# EFFECTS OF CATIONS ON DNA-DEPENDENT RNA POLYMERASE*

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The *in vitro* synthesis of RNA by the DNA-dependent RNA polymerase requires each of the four ribonucleoside triphosphates, DNA, and a divalent cation such as $Mg^{++}$ or $Mn^{++}$.[1] The reaction usually decreases or stops after 30–60 minutes of incubation.[2–6] This is probably due mainly to product inhibition, since long-term RNA synthesis as measured by pyrophosphate liberation occurs when the reaction mixture contains ribonuclease.[7] Inhibition by various RNA's has also been shown in experiments in which the enzyme is preincubated with RNA prior to the addition of DNA template.[8–13]

The present paper describes experiments in which the synthesis of RNA was studied with *E. coli* polymerase in the presence of 0.2 *M* KCl. Under these conditions, it was found that the initial reaction velocity is stimulated about twofold and the synthesis of RNA proceeds for a number of hours. The effects of KCl appear to be multiple. One of these is its influence on the monomer-dimer (or polymer) interconversion of the RNA polymerase.[14–16] It is suggested that this interconversion of the enzyme may account for the stimulation of the initial rate of RNA synthesis. Furthermore, KCl effectively relieves the product inhibition by newly synthesized RNA and restricts abnormal RNA synthesis with T4 DNA template. Recently, Fuchs *et al.* have also observed a stimulation of the RNA polymerase reaction by the addition of salts such as $NH_4Cl$.[17]

*Materials and Methods.*—ATP-8-$C^{14}$ and UTP-2-$C^{14}$ were purchased from Schwarz BioResearch Inc., unlabeled nucleoside triphosphates from Pabst Laboratories (P-L Biochemicals, Inc.), and poly U and poly A from Miles Chemical Company. R17 RNA ($P^{32}$-labeled) was a gift from Dr. R. Gesteland, and sRNA was purchased from Schwarz BioResearch, Inc. The T4 DNA was prepared as described by Bolle *et al.*[18] Calf thymus DNA was purchased from Worthington Biochemical Corporation, and polyoma DNA was kindly provided by Dr. Roger Weil and his coworkers. Spermidine hydrochloride was obtained from Calbiochem., and all other chemicals employed were commercially available analytical grade. RNA polymerase was prepared from *E. coli* MRE 600 by the method of Chamberlin and Berg[19] as modified by Richardson.[20] The first enzyme peak eluted from the hydroxyapatite column was used mainly in the present experiments. The enzyme was stored in 0.2 *M* KCl, 0.05 Tris pH 7.8, 0.005 *M* glutathione or dithiothreitol (Cleland's reagent), and 0.1 mM EDTA at 1–2° and used within a month. Prior to assay, the enzyme was diluted in the same buffer solution unless otherwise indicated. The specific activity ranged from 30 to 160 mμmoles of adenine incorporated per ml per minute per mg protein in the regular polymerase assay with T4 DNA and 0.2 *M* KCl. RNA polymerase concentration was determined by optical density at 280 mμ assuming an absorbancy coefficient ($A^{1\%}_{1\,cm}$) at 280 mμ of 16.9.[21]

*Polymerase assay:* The usual reaction mixture contained 0.10 *M* Tris-HCl buffer, pH 7.8, 0.2 *M* KCl, 12 mM $MgCl_2$, 1.6 mM each of GTP, UTP, and CTP, 1.6 mM ATP-8-$C^{14}$ (specific activity 0.25 μc/mM), 6 μg T4 DNA, 4.8 mM of 2-mercaptoethanol and 6 μg of enzyme in a final volume of 0.25 ml. The reaction mixture was incubated at 35° and stopped by addition of cold 5% trichloroacetic acid. After standing for 10 min at 0°C, the precipitate was collected on a glass filter paper (Whatman GF/C 2.1 cm diameter) and washed with cold trichloroacetic acid and ethanol. The filter was dried and counted in a Tricarb liquid scintillation counter employing PPO-POPOP-toluene scintillation fluid.

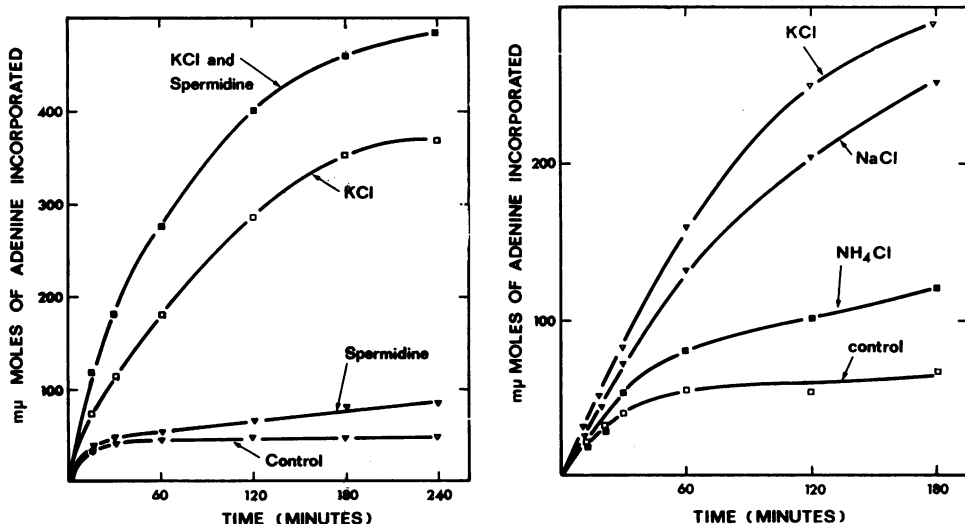
*Hybridization:* Competition-hybridization experiments were carried out as described by

Bolle *et al.*[18] except that a mixture of equal amounts of $H^3$- and $C^{14}$-labeled RNA's was hybridized to the same T4 DNA sample, a modification permitting a more reliable comparison of two RNA's than when separate determinations are carried out with each RNA. The preparation of *in vivo* competitor RNA has been detailed by Bolle *et al.*[18] For the extraction of *in vitro* synthesized RNA, the method of Richardson[21] was employed. Self-hybridization assays were carried out as described by Colvill *et al.*[22]

*Results.—Effects of cations:* The initial rate of RNA synthesis is stimulated about twofold by the addition of 0.2 *M* KCl (Fig. 1). This synthesis is further increased by the addition of 1.2 mM spermidine. In the presence of KCl, RNA synthesis continues for a number of hours and the amount of RNA synthesized becomes tenfold or greater than the controls after four hours. With some enzyme preparations, the reaction continued in a linear manner for five to six hours. The optimal KCl concentration is 0.2 *M* while concentrations higher than 0.25 *M* are inhibitory. At 0.3 *M* KCl, the reaction is inhibited about 70–80 per cent.

The stimulation by 0.2 *M* KCl was not dependent on the order of addition of the various reaction components. No significant difference in the rate as well as the duration of RNA synthesis was found when KCl was added either before or immediately after RNA synthesis had started. In the present experiments, the reaction was initiated by the addition of either enzyme, DNA, or the ribonucleotides.

The optimal magnesium ion concentration in the presence of 0.2 *M* KCl was found to be 10–12 mM. Manganous ions (2 mM) inhibit the reaction about 30 per cent in the presence of 12 mM magnesium ions and 0.2 *M* KCl. With manganous ions alone (2–4 mM), the reaction was stimulated about twofold by the




(*Left*) FIG. 1.—Effect of KCl and spermidine on the synthesis of RNA. The reaction mixtures were essentially as described in the polymerase assay except that the final volume was increased to 0.75 ml with corresponding increase of T4 DNA to 18 μg and RNA polymerase also to 18 μg. Spermidine where indicated was 1.2 mM. Aliquots (0.10 ml) were removed at the times indicated and assayed for radioactivity as described under polymerase assay. Reactions were started by the addition of four trinucleotides.

(*Right*) FIG. 2.—Effect of various salts on RNA Synthesis. The incubation conditions were as described in Fig. 1 except where indicated the following amounts of salts were added: 0.2 *M* KCl, 0.2 *M* NaCl, and 0.1 *M* $NH_4Cl$.

addition of 0.2 $M$ KCl but the rate was only about 70 per cent of the maximal rate found with magnesium ions in the presence or absence of KCl. Mercaptoethanol did not affect the initial rate in the presence or absence of KCl but was required for optimal activity for long incubation periods.

TABLE 1

| Enzyme conc. (μg/ml) | Mμmoles Adenine Incorp./30 min/ml −KCl | +KCl | Relative stimulation |
|---|---|---|---|
| 36 | 17 | 35 | 2.1 |
| 72 | 35 | 66 | 1.9 |
| 144 | 64 | 70 | 1.1 |

The reaction mixtures were as described under polymerase assay except that the concentrations of enzyme were varied as indicated. Reactions were started by the addition of nucleoside triphosphates.

The synthesis of RNA is also stimulated by other salts such as CsCl, NaCl, and $NH_4Cl$. The degree of stimulation, however, varies among the different salts (Fig. 2). CsCl gives the same results as KCl, while NaCl and $NH_4Cl$ are less active. The optimal concentration for KCl, CsCl, or NaCl was found to be 0.2 $M$ while for $NH_4Cl$ it was 0.05–0.10 $M$.

*Binding of enzyme to DNA:* In the synthesis of RNA, the RNA polymerase appears to bind to specific regions on the DNA. These regions presumably involve pyrimidines since RNA synthesis *in vitro* appears to start with a purine.[23, 24] To test whether KCl may influence the $Km$ for T4 DNA, a series of experiments was carried out with increasing levels of DNA (0.5–8.0 μg/ml) at a constant enzyme concentration (22 μg/ml). Initial velocities were measured and the results plotted by the method of Lineweaver and Burk[25]. The $Km$ values were found to be approximately 4 mμmoles of deoxyribonucleotides per ml in the presence or absence of KCl. These values were similar but somewhat lower than those reported by Hurwitz *et al.*[2] The values for $V_{max}$ were 25 and 12 mμmoles of adenine incorporated per ml per 20 minutes incubation in the presence and absence of KCl, respectively.

*Effect of enzyme concentration:* When the weight ratio of enzyme to DNA was increased to very high levels, the usual stimulation by KCl was no longer observed (Table 1). Thus, at enzyme levels of 144 μg per ml, the addition of KCl had little effect on the synthesis of RNA with DNA concentration of 24 μg per ml.

*Effect of various DNA's:* The stimulation by 0.2 $M$ KCl after 30 minutes of incubation occurs with native T4 DNA, calf thymus DNA, and polyoma DNA (Table 2, expt. 1). In contrast, little or no stimulation of RNA synthesis was ob-

TABLE 2

EFFECTS OF KCL WITH DIFFERENT DNA TEMPLATES

| Expt. | DNA template | KCl conc. in the diluting buffer for enzyme ($M$) | Mμmoles Adenine Incorp/15 min/ml −KCl | +KCl | Relative stimulation |
|---|---|---|---|---|---|
| 1 | T4 | 0.2 | 30 | 67 | 2.2 |
| | Calf thymus | 0.2 | 16 | 32 | 2.0 |
| | Polyoma | 0.2 | 78 | 150 | 1.9 |
| | Denatured T4 | 0.2 | 24 | 28 | 1.2 |
| | Denatured calf thymus | 0.2 | 11 | 11 | 0 |
| 2 | Denatured polyoma | 0.2 | 42 | 36 | 0 |
| | Denatured T4 | 0.2 | 6.9 | 8.1 | 1.2 |
| | Denatured T4 | 0.01 | 3.8 | 7.0 | 1.8 |

Incubation conditions were the same as described in polymerase assay except that the following amounts of DNA were added: 100 μg of calf thymus DNA and 7 μg of polyoma DNA. The concentrations of the denatured DNA were identical to those of native DNA of same species. Enzyme was diluted tenfold with either 0.2 or 0.01 $M$ KCl buffer before added to the reaction mixtures. Reactions were started by the addition of enzyme.

served with denatured DNA. In these experiments, the enzyme was diluted in storage buffer which contained 0.2 *M* KCl prior to addition to the reaction mixture. When the enzyme was diluted in buffer solutions containing 0.01 *M* KCl, a stimulation of nearly twofold was observed (Table 2, expt 2). This stimulation, ranging from 1.5- to 2-fold, was always observed for denatured DNA when the enzyme was diluted in buffer solutions containing low levels of KCl.

*Effects of KCl on poly A and poly U synthesis:* With ATP as the only substrate, RNA polymerase catalyzes the synthesis of poly A with DNA or poly U as template.[26, 27] The synthesis of poly A with poly U or T4 DNA as template is shown in Table 3. At low salt concentrations, poly A synthesis is substantial, but when the incubation mixture includes 0.2 *M* KCl, the synthesis is inhibited about 80 per cent. Similar results were observed with poly U synthesis employing poly A as template. In this case, the synthesis of poly U is inhibited about 67 per cent by the addition of 0.2 *M* KCl to the reaction mixture.

*Effects of KCl on inhibition by RNA's:* A number of investigators have reported that various polynucleotides inhibit the RNA polymerase reaction.[8–13, 28] This inhibition is most striking when the enzyme is preincubated with RNA prior to the addition of DNA to the reaction mixture. Similar effects were observed in the present studies employing sRNA, T4 mRNA, and poly U (Table 4). However, in the presence of 0.2 *M* KCl, the usual inhibitory effects of various RNA's were either absent or very low. These experiments suggest that KCl permits the continued synthesis of RNA for a long period of time by relieving the usual product inhibition which occurs at low ionic strength.

*Properties of the RNA synthesized from T4 DNA:* The RNA product synthesized in the presence and absence of KCl was examined for three different properties: (1) relative size, (2) symmetry of copying, and (3) efficiency as a messenger RNA.

TABLE 3

EFFECT OF KCL ON POLY A AND POLY U SYNTHESIS

| Template | Substrate | Mμmoles of Adenine or Uridine Incorp./30 min/ml — −KCl | +KCl | Inhibition (%) |
|---|---|---|---|---|
| Poly U | ATP-$C^{14}$ | 5.8 | 1.0 | 83 |
| T4 DNA | ATP-$C^{14}$ | 3.5 | 0.75 | 79 |
| Poly A | UTP-$C^{14}$ | 2.2 | 0.72 | 67 |

The incubation conditions were as described in polymerase assay except that the reaction mixture contained (1) 1.6 mM of ATP-$C^{14}$ and (2) either 6 μg of T4 DNA, 12 μg of poly A, or 20 μg of poly U. Reactions were started by the addition of enzyme.

TABLE 4

EFFECT OF KCL ON INHIBITION BY VARIOUS RNA'S

| Additions — RNA | KCl | RNA conc. (μg/ml) | Mμmoles of Adenine Incorp./20 min/ml | Inhibition (%) |
|---|---|---|---|---|
| — | — | — | 9.0 | — |
| T4 | — | 5.5 | 1.5 | 83 |
| Poly U | — | 12.0 | 2.8 | 69 |
| sRNA | — | 4.0 | 3.6 | 60 |
| — | + | — | 20.0 | — |
| T4 | + | 5.5 | 17.0 | 15 |
| Poly U | + | 12.0 | 20.0 | 0 |
| sRNA | + | 4.0 | 17.0 | 15 |

The reaction mixtures were as described under polymerase assay except (1) where indicated various RNA preparations in the amount shown were added and (2) the polymerase was preincubated with various RNA preparations for 5 min and the reactions were started by the addition of DNA and the four ribonucleoside triphosphates.

When examined by sedimentation through a sucrose density gradient (5–20%), the RNA synthesized in 20 minutes in the presence of 0.2 *M* KCl gave a sedimentation profile essentially identical to that observed in the absence of KCl, although the amount of product was about twofold greater. The *S* value for the product ranged from 22 to 26 as estimated from a $P^{32}$ R17 RNA marker which has an *S* value of 26. With a two-hour reaction mixture, the RNA synthesized in the presence of KCl had an *S* value of 35 to 45 and in the absence of KCl, 26 to 30.

*In vivo* synthesis of RNA is asymmetric in that any given species of RNA synthesized is complementary to only one of the two strands of the DNA template.[29, 31] RNA synthesized with T2 or T4 DNA template in an *in vitro* system can also be asymmetric.[22, 32, 33] Similarly, the RNA products described in this paper were found to be asymmetric. The results of self-annealing tests indicate that less than 2 per cent of the *in vitro* RNA synthesized on a native T4 DNA template is self-complementary. Results of hybridization experiments in the presence of *in vivo* five-minute RNA also relate to the question of asymmetric synthesis. Curves 1 and 2 of Figure 3 show that more than 90 per cent of the *in vitro* synthesized RNA is displaced from hybrid when the highest concentration of five-minute *in vivo* RNA competitor is used. This finding substantiates the results of the self-hybridization tests and shows that neither conditions of *in vitro* RNA synthesis (i.e., the presence or absence of 0.2 *M* KCl) brings about appreciable transcription of late regions of the T4 DNA template. Similar observations have been made previously for *in vitro* RNA synthesized on T2 DNA template in the absence of 0.2 *M* KCl.[34, 35] Competition by 1-minute 20-second *in vivo* RNA (curves 3 and 4) is incomplete. This indicates that the *in vitro* RNA contains substantial amounts of RNA species which are relatively rare in 1-minute 20-second *in vivo* RNA.

RNA synthesized with native DNA and purified RNA polymerase have been shown to stimulate amino acid incorporation using a cell-free system from microbial source.[36] In the present experiments, the T4 RNA preparations obtained from two-hour incubation mixtures containing 0.2 *M* KCl were found to be very active in promoting amino acid incorporation into polypeptide linkage when used as messenger RNA in an *S*-30 incorporating system from *E. coli*.[37] In a typical ex-

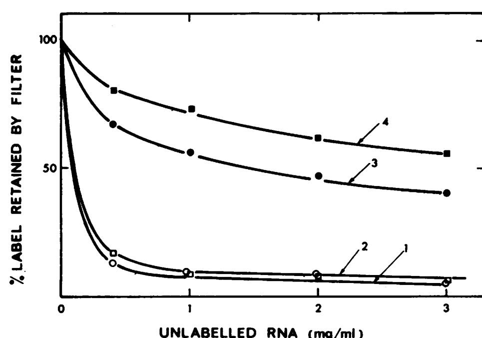



FIG. 3.—Competition of radioactive T4 RNA synthesized *in vitro* by RNA synthesized *in vivo*. *Curves 1 and 3:* $C^{14}$-RNA product from a 90-min *in vitro* synthesis in the presence of 0.2 *M* KCl. *Curves 2 and 4:* $H^3$-RNA product from a 90-min *in vitro* synthesis in the absence of KCl.

For curves 1 and 2, both $C^{14}$- and $H^3$-labeled RNA were added to the same reaction mixture (0.0625 μg/ml for each) and competed with RNA extracted from cells 5 min after infection with T4. For curves 3 and 4, both $C^{14}$- and $H^3$-labeled RNA were added to the same reaction mixture (0.0625 μg/ml for each) and competed with RNA extracted from cells 1 min and 20 sec after infection with T4.

At the concentration of T4 DNA used in the hybridization mixture (30 μg/ml), the DNA is in excess over the labeled RNA. The efficiency of hybridization (% trichloroacetic acid-insoluble radioactive label annealed in the absence of competitor) was about 20% for each RNA. Maximum background relative to the amount annealed in the absence of competitor was about 10% and 2% for the $C^{14}$- and $H^3$-labeled RNA, respectively.

periment, 0.02-ml aliquot samples from a two-hour RNA polymerase reaction mixture (containing 0.4 mg of RNA per ml) were added directly to an *S*-30 amino acid incorporating system.[38] After 30 minutes of incubation, 288 $\mu\mu$moles of $C^{14}$-phenylalanine were incorporated into polypeptides per mg of ribosomal protein. This value corresponds to approximately 5000 $\mu\mu$moles of amino acids incorporated per mg of ribosomal protein per 30 minutes, assuming the polypeptide product contains about 6 per cent phenylalanine.[39] The RNA polymerase reaction mixture incubated for two hours in the absence of KCl contained 0.066 mg of RNA per ml. When a 0.02-ml aliquot of this RNA was added to an amino acid incorporating system, 35 $\mu\mu$moles of $C^{14}$-phenylalanine was incorporated. Thus, on a weight basis the RNA synthesized in the presence or absence of KCl showed very similar activity as messenger RNA, assuming amino acid incorporation is proportional to messenger RNA concentration.

*Discussion.*—The present results with the DNA-dependent RNA polymerase of *E. coli* clearly show that KCl influences both the initial rate and the duration of synthesis of RNA. From the experiments of Fuchs *et al.*,[14] Richardson,[15] and Stevens *et al.*,[16] the RNA polymerase is known to dissociate reversibly into subunits as the ionic strength is increased. The fact that the enzyme is active in both low and relatively high KCl concentrations would suggest that the enzyme is active in both its monomer and dimer (or polymeric) form. Thus as one possibility, the twofold increase in the initial rate of RNA synthesis by 0.2 *M* KCl may be due to the dissociation mainly of active 21*S* dimer units into two active 13*S* monomers. This explanation is strengthened by the observation that at extremely high enzyme concentrations relative to that of DNA, little or no stimulation by KCl is observed. Under these conditions, all the initiation sites on the DNA may be occupied by enzyme in its dimer form. Thus, an increase in concentration of enzyme molecules (by dissociation of dimers into monomers) by the addition of KCl would not influence the initial reaction rate.

The experiments with the single-stranded DNA are also consistent with the monomer-dimer (or polymeric) interconversion of the enzyme. When the enzyme in 0.2 *M* KCl is added to the reaction mixture, it probably binds rapidly to the denatured DNA. Under these conditions, it may never have a chance to form dimers and, consequently, little or no KCl stimulation is observed. However, when the enzyme is first diluted into storage buffer with a low concentration of KCl, dimerization can occur prior to its interaction with DNA and thus a stimulation by KCl is observed.

The effects of salt, however, are not merely due to an increase in ionic strength, since not all salts show the same degree of stimulation. Thus, the effects of KCl on the initial rate may involve factors other than the monomer-dimerization (or polymerization) of the enzyme.

The prolonged synthesis of RNA may be attributed to the relieving of RNA product inhibition by 0.2 *M* KCl which is further potentiated by the addition of spermidine.[9] With some enzyme preparations (Fig. 1) spermidine also increased the initial rate of RNA synthesis and it appears likely that this was due to the presence of some inhibitory RNA present in these preparations. Gros *et al.*[40] previously observed a dependence of salt on RNA inhibition.

Another important function of KCl appears to be for restriction of abnormal

RNA synthesis. This is shown by experiments in which the synthesis of poly A with either T4 DNA or poly U as template was markedly inhibited by the addition of KCl. Thus, the addition of KCl appears to decrease the affinity of RNA polymerase for nonspecific binding sites on the nucleic acid template which lead to subnormal RNA synthesis.

The optimal concentration of KCl observed for polymerase activity was 0.2 *M*. This concentration corresponds very well with the measured intracellular level of KCl for *E. coli*.[41] Thus, it would appear that at KCl concentrations quite similar to those in the cell, the polymerase functions in its monomeric form, synthesizing long chains of RNA asymmetrically, which are active as messenger RNA. Under these conditions, little or no product inhibition occurs, and thus it is likely that the newly synthesized chains are continously released and the initiation of new chain occurs, particularly when the nascent RNA is actively involved in protein synthesis.[42]

It is of interest to compare the present results with binding studies with RNA polymerase and various DNA's as determined by retention on Millipore membrane filters[43, 44] or by sucrose density gradient centrifugation.[45] In the present studies, little difference was found in the *Km* for T4 DNA in the presence and absence of 0.2 *M* KCl, suggesting that the affinity of enzyme for specific sites in DNA is essentially independent of salt concentration. From binding studies, however, it has been observed that in the presence of 0.15–0.20 *M* NaCl, 50 per cent of the RNA polymerase–T7 DNA complex was dissociated and essentially all the complex disappears in 0.3 *M*.[44] Similar effects were observed in the binding of polymerase to λ DNA[45] and calf thymus DNA.[43] Thus, the *Km* determined in the present experiments may not be a measure of the association between the enzyme and the DNA since the complex between enzyme and DNA tends to dissociate poorly,[11] particularly at low salt concentration. However, it is also possible that the binding studies carried out at low ionic strength involve some binding of enzyme in its dimer form as well as some nonspecific enzyme-DNA interaction. The latter possibility seems likely since maximal RNA polymerase activity is observed under conditions where little or no enzyme is bound to DNA when measured by the membrane filtration or centrifugation techniques, and furthermore, KCl inhibits the synthesis of poly A, a reaction involving abnormal enzyme-DNA interaction.

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Abbreviations: ATP, adenosine 5′-triphosphate; UTP, uridine triphosphate; CTP, cytidine 5′-triphosphate; GTP, guanosine 5′-triphosphate.

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