SYNTHESIS OF GUANOSINE TETRAPHOSPHATE (MAGIC SPOT I) IN SACCHAROMYCES CEREVISIAE

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SUMMARY

Guanosine tetraphosphate (Magic Spot I) has been found in formic acid extracts of Saccharomyces cerevisiae subjected to heat shock. The yeast compound comigrates with authentic ppGpp in several chromatographic systems, and further tests confirm its identity with the guanosine-5'-diphosphate-3'-diphosphate found in bacteria. Oxytetracycline inhibits its formation in yeast cells, suggesting that it is produced on the mitochondrial ribosomes.

INTRODUCTION

Guanosine 5' diphosphate-3' diphosphate (ppGpp) controls a remarkably broad range of processes in transcription, translation, and metabolism in Escherichia coli (1-3) and very likely in other bacteria as well (4-6). The possible occurrence of this regulatory nucleotide in eukaryotic cells has therefore received considerable attention. The results of these nucleotide hunting expeditions have been generally negative: the absence of ppGpp has been recounted in studies of yeast (7), Ankystrodesmus (8), Neurospora (8, 9), sea urchin embryos (10, 11), mouse embryos (Silverman, and Atherly, in press), and cultured mammalian cells (12-13); unpublished studies have confirmed the absence of ppGpp in mouse 3T3, human fibroblast, and hamster fibroblast cells (Gallant, Hogan, and Shell, unpublished), mouse ascites cells (Martin, Gallant, and Shell, unpublished), and CHO cells (Lazzarini, unpublished).

Klein (14) has reported a compound chromatographically similar to ppGpp in the slime mold Dictyostelium discoidem, and Rhaese (15) has reported a similar compound in certain mammalian cell lines. In neither of these cases was adequate chemical characterization of the compound(s) in question undertaken. This is a point of some importance, since nucleotides other than ppGpp which comigrate with it in conventional chromatographic separations have been described (16-18). Moreover, the compound reported by Rhaese (15) could not be detected in other studies of one of the same cell lines (13, and Lazzarini, unpublished), which raises the question of mycoplasma contamination in Rhaese's culture. In short, evidence of ppGpp in eukaryotic cells ranges from the negative to the questionable.

In this communication, we report on the occurrence of ppGpp, identified by rigorous criteria, in yeast cells. The nucleotide accumulates in response to heat shock. The mechanism governing its formation in yeast cells is unknown, but evidently involves mitochondrial ribosomes.

MATERIALS AND METHODS

Strains and medium: Saccharomyces cerevisiae A364A used in this study is obtained from Dr. Leland H. Hartwell and was described previously (19). The synthetic complete minimum medium was of Wickerham (20) except the phosphate concentration is 0.5 mM and was supplemented with histidine, tyrosine and lysine at 100 µg/ml and adenosine and uridine at 20 µg/ml.

Chemicals: The following enzymes were purchased from Sigma Chemical Co. at St. Louis, MO.; glyceraldehyde-3-P dehydrogenase/3-P-glycerate phosphokinase mixed enzymes from yeast (50 and 150 units/mg protein respectively), inorganic pyrophosphatase from Baker's yeast (647 units/mg protein). Nucleotides were purchased from P-L Biochemicals, Inc. and ICN Pharmaceuticals. Carrier-free ^{32}P-phosphoric acid were obtained from New England Nuclear and polyethyleneimine-cellulose thin-layer sheets were from Brinkmann Instruments.

Labelling and measurement of nucleotides: Cultures were grown, unless otherwise indicated, at room temperature and under forced aeration. The possibility of bacterial contamination was tested and eliminated by plating samples of 0.1 ml from the medium on a Mac Conkey agar plates or by plating diluted samples on rich nutrient agar plates. To label the nucleotide pool, 1 mCi/ml of ^{32}P-phosphoric acid was added to all cultures growing exponentially at an absorbance of about 0.2 at 720 nm. (An absorbance of 0.2 corresponds to about 3.4×10^6 cells/ml). Starting of experiments and removal of samples for nucleotide determination were carried out at least one cell doubling after addition of isotope to the medium to ensure complete equilibrium between the nucleotide pools and exogenous ^{32}P-phosphoric acid. For determination of nucleotide level, 100-µl samples were extracted with 5 µl 2 M formic acid followed by two-dimensional thin-layer chromatography on polyethyleneimine-cellulose sheets (21). The sheets were developed with 2 M formic acid containing 1.5 M LiCl in the first dimension followed by 1.5 M KH_2PO_4 in the second dimension (18). The desalted chromatograms were radioautographed and radioactive spots were cut out and counted in a Pakard Tri-Carb liquid scintillation spectrometer.

Purification of ppGpp: Radioactive spots corresponding to ppGpp from 10 to 14 chromatograms were cut out (yielding about 10^4 cpm per preparation) and eluted in a small QUIK-SEP absorption column (Isolab Inc. Akron, Ohio) according to the following steps: first washed with 2 ml methyl alcohol and then 2 ml 0.01 M Tris·HCl buffer, pH 7.4 followed by 3 consecutive elutions with 2 ml (each) of 2 M triethylammonium bicarbonate (pH 8.0). The combined TEA-bicarbonate elutes were lyophilized and resuspended in distilled water. As thus isolated, ppGpp was shown to be homogenous in 2 different systems of development on PEI-cellulose sheets.

In Vitro enzymatic conversion of ppGpp to pppGpp: Procedures were of Glynn and Chappell (22) with modifications. ppGpp can substitute ADP as acceptor

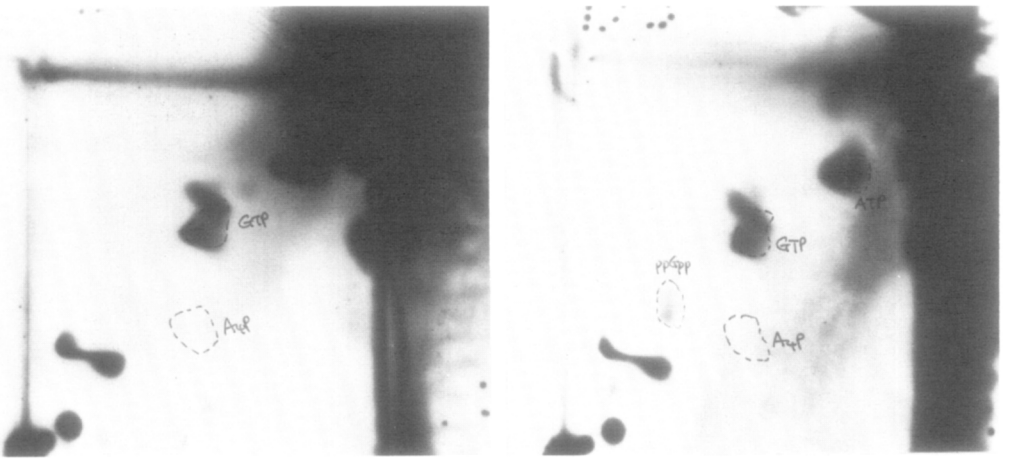

FIGURE 1. Autoradiograph of two-dimensional thin-layer chromatograms of formic acid extracts (20 µl) of yeast grown at room temperature (left) and after 6 minutes at 38° (right). Nucleotides were extracted in formic acid and resolved on thin-layer of PEI-cellulose together with non-radioactive marker nucleotides as described in MATERIALS AND METHODS and previously (21). The chromatograms were exposed to Kodak non-screen X-ray films for 72 hours. Dotted circles represent the positions where marker nucleotides migrated.

in the exchange reaction between inorganic phosphate and ATP catalyzed by glyceraldehyde-3-P dehydrogenase and 3-p-glycerate phosphokinase:

$$\text{Glyceraldehyde-3-P} + \text{Pi} + \text{NAD} \longrightarrow \text{1,3-PP-glycerate} + \text{NADH}$$

$$\text{1,3-PP-glycerate} + \text{ADP(ppGpp)} \longrightarrow \text{3-P-glycerate} + \text{ATP(pppGpp)}$$

The equilibrium position of the second reaction is far to the right, so that ADP (or ppGpp) can be present only in catalytic amounts. 10 µl reaction mixture contained the following: Tris·HCl buffer at pH 7.4 (0.3 mmole), $MgSO_4$ (0.05 mmole), KCl (0.5 mmole), KH_2PO_4 (1 µmole), NAD (1 µmole), glyceraldehyde-3-P (10 µmole), ^{32}P-phosphoric acid (0.2 µCi), glyceraldehyde-3-P dehydrogenase and 3-P-glycerate phosphokinase at 0.1 and 0.3 units respectively. Reaction mixture was incubated at 37° for 60 minutes and followed by two-dimensional thin-layer chromatography as described before (21).

Hydrolysis of ppGpp by Zinc-activated yeast inorganic pyrophosphatase: Purified ppGpp was hydrolyzed in 100 µl containing 10 mM Tris·HCl, pH 7.0, 0.4 mM $Zn(OH)_2$ and 10 units of yeast inorganic pyrophosphatase at 37° for 10 hours. Nucleotides were resolved on PEI-cellulose thin-layer sheet.

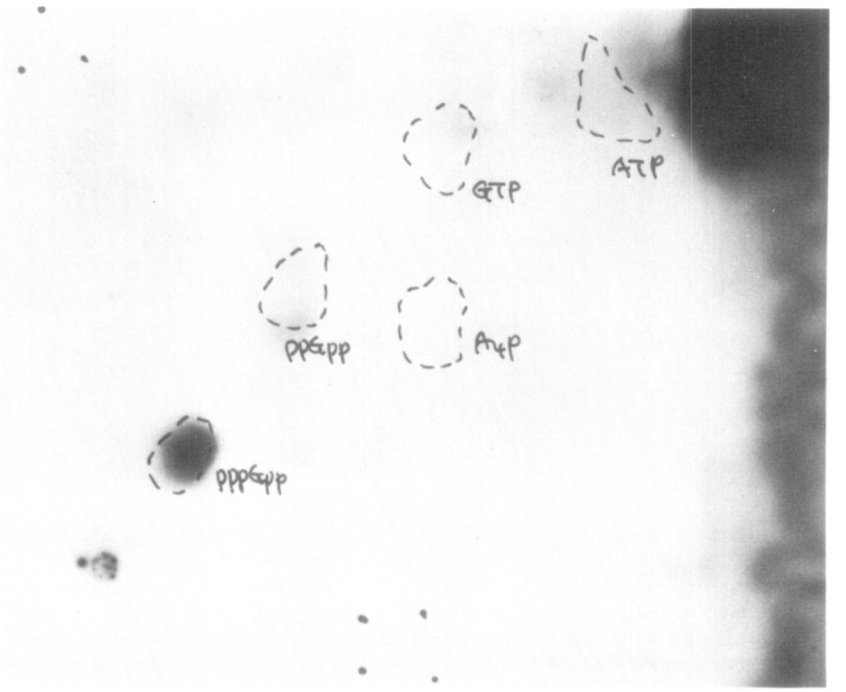

FIGURE 2. Conversion of yeast ppGpp to pppGpp by glyceraldehyde-3-P dehydrogenase and 3-P-glycerate phosphokinase. ppGpp was purified by preparative thin-layer chromatography from yeast culture which has been shifted from room temperature to 38° for six minutes. Experimental details were described in MATERIALS AND METHODS. Dotted circles represent the positions where marker nucleotides migrated. Radioactivity in pppGpp and ppGpp spots are 806 and 136 cpm respectively. The chromatogram was exposed to Kodak nonscreen X-ray films for six days.

Alkaline hydrolysis of yeast ppGpp: To 60 µl sample containing radioactive ppGpp, 6 µl each of 0.1 M EDTA and 2 N KOH were added. After overnight incubation at 37°, approximately 8 µl 2 M formic acid was added to the reaction mixture. Nucleotides were then separated by thin-layer chromatography.

RESULTS

FIGURE 1 shows a radioautogram of ^{32}P labelled compounds extracted from yeast cells after heat shock and resolved by two dimensional thin-layer chromatography. A compound which comigrates exactly with marker ppGpp is evident. This compound was quantitatively adsorbed by activated charcoal,

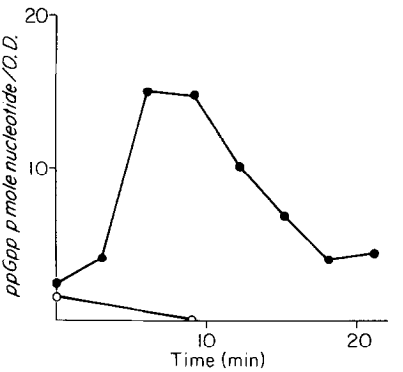

FIGURE 3. Formation of ppGpp by yeast during temperature shift-up. A364A was grown in synthetic minimum medium and labeled with ^{32}P-phosphoric acid at 1.0 mCi/mL at room temperature of about 23° for at least one cell generation before being shifted to 38°. 100 µl samples were removed at times indicated. For other information see MATERIALS AND METHODS. In the oxytetracycline experiment, the drug was added 10 minutes before temperature change and to a final concentration of 2 mg/ml. Closed circles represent ppGpp produced without oxytetracycline treatment; open circles, with oxytetracycline.

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and was also labelled by ^{14}C-adenine. Given these favorable preliminary indications, we isolated ^{32}P-labelled material by preparative thin-layer chromatography and subjected it to more stringent tests.

The isolated compound comigrated with authentic ppGpp in the separation system of FIGURE 1 both on Merck and Brinckmann PEI-cellulose plates, which in our hands give rather different separations, and also in the double-step two dimensional separation described by Randerath (21, FIGURE 88). These chromatographic properties distinguish the compound from pppG, ppppG, pppA, ppppA, ppGp, pGpp, pppGpp, ppApp, and pppApp, all of which run differently in one or both of the seperation systems tested. The compound was insensitive to periodate oxidation, like ppGpp, indicating an esterified 2' or 3' position.

The isomer pppGp is chromatographically indistinguishable from ppGpp and is also periodate resistant. However the two isomers differ drastically in their sensitivity to alkaline hydrolysis, providing a simple way to distinguish them. Alkaline treatment of the yeast nucleotide hydrolyzed it at the same rate and to the same degradation product as alkaline hydrolysis

of authentic ppGpp. Similarly, treatment with zinc activated pyrophosphatase (or prolonged storage) yielded two identical breakdown products with the yeast nucleotide and with authentic ppGpp. The major breakdown product comigrates with pGpp; the minor one is unknown, and could be a cyclic isomer.

The Glynn-Chapell (22) reaction provides a highly specific enzymatic test; ppGpp can be phosphorylated to pppGpp in this reaction, but the 2' isomer of ppGpp cannot (23; Cashel, personal communication; Sy, personal communication). FIGURE 2 shows that the yeast compound passed this test as well. We conclude that the compound accumulated by yeast cells after heat shock is the same guanosine 5'-diphosphate 3'-diphosphate found in bacteria.

FIGURE 3 shows the kinetics of ppGpp accumulation in yeast cells following shift from room temperature to 38°. The peak level attained by ppGpp was about 10% that of pppG. The nucleotide's accumulation is completely blocked by oxytetracycline.

DISCUSSION

In E. coli, the ribosome-linked synthesis of ppGpp is inhibited by the tetracyclines (24, 25). Since oxytetracycline inhibits ppGpp formation in yeast cells (FIGURE 3), it is likely that the nucleotide is produced by the mitochondrial ribosomes which are sensitive to these antibiotics and resembly prokaryotic ribosomes in other respects as well (26). In fact, Richter (27) has shown that the stringent factor of E. coli, the enzyme responsible for ppGpp synthesis (25), can be activated by mitochondrial ribosomes from yeast. Our results imply that yeast cells contain an enzyme analagous to the stringent factor which is resonsible for endogenous ppGpp synthesis and which also depends in some way on activation by the tetracycline-sensitive mitochondrial ribosomes.

The mechanism by which heat shock induces ppGpp synthesis is unknown. It clearly does not involve amino acid starvation, since transfer from 23° to 38° increases the growth rate of our yeast strain. Moreover, a derivative

of this strain with a temperature sensitive isoleucine activating enzyme produced no more ppGpp upon temerature shift than the wild type parental strain. Thus, the signal for ppGpp accumulation in yeast is probably something other than uncharged tRNA, which explains Kudrna and Edlin's failure to detect the nucleotide in amino acid starved yeast cells (7).

Nonetheless, the signal, whatever it is, apparently operates through some activity of the mitochondrial ribosomes. It is noteworthy, in this respect, that yeast mitochondrial prctein synthesis falls sharply with increasing temperature above 30°, whereas cytoplasmic protein synthesis is maximal at 36° (28).

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