PRESENCE OF MAGIC SPOT IN *DICTYOSTELIUM DISCOIDEUM*

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1. Introduction

When rel$^+$ *Escherichia coli* are transferred to a nutritionally deficient media, they undergo various metabolic changes, including a preferential restriction of ribosomal and transfer RNA synthesis [1–3]. This response to step-down conditions is preceded by the accumulation of the nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) which are thought to participate in the amino acid control of RNA synthesis [4–6]. This possibility has prompted several laboratories to examine eukaryotes for the existence of these compounds [7, 8] but, to date, these attempts have been unsuccessful. The inability to detect ppGpp in the systems studied may have been due to the failure to establish step-down conditions of physiological significance. In contrast, these conditions are clearly defined for *Dictyostelium discoideum* since the life cycle of this organism includes a differentiation phase which is initiated by starvation [9]. Under such conditions, amoebae stop growing, aggregate, and finally become fruiting bodies and spore cells. This developmental sequence is characterized by a modification of DNA transcription [10], general RNA and protein degradation, [11, 12] and the appearance of new enzymes and membrane constituents [13, 14]. Since several metabolic changes occurring in starved amoebae resemble those of bacteria, they could involve the same regulatory mechanisms. Therefore the presence of ppGpp was examined in this organism. Data are presented here which demonstrate its existence in *D. discoideum* and its accumulation during differentiation.

2. Materials and methods

2.1. *Culture conditions*

Ax-2 amoebae were grown in suspension cultures in HL-5 medium [14] supplemented with 100 μg/ml penicillin and streptomycin. Exponential cells were diluted into the same medium buffered with 5 mM 2-*N*-morpholinoethanesulfonate (MES) pH 6.2 instead of 2 mM phosphate buffer, pH 6.2. In prelabeling experiments, 200 μCi/ml of $^{32}P_i$ were added at this time. The cells were grown overnight (2–3 generations) at 21°C, centrifuged at room temperature at 800 *g* for 2 min, and resuspended at a density of 1.2×10^7 cells/ml in 5 mM MES, pH 6.2.

2.2 *Determination of radioactivity in Dictyostelium Magic Spots (DMS)*

The accumulation of $^{32}P_i$ into DMS was determined by thin layer chromatography after the extraction of acid-soluble pools with ice-cold formic acid [5]. In a typical experiment, an aliquot of cells was added to an equal volume of 4 M formic acid, pH 3 and incubated at 0°C for 30 min. The sample was then centrifuged at 5000 *g* for 20 min and the supernatant spotted onto polyethyleneimine cellulose (PEI) sheets (Brinkmann Instruments, Inc., Westbury, New York). Chromatograms were usually developed in 1.5 M phosphate, pH 3.4 [5], DMS was located by autoradiography, and its radioactivity determined by liquid scintillation counting. When a two-dimensional system

was used, chromatograms were first developed in 2 M $LiCl_2$ soaked 20 min in 800 ml methanol, air-dried and then developed in the second dimension with 1.5 M KH_2PO_4 [15].

3. Results

When amoebae are suspended in 20 mM phosphate buffer, pH 6.2, for 6 hr they become 'competent', i.e. when placed on a solid support they aggregate and differentiate within a minimal time span [16]. Development of the competent state is associated with the appearance of specific antigens [17] as well as changes in cAMP [18] and phosphodiesterase levels [18, 19]. Since competence is similarly obtained in either 20 mM phosphate or 5 mM MES buffer, pH 6.2 [20], the presence of ppGpp was determined in axenically grown cells which had been labeled with $^{32}P_i$ during growth and then resuspended in 5 mM MES buffer. Under these conditions, formic acid extracts prepared as described by Cashel [5] contain a compound, referred to as *Dictyostelium* Magic Spot (DMS), which migrates on polyethyleneimene cellulose sheets with the same R_f as bacterial ppGpp (fig. 1). For these experiments, a $^{32}PO_4$-labeled extract of *E. coli* C600 (rel^+) which has been starved for isoleucine by the addition of excess valine [21] was employed as a marker. Although the bacterial spots migrate slightly ahead of those of the amoebae, they co-migrate when extracts are mixed prior to chromatography. In extracts prepared from amoebae, a large amount of radioactivity is associated with the origin and may represent the products which retard nucleotide migration. Therefore attempts were made to partially purify extracts before chromatography. Variation of cell incubation time in formic acid, and phenol–chloroform–ether treatment of resulting extracts [22] did not alter the patterns obtained. The results, however, further indicate the nucleotide nature of the remaining compounds.

More direct evidence concerning the chemical nature of DMS was obtained using partially purified preparations. Elution of DMS from PEI sheets with 4 M $LiCl_2$ and precipitation with concentrated NH_4OH [23] yields a compound which quantitatively adsorbs to activated charcoal. Radioactivity eluted from charcoal with 3% NH_4OH re-chromatograms as a single spot in 1.5 M KH_2PO_4. Unfortunately, the low level of radioactivity in DMS and its inefficient elution from charcoal (20%, as reported for bacterial ppGpp [23]) makes further purification difficult. Therefore, other characterization experiments were performed using unpurified formic acid extracts.

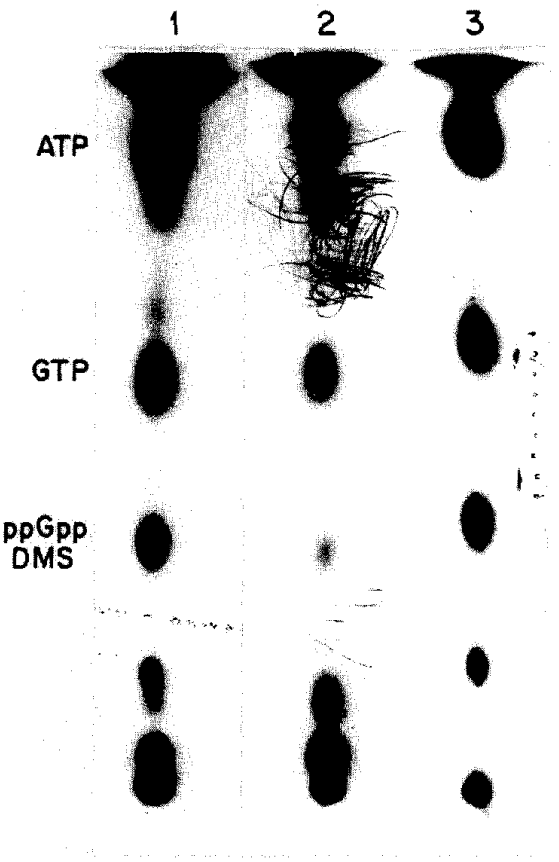

Fig. 1. One-dimensional chromatography of formic acid extracts prepared from bacteria, and amoebae suspended in 5 mM MES for 2 hr: column 1, mixed extracts prepared from amoebae and bacteria; column 2, amoeboid extract; column 3, bacterial extract.

If two-dimensional chromatograms of extract prepared from amoebae and bacteria are compared, DMS and ppGpp are found to migrate similarly [20]. When purified, non-radioactive, bacterial ppGpp is added to an amoeboid extract, ultraviolet-absorbing material and radioactivity identified as DMS co-migrate in both one- and two-dimensional systems (fig. 2). A small amount of ultraviolet-absorbing material was detected with the spot appearing to the right of GTP and may represent either a degradation product or an impurity.

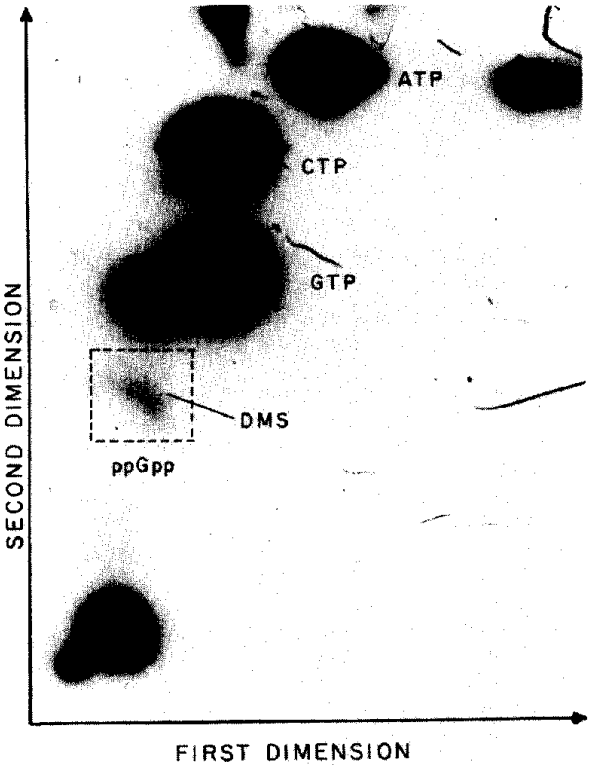

Fig. 2. Two-dimensional chromatography of formic acid extracts prepared from amoebae incubated in 5 mM MES buffer to which was added purified ppGpp.

Susceptibility of DMS to phosphomonoesterase digestion and nitrous acid oxidation was then compared to that of ppGpp, using the procedures described by Cashel and Kallacher [23]. When extracts prepared from amoebae are incubated with enzyme, 50% of DMS, as determined by its radioactive content, is destroyed after 1 hr. ppGpp is similarly destroyed when unpurified bacterial extracts are incubated with enzyme. The time course of nitrous acid oxidation of DMS was also found to parallel that of bacterial ppGpp (data not shown). In both cases, the radioactivity associated with the origin does not change.

Since all present data indicate that DMS is indeed ppGpp, the appearance of this nucleotide was examined in amoebae incubated under various conditions. The accumulation of radioactivity in DMS by prelabeled cells suspended in 5 mM MES buffer is shown in fig. 3. DMS is maximally labeled at 2 hr, after which time its radioactive content declines. In comparison,

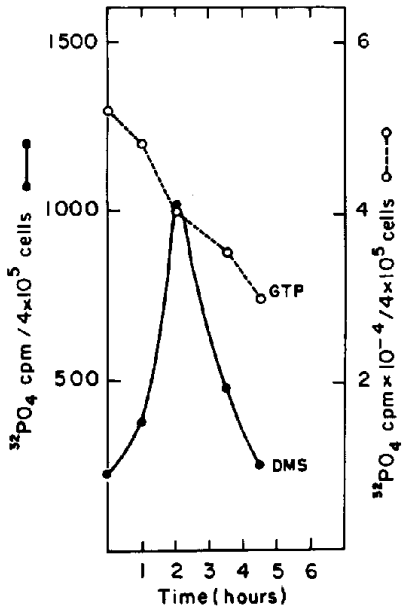

Fig. 3. Accumulation of DMS during starvation.

the radioactivity associated with the origin continuously increases, and with GTP, decreases.

To determine if increased synthesis or decreased degradation accounts for the accumulation of DMS, the kinetics of radioactive incorporation into this compound were examined (fig. 4). When cells are placed in 5 mM MES buffer containing 200 μCi/ml $^{32}P_i$, radioactivity in DMS appears after 40 min of incubation, and increases for approximately another 50 min. The GTP pool, however, is labeled within 5 min, and is radioactively saturated by 30 min. When cells are incubated in buffer 60 min prior to $^{32}P_i$ addition, peak radioactivity in DMS is found within 15 min. These results indicate that maximal synthesis of DMS occurs at the time it is first detected in the extracts.

Synthesis of DMS was next examined in exponentially growing cells. Amoebae were incubated in HL-5 growth media buffered with either 2 mM phosphate or 5 mM MES and containing up to 1 mCi/ml $^{32}P_i$. Under these conditions, the GTP pool is saturated with label within 15 min, but autoradiograms prepared at various times during a 3-hr incubation showed no spot corresponding to DMS. Cells were then grown for two generations in either media containing 200 μCi/ml $^{32}P_i$. Again, chromatography of extracts obtained from both

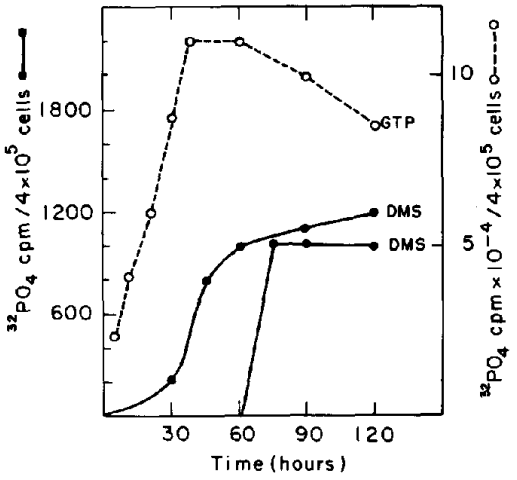

Fig. 4. Kinetics of DMS labeling.

cultures revealed no radioactive DMS.

The data, therefore, indicate that *D. discoideum* accumulate DMS in response to starvation. The interim between the time cells are placed in buffer and the time they synthesize DMS may reflect the period required for amoebae to exhaust a specific nutrient(s), or otherwise reach critical concentrations of metabolites. If this is true, the state of starvation could then be defined by the appearance of DMS.

If DMS serves a regulatory function in *D. discoideum*, then it is of interest to compare its appearance with that of other effectors which are thought to play essential roles in differentiating amoebae, such as cAMP [14, 25]. Variations in the concentration of this nucleotide during the developmental phase have been reported [18, 25] but these changes occur several hours after DMS synthesis. It is possible to obtain more information concerning the role of DMS and its relationship to cAMP by examining developmental mutants of *D. discoideum*. This work is currently in progress.

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