pH 3.4) as a solvent following the procedure described by Cashel [12]. Routinely, 10 μl samples of formic acid extracts were analysed. The chromatograms were usually developed for 2 h in the solvent, dried and autoradiographed with Ilford Rapid R type X-ray film. Exposure was for 3–4 days. Authentic ^{32}P-labelled ppGpp was a generous gift of Dr Dietmar Richter.

3. Results and discussion

Starvation of 3T3 cells for 30–32 hours in a medium containing 0.5% serum results in cells arrested in G1 phase of the cell cycle [13,14]. Stimulation of serum-starved cells with a medium containing 10% serum results in the resumption of DNA synthesis [9]. We have confirmed the established data [9] that it takes nearly 15 hours for the 3T3 cells to resume DNA synthesis following serum stimulation, and found that a 12 h serum stimulation is enough to sustain the DNA synthesis (not shown). There is considerable evidence [7–9] that by about 9–10 h post serum stimulation the cells actively synthesize ribosomal RNA and ribosomes. Therefore, having established the conditions for synchrony in our hands we starved 3T3 cells for isoleucine for various lengths of time after 9 h of serum stimulation, when ribosomal RNA synthesis would be maximal.

Mammalian cells have a significant protein turnover even in the growing state, and as a result there is a continual supply of amino acids even when they are omitted from the media [15]. In order to ensure that true isoleucine deficiency was being produced, we used an analogue of isoleucine, *O*-methyl threonine which is known to cause an accumulation of uncharged isoleucyl-tRNA in vivo by inhibiting the isoleucine charging reaction [11]. The dose response of the growth curve at various levels of *O*-methyl threonine is shown in fig.1. In the upper range of concentrations, cells detached from the plates and could not be rescued by replating in normal medium. We therefore selected concentrations of 2 mM and 4 mM *O*-methyl threonine for subsequent experiments. At these concentrations, the effect on 3T3 cells was reversible.

Figure 2 shows the results of a typical experiment. As can be seen, we could not detect the production of ppGpp even under severe conditions of amino acid starvation of synchronized 3T3 cells. Exposure of cells to as high as 20 mM *O*-methyl threonine also did not elicit the production of ppGpp under similar conditions. The data presented in fig.2 are from a typical 3 hour pulse with [^{32}P]phosphate; however, variation of ^{32}P-labelling period from 1 to 14 h gave similar results. The labelling periods covered points in the cycle between the beginning of S phase to the end of S phase and into G2 phase.

It is conceivable that during the establishment of

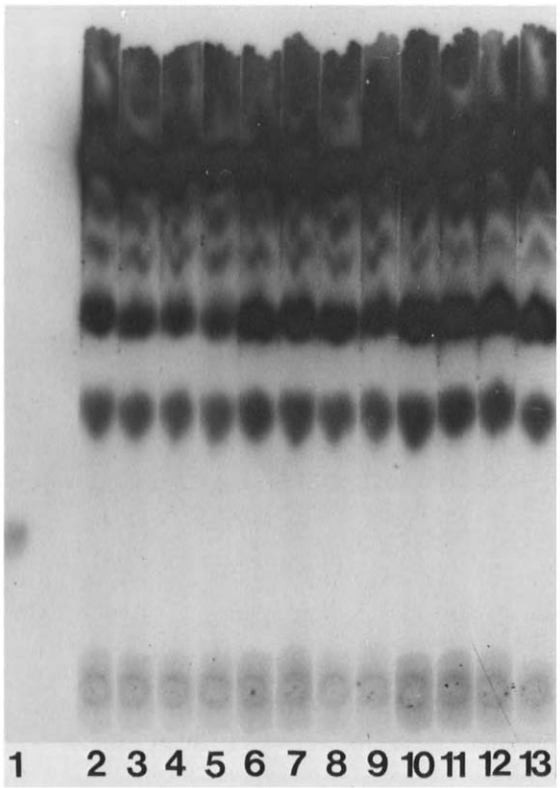

Fig.2. Autoradiogram of formic acid extracts of 3T3 cells treated with *O*-methyl threonine. Synchronised 3T3 cells were treated with *O*-methyl threonine for various times indicated, and formic acid extracts were prepared and chromatographed as described in Materials and methods. Samples in columns from left to right: 1. authentic ppGpp, 2. zero time control, 3. zero time ileu$^-$, 4. zero time treated with 2 mM *O*-methyl threonine, 5. zero time treated with 4 mM *O*-methyl threonine. Columns 6–9 same as 2–5 but treated with *O*-methyl threonine for 4 h before ^{32}P-labelling. Columns 10–13 same as 2–5 but treated with *O*-methyl threonine for 12 h before ^{32}P-labelling.

3T3 as a cell line, the ability to produce ppGpp may have been lost, and thus our negative results do not exclude the existence of ppGpp in mouse fibroblasts. In order to check this possibility, primary cultures of embryonic fibroblasts were prepared from 16–17-day-old Balb/c mouse embryos. When these embryonic fibroblast cultures were tested at the third passage level under the conditions described in fig.2, we could not detect the production of ppGpp. Direct treatment with *O*-methyl threonine or following serum starvation (40 h) and serum stimulation gave identical results (data not shown). From these observations we conclude that the stringent control mechanism to elicit the production of ppGpp on amino acid starvation does not exist in mouse fibroblasts. By cutting out the PEI cellulose strips after chromatography and direct counting with an authentic ^{32}P-labelled ppGpp, we have found that our limit of detection by autoradiography is of the order of 0.5–1% of the counts in GTP. Thus, we conclude that even if ppGpp were present, it does not increase to levels higher than 0.5% of the GTP.

While our experiments were in progress, a report [16] was published describing the presence of ppGpp and pppGpp along with another highly phosphorylated nucleotide in Chinese hamster ovary cells (CHO) and baby hamster kidney cells (BHK). The same report also described the presence of pppGpp and another highly phosphorylated nucleotide in the human diploid cell culture WI38. We subjected the BHK and WI38 cells directly to *O*-methyl threonine treatment at 2 mM and 4 mM concentrations for periods up to 14 h and failed to detect the production of ppGpp (data not shown). It must be pointed out that we do observe the presence of some phosphorylated compound remaining at the origin of chromatograms with all the three different kinds of mammalian cells we have tested (see fig.2). However, this radioactivity does not represent ppGpp artifactually adsorbed at the origin, as the authentic ppGpp migrates with the same R_F either alone or mixed with formic acid extracts (not shown). The nature of radioactively labelled material at the origin is not identified. Thus, we have not detected the appearance of any unusual nucleotides, such as ppGpp on severe amino acid starvation of two different mammalian cell lines, namely 3T3 and BHK, nor in a human diploid cell culture WI38 and in early passage mouse embryo fibroblast cultures. It should be noted that our results are consistent with earlier reports of failure to detect ppGpp and pppGpp production in HeLa [4], 3T3 [5] and CHO cells [6].

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