Nucleotide Specificity of Stringent Factor and the Synthesis of Analogs of Guanosine 5′-Diphosphate 3′-Diphosphate and Guanosine 5′-Triphosphate 3′-Diphosphate†

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ABSTRACT: The ribosome-dependent stringent factor reaction was found to be nonspecific with regard to the number of phosphate groups linked to the 5′ position of guanosine nucleotides. Both GMP and guanosine 5′-tetraphosphate could accept a pyrophosphoryl group from ATP although at a much lower rate than GDP or GTP. Guanosine 5′-monophosphate 3′-diphosphate and guanosine 5′-tetraphosphate 3′-diphosphate were the products of these reactions. Furthermore, 3′-linked analogs of guanosine 5′-diphosphate 3′-diphosphate and guanosine 5′-triphosphate 3′-diphosphate were synthesized from the corresponding ATP analog, adenosine 5′-*O*-(3-thiotriphosphate). The stringent factor catalyzed reverse reaction was found to be specific for guanosine 5′-diphosphate 3′-diphosphate, and was essentially inactive to the isomeric form, guanosine 5′-diphosphate 2′-diphosphate.

The in vitro synthesis of guanosine polyphosphates catalyzed by the *Escherichia coli* stringent factor (Haseltine et al., 1972; Sy et al., 1973) requires the presence of the ribosome-mRNA-uncharged tRNA complex for maximal activity (Pedersen et al., 1973; Haseltine and Block, 1973). The following reaction occurs on this ribosomal complex:

$$(p)pp^{5'}G + ppp^{5'}A \longleftrightarrow (p)pp^{5'}G^{3'}pp + p^{5'}A$$

It proceeds by a pyrophosphate transfer from ATP to the 3′ position of GDP or GTP (Sy and Lipmann, 1973). The same ribosome-mRNA-uncharged tRNA and stringent factor complex has also been found recently to catalyze the reverse reaction (Sy, 1974). Furthermore, it was reported from this laboratory (Sy et al., 1973) that, in the absence of the ribosomal complex, the stringent factor catalyzes a very slow pyrophosphate transfer, the rate of which may be stimulated tenfold by the presence of 20% methanol.

Cochran and Byrne (1974) reported that stringent factor has a rather restricted requirement for the base and ribosyl moieties, as only ITP was found to replace GDP and GTP as pyrophosphate acceptor. CTP, UTP, ATP, and deoxyguanosine di- or triphosphates failed to accept pyrophosphate. As pyrophosphate donor, only dATP was found to replace ATP in the reaction; neither adenylyl imidodiphosphate nor adenylyl(β,γ-methylene) diphosphonate reacted (Cashel, 1974).

I wish to report here that (1) the enzyme shows response to two additional members of 5′-phosphate group linked in the guanosine nucleotides; although GTP and GDP are the best acceptor molecules, both GMP and guanosine 5′-tetraphosphate have acceptor activity, (2) a 3′-thiopyrophosphate analog of (p)ppGpp[1] can be synthesized using ATPγS as donor, and (3) only the 3′ isomer of ppGpp, but not the 2′ isomer, acts as pyrophosphoryl donor to AMP in the stringent factor catalyzed reverse reaction.

Materials and Methods

Salt-washed ribosomes and fraction II stringent factor were obtained from *E. coli* (K-19 stringent) as described (Sy et al., 1973). [α-^{32}P]GTP and [γ-^{32}P]ATP were obtained from ICN. [β-^{32}P]ATP was synthesized from [γ-^{32}P]ATP as described (Sy and Lipmann, 1973). Carrier-free [^{32}P]phosphate was obtained from Schwarz/Mann. Yeast 3-phosphoglycerate kinase, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, DL-glyceraldehyde-3-phosphate diethylacetal, and ATPγS were purchased from Boehringer Mannheim. ppG$^{3'}$pp and ppG$^{2'}$pp, obtained by chemical synthesis (Simoncsits and Tomasz, 1974), were a gift from Dr. J. Tomasz. Guanosine 5′-monophosphate was obtained from P-L Biochemicals, and guanosine 5′-tetraphosphate, from Sigma.

Synthesis of the Various Guanosine Polyphosphates. ppGpp and pppG*pp were prepared as described (Sy and Lipmann, 1973), ppppG*pp and pG*pp were prepared as follows: 50 μl of reaction mixture containing 50 m*M* Tris-OAc (pH 8.1), 5 m*M* dithiothreitol, 20 m*M* $Mg(OAc)_2$, 1 m*M* [β-^{32}P]ATP (175 Ci/mol), 45 μg of washed ribosomes, 1 μg of fraction II stringent factor, 5 μg of poly(A,U,G), and 15 μg of tRNA was incubated at 30° for 45 min with either 2 m*M* of ppppG to obtain ppppG*pp, or 2 m*M* pG for pG*pp. Reactions were stopped by the addition of 1 μl of HCOOH. After centrifugation, the resulting supernatant was neutralized and diluted with 1 ml of 50 m*M* triethylamine-HCO_3 (pH 7.7), and chromatographed on a DEAE-cellulose column as described (Sy et al., 1973; Sy, 1974). pG*pp was eluted from the column with 0.3 *M* triethylamine-HCO_3 (pH 7.7) and ppppG*pp with 0.5 *M* triethylamine-HCO_3. Both fractions were then lyophilized to remove salts and were redissolved in water. Aliquots of the isolated nucleotides were stored in liquid nitrogen until used. As with ppGpp and pppGpp, storage in liquid nitrogen considerably slowed the spontaneous breakdown of the 3′-pyrophosphate bond in pGpp and ppppGpp.

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[1] Abbreviations used are: pGpp, guanosine 5′-monophosphate 3′-diphosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate; pppGpp, guanosine 5′-triphosphate 3′-diphosphate; ppppGpp, guanosine 5′-tetraphosphate 3′-diphosphate; ATPγS, adenosine 5′-O-(3-thiotriphosphate). An asterisk before the 'p', e.g., *p, denotes a ^{32}P label in that position; an 's' superior to the 'p', e.g., ^{s}p, denotes a phosphothioate in that position.

Table I: Synthesis of Various Guanosine Polyphosphates.[a]

Nucleotide Added	Nucleotide Products Formed (nmol)			
	pG*pp or GT*P	ppGp*p	pppGp*p	ppppGp*p
pG	1.0	0.1	0	0
ppG	0.3	7.9	5.1	0
pppG	0.2	3.1	10.3	0.1
ppppG	0	0	0.9	1.3

[a] Reaction mixtures were incubated at 30° for 60 min and contained in 50 μl: 40 m*M* Tris-OAc (pH 8.1), 4 m*M* dithiothreitol, 20 m*M* $Mg(OAc)_2$, 2 m*M* [γ-^{32}P]ATP (500 mCi/mol), 45 μg of washed ribosomes, 1.4 μg of fraction II stringent factor, 5 μg of poly(A,U,G), 15 μg of tRNA, and various guanosine 5′-phosphates at 0.4 m*M*. Reactions were stopped by the addition of 1 μl of HCOOH, the resulting mixtures were centrifuged, and 5 μl of the resulting supernatants were chromatographed on polyethyleneimine-cellulose as described (Sy and Lipmann, 1973). The developed chromatograms were radioautographed and the radioactive spots were cut out and counted.

Assay for the Reverse Reaction to Compare Unlabeled ppG3′pp and ppG2′pp. The reverse reactions were performed as described (Sy, 1974). The reaction mixture was incubated at 30° and contained in a final volume of 100 μl: 40 m*M* Tris-OAc (pH 8.4), 4 m*M* dithiothreitol, 12 m*M* $Mg(OAc)_2$, 20 μg of poly(A,U,G), 20 μg of tRNA, 75 μg of ammonium chloride washed ribosomes, 6 μg of fraction II stringent factor, and 10^{-4} *M* of either ppG3′pp or ppG2′pp, both unlabeled. At the indicated time points, 20 μl of the reaction mixture was removed and the reaction stopped by heating in a boiling water bath for 3 min. After the precipitate was removed by centrifugation, 15 μl of the supernatant was assayed for GDP formation. To recognize the GDP formed it was converted to [γ-^{32}P]GTP by phosphorylation in the presence of $^{32}P_i$, and was used instead of ADP as acceptor in the coupled glyceraldehyde-3-phosphate dehydrogenase–3-phosphoglycerate kinase system. The thus obtained [γ-^{32}P]GTP was counted after polyethyleneimine-cellulose thin layer chromatographic separation. The ^{32}P transfer to GDP was carried out in a 20-μl incubation medium containing 50 m*M* Tris-OAc (pH 8.0), 1.25 m*M* dithiothreitol, 10 m*M* $Mg(OAc)_2$, 0.125 m*M* EDTA, 1.25 m*M* NAD^+, 0.25 m*M* DL-glyceraldehyde 3-phosphate, 0.125 μg each of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase, and 0.9 m*M* [^{32}P]PO_4 (41 Ci/mol). Incubations were at 30° for 5 min, and the reactions were terminated by addition of 1 μl of formic acid (88%). The acidified samples were applied to polyethyleneimine-cellulose plates and developed with 0.75 *M* KH_2PO_4 (pH 3.4) (Cashel et al., 1969). The developed chromatograms were then radioautographed and the GTP region cut out and counted. Under these conditions, the assay is linear for GDP from 100 to 1000 pmol.

Results

Table I indicates that under the standard assay conditions for guanosine polyphosphate synthesis, using [γ-^{32}P]ATP as the pyrophosphate donor, stringent factor fraction II, and with GTP as substrate, the predominant nucleotide formed was pppG*pp, whereas ppG*pp predominated when the substrate was GDP. In the presence of guanosine 5′-monophosphate, a ^{32}P-marked nucleotide was produced that migrated close to GTP. In the presence of guanosine 5′-tetraphosphate, the stringent factor ribosomal system synthesized a nucleotide that migrated slower than pppGpp in the polyethyleneimine-cellulose thin layer chromatography system developed with 1.5 *M* KH_2PO_4 (pH 3.4) (Cashel and Kalbacher, 1970). No radioactive spot that moved slower than ATP was formed in the absence of guanosine nucleotides. These two nucleotide products were presumed to be ppppGp*p and pGp*p, respectively, by analogy to the position of the GDP and GTP reaction products, and their structures were confirmed in the experiments discussed below. As indicated in Table I, these two nucleotides were formed only at 10% of the rate of ppGpp and pppGpp.

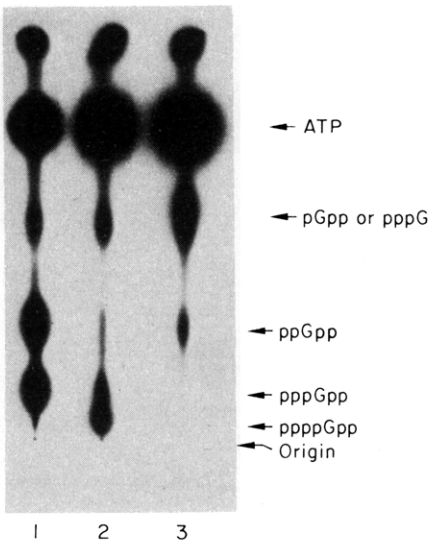


FIGURE 1: Synthesis of ppG*pp, pp*pGpp, and ppppG*pp. Assay conditions were as described under Materials and Methods. To stop the reaction, 1 μl of HCOOH was added and, following centrifugation, 1 μl of the resulting supernatant was chromatographed as described (Sy and Lipmann, 1973). (1) 2 mM GTP as acceptor molecule; (2) guanosine 5′-tetraphosphate; (3) 2 m*M* GMP. The developed chromatograph was then radioautographed.

The biosynthesis of ppGpp occurs by way of a pyrophosphoryl transfer from ATP to the 3′ position of GDP (Sy and Lipmann, 1973). To find out whether pGpp and ppppGpp were made by an identical pyrophosphoryl transfer mechanism, experiments similar to those in Table I were performed using [β-^{32}P]ATP as the donor. Figure 1 indicates that, as in [γ-^{32}P]ATP, pG*pp is made when GMP is added, and ppppG*pp is made when ppppG is added. To characterize these two nucleotides further, pG*pp and ppppG*pp were purified by DEAE-cellulose chromatography as described under Materials and Methods. Figure 2 shows autoradiograms of two-dimensional thin layer chromatography of the isolated nucleotides. pG*pp migrates faster than GTP and has a mobility similar to that of GDP in the neutral borate system (first dimension). However, it has the same mobility as GTP in the pH 3.4 KH_2PO_4 buffer system (Figure 2A). ppppG*pp migrates slower than pppGpp in both the neutral borate and pH 3.4 phosphate system (Figure 2B). Their chromatographic behavior in borate is analogous to that of ppGpp and pppGpp (Cashel and Kalbacher, 1970) and indicates the absence of two adjacent hydroxyl groups in pGpp and ppppGpp.

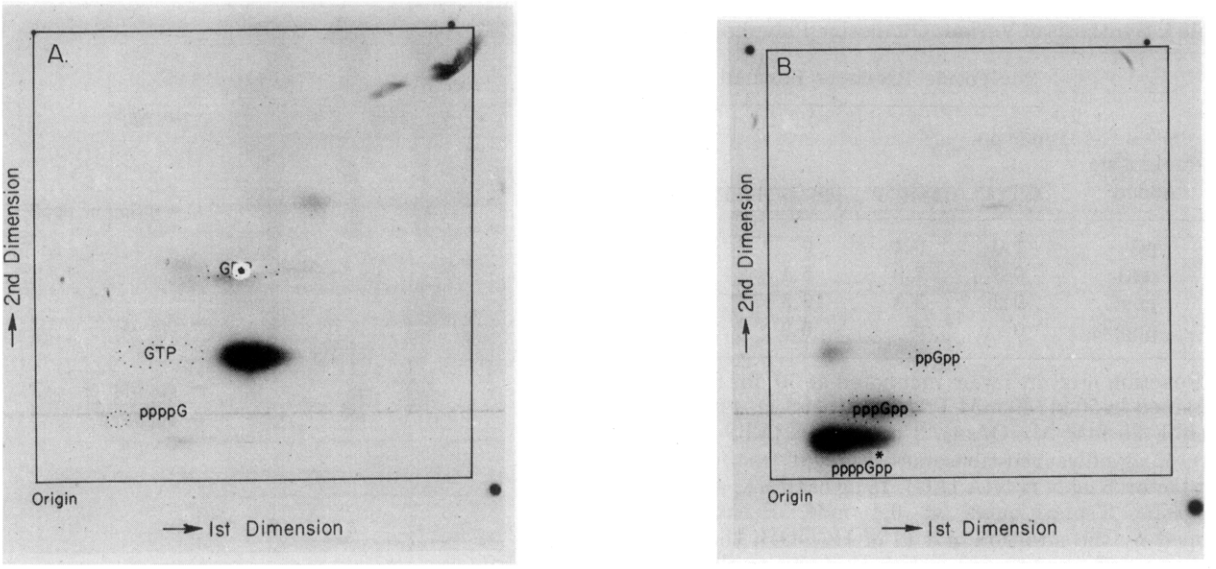


FIGURE 2: Two-dimensional chromatography of pG*pp and ppppG*pp. pG*pp and ppppG*pp, isolated as described under Materials and Methods, were spotted on polyethyleneimine cellulose sheets and chromatographed in the following two-dimensional systems. (A) For pG*pp, the first dimension is 3.3 *M* $HCOONH_4$ and 4.2% boric acid brought to pH 7 with NH_4OH; the second dimension is 0.75 *M* KH_2PO_4 (pH 3.4). (B). For ppppG*pp, the first dimension is also the neutral borate system; the second dimension is 1.5 *M* KH_2PO_4 (pH 3.4). The chromatograms were radioautographed and the marker nucleotides visualized under ultraviolet light.

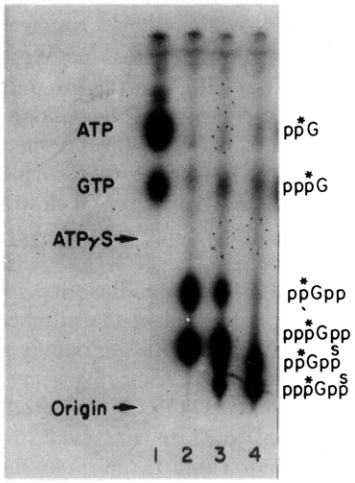
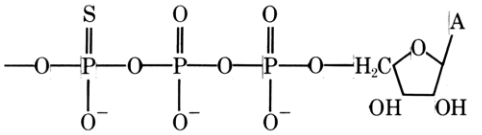


FIGURE 3: Synthesis of ppGp^{s}p and pppGp^{s}p. Assay conditions were similar to those for the synthesis of pG*pp and ppppG*pp as described under Materials and Methods. The incubation mixture contained in 50 μl: 40 m*M* Tris-OAc (pH 8), 4 m*M* dithiothreitol, 20 m*M* $Mg(OAc)_2$, 2 μg each of poly(A,U,G) and tRNA, 0.2 m*M* [α-^{32}P]GTP (24 Ci/mol), 3.5 μg of fraction II stringent factor, 75 μg of ammonium chloride washed ribosomes, and in (1) no further addition, in (2), 4 m*M* ATP, in (3), 4 m*M* each of ATP and ATPγS, and in (4), 4 m*M* ATPγS. Incubations were at 30° for 1 hr, and the reactions were stopped by the addition of 1 μl of formic acid. After centrifugation, 2 μl of the resulting supernatant was chromatographed on polyethyleneimine cellulose as described (Sy et al., 1973), and the developed chromatogram was then radioautographed overnight.

The 3′-pyrophosphoryl bond of ppGpp is more labile to alkali (0.33 *N* KOH, 30°) and mild acid (1 *N* HCl, 30°, 1 hr) hydrolysis than the 5′-pyrophosphoryl bond. Under these conditions, ppGpp is converted into ppG$^{2'+3'}$p (Sy and Lipmann, 1973). When pp*pGpp, pG*pp, and ppppG*pp were subjected to these mild hydrolysis conditions, they lost only one phosphate group and chromatographed on polyethyleneimine-cellulose developed with 1.5 *M* $KHPO_4$ corresponding to pp*pGp, pG*p, and ppppG*p. On prolonged acid hydrolysis, pp*pGp and ppppG*p were further hydrolyzed and were converted into *pGp and pG*p, respectively.

The ATP analogs adenylyl imidodiphosphate and adenylyl(β,γ-methylene) diphosphonate were found to be inactive in the guanosine polyphosphate synthesis system (Cashel, 1974). As shown in Figure 3, however, the γ-phosphothioate ATP analog (Goody and Eckstein, 1971) was active:



The two spots synthesized in the presence of ATPγS corresponding to ppGp^{s}p and pppGp^{s}p chromatographed with a much slower R_f than ppGpp and pppGpp due to the increase in ionization of the phosphothioate ions.

It was shown (Sy, 1974) that the stringent factor catalyzed ribosomal and nonribosomal reactions are reversible. ppG3′pp and ppG2′pp were tested to determine the positional specificity of the reverse reaction (Figure 4), and it was found that only ppG3′pp was used as substrate in the reverse reaction and not the isomeric form, ppG2′pp. The slight hydrolysis of ppG2′pp may be due to the presence of contaminating ppG3′pp. This result further supports a 3′-pyrophosphoryl structure for ppGpp (Sy and Lipmann, 1973; Que et al., 1973).

Discussion

Experiments with GMP and guanosine 5′-tetraphosphate as substrate indicate that the stringent factor is preferential in its recognition of the number of phosphate groups present in the 5′ position of the guanosine nucleotides. However, both GMP and guanosine 5′-tetraphosphate were used at 10% of the rate of GTP and GDP. The structures of pGpp and ppppGpp were inferred by analogy to those of ppGpp and pppGpp from their mode of synthesis (Table I and Figure 1), their chromatographic behavior (Figure 2), and their sensitivity to alkali and acid.

Biosynthetic studies have so far yielded two types of ppGpp or pppGpp analogs: (1) the base analog with re-

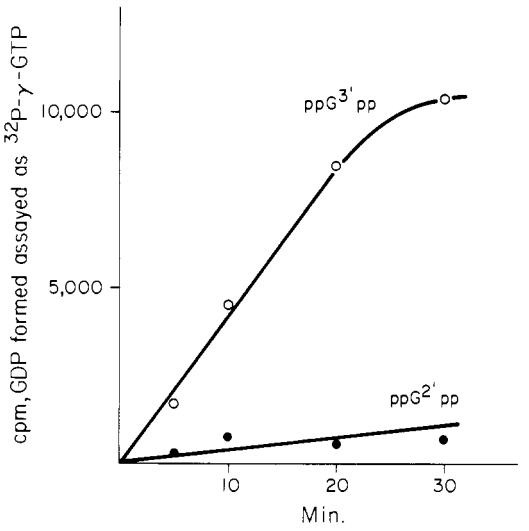


FIGURE 4: Reverse reaction. Assays were as described under Materials and Methods using 10^{-4} *M* unlabeled ppG$^{3'}$pp and ppG$^{2'}$pp. The GDP formed in the reverse reaction (ppGpp + pA→ppG + pppA) was recognized through phosphorylation to form [γ-^{32}P]GTP with glyceraldehyde-3-phosphate dehydrogenase-3-phosphoglycerate kinase coupled system in the presence of $^{32}P_i$. The [γ-^{32}P]GTP formed was separated from other products by polyethyleneimine cellulose chromatography developed with 0.75 *M* KH_2PO_4 (pH 3.4) (Cashel et al., 1969).

placement of inosine for guanosine in ppGpp and pppGpp (Cochran and Byrne, 1974), and (2) the 5′-phosphate analog with substitution of the 5′-β-γ-methylene diphosphonate group in pppGpp (Cashel, 1974). Our studies have now yielded two additional 5′-phosphate analogs of ppGpp and pppGpp with one shorter and one longer 5′-pyrophosphoryl chain (pGpp and ppppGpp). The synthesis of the 3′-pyrophosphoryl derivatives of ppGpp and pppGpp was also studied with different ATP analogs. The 3′-terminal phosphothioate derivatives of ppGpp and pppGpp were synthesized by using ATPγS. These 3′ analogs may be useful in enzymatic degradation studies considering the resistance of ATPγS to alkaline phosphatase (Goody and Eckstein, 1971). The nonacceptance of pyrophosphoryl from ppG$^{2'}$pp by 5′-AMP in the reverse reaction catalyzed by the stringent factor ribosome complex further confirmed our previous enzymatic degradation determination of the 3′ position of the pyrophosphoryl group in ppGpp (Sy and Lipmann, 1973).

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