Short Communication

An Improved Radioimmunoassay for Guanosine-5'-diphosphate-3'-diphosphate (ppGpp) and Its Application to Search for ppGpp in Rat Embryo and Brain

Yasutaro HAMAGISHI, Toshio KIYOSAKI,
Toshikazu OKI, Hiroshi TONE
and Tomoyuki ISHIKURA

Central Research Laboratories,
Sanraku-Ocean Co., Ltd.,
Johanan, Fujisawa, Kanagawa 251, Japan

Received June 12, 1981

The presence of ppGpp in various prokaryotes has been proven, but its presence in eukaryotes is still controversial.¹⁾ In this communication, determination of ppGpp in rat embryo and brain by a radioimmunoassay (RIA) is described. As previously reported,^{2,3)} a RIA for ppGpp was developed to permit direct determination at a concentration of 10 nM, which was more accurate and convenient than the ³²P-labeling method. The RIA for ppGpp used in this experiment was improved as follows: (a) use of a more-sensitive anti-ppGpp-antibody, which was prepared from rabbits immunized with 8-(6-aminoethyl)amino-ppGpp-bovine serum albumin (BSA) conjugate, than that in the previous method, (b) use of simple procedures of extraction and purification of ppGpp from bacterial cells and animal tissues, and (c) use of a high concentration of sodium salt for the antigen-antibody reaction (0.25 M NaCl-50 mM phosphate buffer, pH 8.4).

Three male New Zealand White rabbits were sensitized by an injection of 1.5 ml of the emulsion into the foot pads and body (2.0 mg of conjugate per ml of emulsion prepared with complete Freund's adjuvant), and then boosted weekly with the same dose of the conjugate using the same injection procedure (total of 4

Fig. 1. Standard Immunoassay Curve for ppGpp.

Experimental details are given in the text. $Cx/Co \times 100$ was plotted on the ordinate (probit percent scale) and the concentration of ppGpp on the abscissa (logarithmic scale).

$Co = [^3H]ppGpp$ bound in the absence of unlabeled ppGpp (5,217 dpm), and $Cx = [^3H]ppGpp$ bound in the presence of unlabeled ppGpp.

booster injections). The blood was collected from the carotid artery without anesthetization 7 days after the final booster injection. The RIA for ppGpp was performed according to the following procedure: 200 μ l of sample or appropriate standards dissolved in 50 mM phosphate buffer (pH 8.4) containing 0.25 M NaCl, 50 μ l of [³H]ppGpp (1.67×10^4 dpm, 3.4×10^4 dpm/pmol) and 50 μ l of antiserum in saline at a dilution rate of 1:10 were thoroughly mixed and incubated for 60 min at 4~5°C. After incubation, 200 μ l of dextran-coated charcoal suspended in 50 mM phosphate buffer (pH 8.4) was added to remove free ³H-labeled and cold ppGpp. Radioactivity of the supernatant collected by decantation was measured in Bray's cocktail using an Aloka scintillation spectrometer, model 653.

A typical standard immunoassay curve is shown in Fig. 1. Displacement of [³H]ppGpp by cold ppGpp was linear over the concentration range of 1~10 pmol/assay-tube. The cross-reactivity of antibody against structurally related nucleotides is summarized in Table I. pppApp, ppApp, ATP, 3',5'-cyclic GMP, GTP and ppppG exhibited negligible cross-reactivity. However, this antibody could not distinguish ppGpp from pppGpp and

TABLE I. CROSS-REACTIVITIES OF VARIOUS NUCLEOTIDES IN THE RIA FOR ppGpp

Experimental details are given in the text.

Nucleotides	Cross-reactivity (%) ^a
Guanosine-5'-triphosphate-3'-diphosphate (pppGpp)	100
Guanosine-5'-diphosphate-3'-diphosphate (ppGpp)	100
Guanosine-5'-monophosphate-3'-diphosphate (pGpp)	100
Guanosine-3'-diphosphate (Gpp)	5.63
Guanosine-5'-monophosphate-3'-monophosphate (pGp)	0.033
Guanosine-5'-diphosphate-3'(2')-monophosphate (ppGp)	6.43
Guanosine-5'-diphosphate (GDP)	0.063
Guanosine-5'-triphosphate (GTP)	0.034
Guanosine-5'-tetrphosphate (ppppG)	<0.027 ^b
Guanosine-3'(2')-monophosphate (3'(2')-GMP)	<0.027 ^b
Guanosine-5'-monophosphate (5'-GMP)	<0.027 ^b
Guanosine-3',5'-cyclic monophosphate (cyclic GMP)	<0.027 ^b
Deoxyguanosine-5'-diphosphate (dGDP)	<0.027 ^b
Deoxyguanosine-5'-triphosphate (dGTP)	<0.027 ^b
Guanosine	<0.0027 ^c
Adenosine-5'-triphosphate-3'-diphosphate (pppApp)	<0.027 ^b
Adenosine-5'-diphosphate-3'-diphosphate (ppApp)	<0.027 ^b
Adenosine-5'-monophosphate (5'-AMP)	<0.0027 ^c
Adenosine-5'-triphosphate (ATP)	<0.0027 ^c

^a Cross-reactivity is calculated as [homogeneous nucleotides]/[test nucleotides] × 100, where [homogeneous nucleotides] is the concentration of ppGpp required to displace 50% of [³H]ppGpp from antibody, and [test nucleotides] is the concentration of nucleotides listed required to cause 50% displacement of [³H]ppGpp in the same assay.

^b ppppG, 3'(2')-GMP, 5'-GMP, cyclic GMP, dGDP, dGTP, pppApp and ppApp did not inhibit the binding of [³H]ppGpp to antibody even at 10⁻⁸ mol/assay-tube.

^c Guanosine, 5'-AMP and ATP did not inhibit the binding of [³H]ppGpp to antibody even at 10⁻⁷ mol/assay-tube.

pGpp. Therefore, the chromatographic removal of cross-reactive nucleotides is necessary for the determination.

ppGpp was extracted with 0.1 N HCl from *Escherichia coli*, *Saccharomyces cerevisiae* and animal tissues according to the method of Cashel and Gallant⁴⁾ or Marmont *et al.*⁵⁾ with minor modification. The acidic extracts containing [³H]ppGpp were neutralized with 14% aqueous ammonia, and then applied to a column of DEAE-Sephadex A-25 (1.0 × 3.0 cm, Cl⁻ form). The column was rinsed with 50 ml of water, and GDP and pGpp were eluted with 0.1 M NaCl-50 mM phosphate buffer (pH 8.4). ppGpp was subsequently eluted with 0.25 M NaCl-50 mM phosphate buffer (pH 8.4). Fractions containing ppGpp were directly subjected to RIA.

The levels of ppGpp in *E. coli*, *S. cerevisiae*, rat embryo and brain, and mouse tumor

Sarcoma-180 are summarized in Table II. ppGpp was detected in *E. coli* and *S. cerevisiae*, but not in embryo, brain or S-180 tumor. These results indicate that the basal level of ppGpp in higher eukaryotic cells is very low (<0.270 pmol/mg wet weight of rat embryo).

REFERENCES

- 1) R. H. Silverman and A. G. Atherly, *Microbiol. Rev.*, **43**, 27 (1979).
- 2) H. Tone, Y. Hamagishi, T. Oki and T. Inui, *Agric. Biol. Chem.*, **43**, 2007 (1979).
- 3) Y. Hamagishi, T. Oki, H. Tone and T. Inui, *J. Biochem.*, **88**, 1785 (1980).
- 4) M. Cashel and J. Gallant, *Nature (New Biol.)*, **221**, 838 (1969).
- 5) P. Marmont, A. Hershko, R. Kram, L. Schachter, J. Lust and G. M. Tomkins, *Biochem. Biophys. Res. Commun.*, **48**, 1378 (1972).

TABLE II. LEVELS OF ppGpp IN ACID EXTRACTS OF *E. coli*, *S. cerevisiae*, RAT EMBRYO AND BRAIN, AND MOUSE S-180 TUMOR

Tissues and cells		ppGpp assayed (pmol) ^a	Recovery (%) ^b	Concentration
<i>E. coli</i> CP78 ^c	1.55 A_{660} unit	120.1	—	77.48 pmol/ A_{660} unit
<i>S. cerevisiae</i> ^d	82.08 A_{660} unit	94.9	—	1.16 pmol/ A_{660} unit
Rat embryo ^e	600 mg wet weight	Not detected	—	<0.270 pmol/mg wet wt ^g
	600 mg wet weight			
Rat brain	+		99.0	
	200 pmol cold ppGpp	198.0		
	377 mg wet weight	Not detected	—	<0.67 pmol/mg wet wt ^g
	467 mg wet weight			
S-180 ^f	+		111.0	
	200 pmol cold ppGpp	222.0		
	1.16 $\times 10^9$ cells	Not detected	—	
	1.16 $\times 10^9$ cells			
	+			
	200 pmol cold ppGpp	227.0	113.5	

^a These values were corrected for the recovery of [³H]ppGpp (4.3×10^4 dpm), which was added to tissues or cells prior to acid extraction, during the procedures of extraction and purification.

^b Recovery of 200 pmol cold ppGpp added to tissues or cells prior to acid extraction.

^c *E. coli* CP78 (*rel*⁺) was cultivated at 30°C for 4.5 hr in Tris-glucose minimal medium containing 0.2% casamino acid and thiamine (10 μ g/ml).

^d *S. cerevisiae* was cultivated at 30°C for 8 hr in 1% glucose-Yeast Nitrogen Base (Difco Lab.) medium.

^e The embryo was isolated from the rat on day 16 of gestation.

^f Sarcoma-180 cells were collected from the ascites fluid (one week after transplantation).

^g Indicated value shows the lower limit of detection.