RIBOSOMAL SYNTHESIS OF GUANOSINE TETRA- AND PENTAPHOSPHATE WITH mRNAs OF DIFFERENT CHAIN LENGTH

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1. Introduction

Synthesis of guanosine tetra- and pentaphosphate (ppGpp and pppGpp) by 70 S ribosomes requires the stringent factor, a natural or synthetic mRNA and uncharged tRNA bound to the ribosomal A-site [1–3]. $tRNA^{Phe}$ or other single tRNA species stimulate the reaction only if a specific polynucleotide containing the appropriate codon is present [1,2]. In the experiments reported here the minimal length of a template required for activity in the synthesis of ppGpp and pppGpp was determined. We tested the effect of oligo(U) fragments of different chain length on the reaction with purified $tRNA^{Phe}$ and found that oligomers containing less than 2 codons (6 nucleotides) do not stimulate formation of ppGpp and pppGpp. The system showed optimal activity when templates with a sequence of 10 or more nucleotides were added.

2. Materials and methods

[α-^{32}P]GTP (spec. act. 4–7 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, England. Purified uncharged $tRNA^{Phe}$ from *Escherichia coli* and poly(U) were purchased from Boehringer Mannheim, FRG.

The oligonucleotides $(U)_2$ (uridyl-(3′→5′)-uridine) to $(U)_8$ (uridyl-$(3'\rightarrow5')_7$-uridine) were commercial preparations from Boehringer Mannheim, FRG. $(U)_9$ to $(U)_{17}$ were synthesized as described [4] and kindly provided by Dr H. G. Gassen. Stringent factor was prepared from *Escherichia coli* strain CGSC as reported [5]. 30 S and 50 S ribosomal subunits from *E. coli* A19 cells were isolated by zonal centrifugation [6] of an 'S30' preparation through a sucrose gradient (0–38% sucrose in 10 mM Tris–HCl, pH 7.8, 0.3 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM mercaptoethanol).

The in vitro system for synthesis of ppGpp and pppGpp contained in vol. 25 μl: 20 mM Tris–HCl, pH 7.8, 30 mM magnesium acetate, 2 mM dithiothreitol, 4 mM ATP (pH 6–7), 0.4 mM [α-^{32}P]GTP (spec. act. 30 Ci/mol), 0.2 A_{260} units of 30 S subunits, 0.2 A_{260} units of 50 S subunits, 4 μg stringent factor, 0.015 A_{260} units of uncharged $tRNA^{Phe}$, and poly(U) or oligo(U) fragments as indicated in the figures. The reaction mixtures were incubated for 90 min at 30°C, then 1 μl 100% formic acid was added to each sample. After 4 min centrifugation at 8000 rpm and 20°C aliquots from the supernatants were spotted on thin-layer chromatograms (cellulose, 0.1 mm layer; CEL 300 PEI from Macherey and Nagel). The chromatograms were developed and subjected to autoradiography as described. The radioactive spots were cut out and counted, and the percentage of the total radioactivity present in the ppGpp and pppGpp spots was calculated.

3. Results and discussion

In vitro synthesis of the two guanosine polyphosphates ppGpp and pppGpp in a purified system with uncharged $tRNA^{Phe}$ shows a strict requirement for a mRNA containing the specific codon UUU [1,2]. It has been reported that a template consisting of only one codon, i.e., a trinucleotide, is not sufficient to

promote the stringent reaction [2,8]. In order to determine the minimal length of a polynucleotide required for functionality, we tested the effect of oligo(U) fragments of different chain length on $tRNA^{Phe}$-dependent formation of ppGpp and pppGpp. Figure 1a–j shows the result of a series of experiments with increasing amounts of the oligonucleotides $(U)_2$ to $(U)_{11}$. In the experiment of fig.2a saturating amount of $(U)_2$ to $(U)_{17}$ or poly(U) was added.

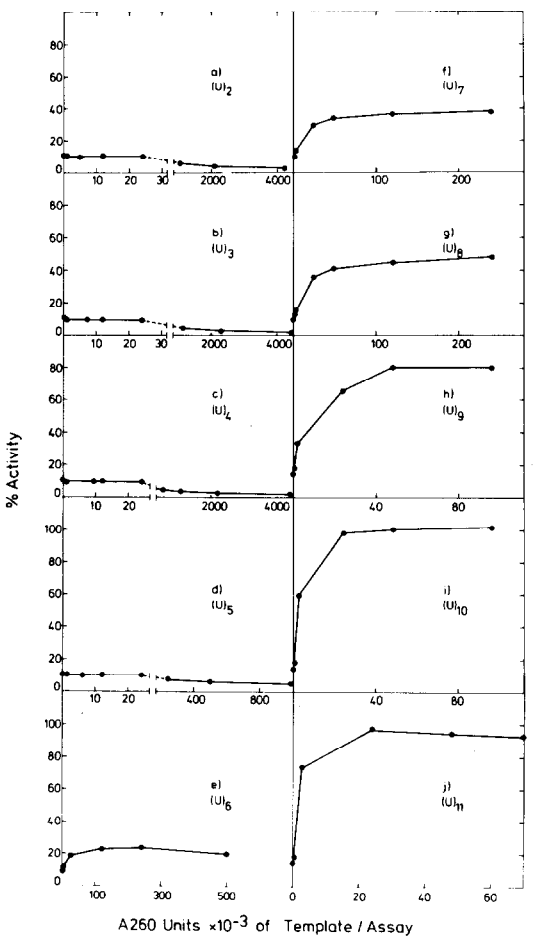

Fig.1. $tRNA^{Phe}$ dependent synthesis of ppGpp and pppGpp with increasing amounts of the oligonucleotides $(U)_2$ to $(U)_{11}$. Reaction conditions are described in Materials and methods. 100% activity corresponds to the amount of ppGpp and pppGpp synthesized in the presence of 24×10^{-3} A_{260} units of poly(U). In the poly(U) system usually 70% of the GTP was converted to ppGpp and pppGpp.

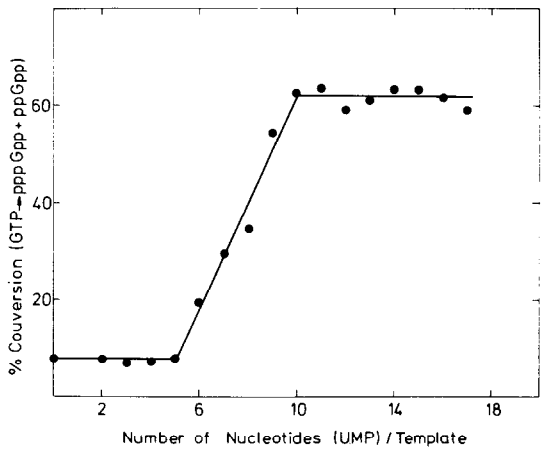

Fig.2. Effect of the oligonucleotides $(U)_2$ to $(U)_{17}$ on ribosomal synthesis of ppGpp and pppGpp. The reaction was carried out as described in Materials and methods. The system contained saturating amounts of template. The following A_{260} units of oligonucleotides were added per assay: $(U)_2$ 0.48×10^{-3}; $(U)_3$ 0.72×10^{-3}; $(U)_4$ 0.96×10^{-3}; $(U)_5$ 1.2×10^{-3}; $(U)_6$ 240×10^{-3}; $(U)_7$ 120×10^{-3}; $(U)_8$ 120×10^{-3}; $(U)_9$ 48×10^{-3}; $(U)_{10}$ to $(U)_{17}$ and poly(U) 24×10^{-3}. In presence of poly(U) 67% of the GTP was converted to ppGpp and pppGpp.

In the presence of the lower oligomers $(U)_2$ to $(U)_5$ the activity does not exceed the background value observed in the absence of template (fig.1,2). Addition of more than 30 pmol. $(U)_2$ to $(U)_5$ results in inhibition (fig.1a–d). The higher oligonucleotides stimulate formation of ppGpp and pppGpp. From $(U)_6$ to $(U)_{10}$ the activity at saturating concentrations of oligonucleotide increases, and at 10 or more nucleotides per template a plateau corresponding to poly(U) activity is observed (fig.1e–j and fig.2).

The reported results establish that two codons are the minimal requirement for a mRNA to permit tRNA and stringent factor dependent synthesis of ppGpp and pppGpp in a ribosomal system. This observation is in full accord with the previous finding that a trinucleotide is not of sufficient length to catalyze the synthesis of ppGpp and pppGpp [2,8]. The necessity for the presence of two codons on the ribosome may implicate a requirement for mRNA interaction at the ribosomal A- and P-site before the stringent factor is bound or activated on the ribosome. This interpretation would be in agreement with experiments demonstrating

(i) That a peptidyl-tRNA bound to the ribosomal P-site stimulates aminoacyl-tRNA binding to the ribosomal A-site [9].
(ii) That the tRNA fragment TpψpCpGp bound to the ribosomal A-site optimizes the interactions of fMet-tRNA$_f^{Met}$ with the ribosomal P-site [10].
(iii) That the stringent factor interacts only with those 70 S particles which have previously complexed with mRNA [5].

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