The effects of nucleoside triphosphates and oligoribonucleotides on the initiation of synthesis of messenger RNA of the T4 phage-specific enzyme, deoxynucleotide kinase, have been studied. The procedure involved incubation of T4 DNA, purified RNA polymerase from Escherichia coli, and selected nucleotide compounds during a brief period to permit initiation of RNA synthesis. Further initiation was arrested by the addition of rifampicin, and completion of the transcription of the newly initiated RNA was permitted to take place in the presence of the full complement of nucleoside triphosphates.

After translation of the messenger RNA into phage-specific enzymes, the measured activities of the latter were considered to be proportional to the amount of initiation that had occurred in the first incubation period. The effectiveness of individual nucleoside triphosphates, when present singly or in combination during the initiation period, was compared to that when all four nucleoside triphosphates were available. ATP alone was extremely effective as an initiator of the synthesis of the messenger RNA for deoxynucleotide kinase. The addition of UTP to ATP not only enhanced the magnitude of initiation but also affected the kinetics of ATP interaction with T4 DNA and RNA polymerase during the initiation period.

Several oligoribonucleotides including a series ApA to ApApApA, UpU to UpUpUpU, and the heteropolymers, ApUpU and ApApApU, were tested as initiators of kinase mRNA synthesis. A sequence of nucleotides in the promoter region of T4 DNA for the deoxynucleotide kinase gene has been proposed as a result of these experiments.

We have previously reported studies on the initiation characteristics of five T4 phage specific messenger RNAs (1). One of these messages, namely that for deoxynucleotide kinase, was unique among those studied in that it alone was able to be initiated in the presence of a single nucleoside triphosphate. The presence of ATP alone during the initiation phase of RNA synthesis yielded up to 50% of the kinase message observed when all four nucleoside triphosphates were present during the initiation period.

Recently, Downey et al. (2, 3) and Hoffman and Niyogi (4) have examined the promoter regions of T4 DNA using diribonucleotide monophosphates to stimulate the synthesis of RNA. Based on the observation that certain oligoribonucleotides can stimulate the transcription of both synthetic and natural templates (2-6), they have attempted to sequence the promoter regions by use of dinucleotide monophosphates on the assumption that the latter are complementary to the bases in the initiation regions for RNA synthesis from a T4 DNA template. In similar experiments designed to study the effect of dinucleotide monophosphates on the stimulation of RNA synthesis, Minkley and Pribnow (7) have demonstrated that three closely positioned promoters in the early region of T7 DNA can be selectively activated by CpA, CpC, ApU, or ApC. The function of the dinucleoside phosphates has been partially clarified in recent publications (8, 9).

In this paper we report experiments with oligoribonucleotides performed in a similar manner in an attempt to gain information about the promoter region (10) of a single T4-specific monocistronic message (11), that of deoxynucleotide kinase.

**EXPERIMENTAL PROCEDURES**

**Materials**

Uridine diphosphate, [14C]glucose, [14C]formaldehyde, [3H]deoxycytidine monophosphate, [α-32P]ATP, and 32PO4, were purchased from New England Nuclear Corp.; L-amino acids from Schwarz/Mann; ATP, UTP, GTP, CTP, phosphoenolpyruvate, pyruvate kinase, hexokinase, dithiothreitol, tetrahydrofolic acid, GpC, CpC, ApG, CpA, Cpa, ApA, ApU, ApApC, and alkaline phosphatase from Sigma Chemical Corp.; ApUpU, UpU, UpUpUpU, and polynucleotide kinase from Miles Laboratory, No. 29 glass-fiber filters from Schleicher and Schuell, oligoribonucleotides of adenine from Boehringer Mannheim Corp.; and rifampicin from Calbiochem. Actinomycin D has been kindly supplied by Dr. Walter Gail of Merck, Sharp and Dohme Research Labs, and the oligoribonucleotides, ApApApU and ApApU, were a generous gift from Dr. Leon Heppel of Cornell University.

**Methods**

**Synthesis of Oligoribonucleotides Labeled with 32P**

γ-labeled ATP of high specific activity (15 to 18 Ci/mmol) was prepared according to the procedure described by Glynn and Chappell (12) and used for the synthesis of the 5′-labeled oligonucleotides by the procedure involving the enzyme polynucleotide kinase (13). The labeled compounds were purified by chromatography on DEAESephadex A-25 columns (1 × 13 cm).

ATP containing 32P in the α position (2.5 Ci/mmol) was used to synthesize internally labeled oligonucleotides. The reaction mixture, which consisted of RNA polymerase (70 µg/ml), T4 DNA (30 µg/ml), magnesium acetate (8.75 mM), Tris/acetate, pH 7.8 (65 mM), [α-32P]ATP (0.4 mM), and either ApA or ApApA (0.4 mM), was incubated for 5 min at 25°. The reaction was stopped by placement of...
the reaction vessels in a boiling water bath for 1 min. After cooling, alkaline phosphatase was added to the reaction mixture to make a concentration of 30 units/ml and the incubation continued for 2 h at 37°C. Thereafter, the tri- and tetraoligonucleotides were isolated on columns of DEAE-Sephadex A-25 as previously described (13).

**Verification of Purity of Oligonucleotides**

We have checked the purity of the polynucleotides that have been particularly effective in stimulating initiation. Specifically, the series of polyadenylates and polyuridylates as well as ApUpU have been examined by the following procedure. The individual polynucleotides were subjected to thin-layer chromatography on DEAE-Sephadex A-25 (1 x 13 cm) (13) with elution by a linear gradient of NaCl (0.08 to 0.4 M containing 7 M urea) in each case greater than 95% of the polynucleotide was found at the expected elution volume relative to that of ATP (13). This procedure was used for the oligonucleotides in the series of ApA to ApApApApA, of UpU to UpUpUpUpU, and of ApUpU.

Then ApA, ApApA, ApApApA, and ApApApApA as collected from the column were phosphorylated in position 5' with γ-labeled ATP and reasayed by homochromatography (14). In each instance the radioactivity associated with the polynucleotide was located primarily in a single spot with less than 5% of other products.

**Preparation of DNA**

DNA to be used as template was isolated from T4D phage stocks containing 1 to 2 x 10^9 phages/ml by the phenol extraction procedure described by Thomas and Abelson (17).

**Cell-free Extract**

The S-30 extract used for the translation of deoxynucleotide kinase messenger RNA for the production of enzymatically active protein was prepared from *E. coli* RNAse I as described by Burgess and Thomas (15) through the high salt glycerol gradient step and stored at -20°C in a salt buffer (M). When the preparation was subjected to electrophoresis (16) on polyacrylamide gel columns, the bands representing the subunits αββ′α and a fifth minor band were the only protein components present in the electropherogram.

**Preparation of RNA Polymerase**

Escherichia coli RNA polymerase holenzyme was prepared from *E. coli* RNAse I as described by Burgess and Thomas (15) through the high salt glycerol gradient step and stored at -20°C in a salt buffer (M). When the preparation was subjected to electrophoresis (16) on polyacrylamide gel columns, the bands representing the subunits αββ′α and a fifth minor band were the only protein components present in the electropherogram.

**Preparation of mRNA**

DNA to be used as template was isolated from T4D phage stocks containing 1 to 2 x 10^9 phages/ml by the phenol extraction procedure described by Thomas and Abelson (17).

**Measurement of Initiation of mRNA Synthesis**

The determination of deoxynucleotide kinase activity in the final stage is known to be proportional to kinase mRNA and is therefore believed to be a measure of the extent of initiation of RNA synthesis. The procedure to measure the extent of initiation of synthesis of a particular mRNA in the presence of oligoribonucleotides or nucleoside triphosphates or both, at the concentrations indicated in the legends of respective experiments. Initiation of RNA synthesis was terminated by the addition of rifampicin at a final concentration of 67 μg/ml, and the mixture was allowed to remain at 25°C for an additional minute before cooling in ice in order to synchronize the start of transcription in all vessels after addition of nucleoside triphosphates (Stage II).

**Stage I** — This stage consisted of the initiation of RNA synthesis at 25°C for 3 min in a 0.04-ml reaction mixture containing 65 mM Tris/acetate (pH 7.8), 8.75 mM magnesium acetate, 2.0 mM dithiothreitol, 30 μg/ml of T4 DNA, 70 μg/ml of RNA polymerase, and oligoribonucleotides or nucleoside triphosphates. The concentration of bacterial RNA polymerase was approximately that described by Burgess (15) for enzyme prepared through the glycerol gradient step. The oligoribonucleotides or nucleoside triphosphates have been subjected to chromatography on columns of DEAE-Sephadex A-25 as previously described (13).

**Stage II** — The elongation of RNA chains was effected by the addition of the nucleoside triphosphates previously omitted, or by the addition of those initially present to 0.4 M, thus increasing the reaction volume to 0.65 ml. The incubation mixture was placed at 25°C, and the elongation of RNA chains was allowed to proceed for 15 min.

**Stage III** — The translation of kinase mRNA was accomplished by adding the reagents necessary for protein synthesis, which included actinomycin D to stop further chain elongation plus the S-30 fraction. Incubation was at 30°C for 30 min with the final incubation mixture (0.1 ml) for protein synthesis containing 52 mM Tris/acetate (pH 7.8), 7.7 mM magnesium acetate, 1.6 mM dithiothreitol, 6.3 mM magnesium acetate, 2.2 mM ATP, 0.2 mM concentrations each of 30 L-amino acids, 50 mM potassium acetate, 50 mM ammonium acetate, 30 μg/ml actinomycin D, 5.5 mg/ml of S-30 protein, and the oligoribonucleotide or nucleoside triphosphate, or both, remaining from Stages I and II.

**Evaluation of Factors Involved in Determination of Efficiency of Initiating Compounds**

At the present stage of development of the problem of measuring messenger RNA activity for the synthesis of individual proteins (or enzymes) in complex mixtures of RNA species, the above assay seems to be the best approach for ascertaining information about certain events in the synthesis, etc. (i.e. translation, etc.) of messenger RNA. Because of the relative complexity of the assay, we have made a thorough examination of the factors influencing the ultimate yield of enzyme activity in this four-step procedure. The considerable variation seen in individual experiments, when samples are run under apparently identical conditions, may be attributed almost solely to the activity of the ribosomal S-30 preparation. For reasons that are not presently understood identical samples of messenger RNA for all the enzymes studied may be translated at different rates by different preparations of S-30 fraction. However, although the absolute yields of enzyme vary from one ribosomal preparation to another, the relative values for a particular S-30 fraction are meaningful and give an accurate estimation of the relative amounts of mRNA present in a test sample. With different ribosomal preparations we have on several occasions measured the linear relationship between the amount of enzyme RNA and the amount of dTTP utilized for the initiation of synthesis of enzyme. In all cases tested we have kept within this linear, or near linear, range of RNA concentration in conducting the assays reported in these investigations.

A study has also been made of the conditions relating to the initiation and transcription of the T4 kinase cistron. The concentrations of RNA polymerase and T4 DNA were chosen on the basis of the optimal production of kinase mRNA. There was also a correspondence of incorporation of 32P-labeled ATP into RNA. The specific activity of RNA polymerase was approximately that described by Burgess (15) for enzyme prepared through the glycerol gradient step. In the conditions of our assay procedure with T4 DNA and four nucleoside triphosphates present, the enzyme catalyzes the incorporation of 32P-labeled ATP into RNA at a rate of approximately 100 cpm/min of mg of protein. Under the conditions of our assay this enzyme activity would have the potential of transcribing the equivalent of the T4 DNA molecules twice. In our calculations to estimate the maximal putative incorporation of initiators into kinase mRNA we require that the kinase cistron be transcribed only once. In a previous communication we have shown that the kinase gene is transcribed at a rate of seven nucleotides/s and requires a total of approximately 3 min for completion at 25°C. The 15-min period allowed for transcription in the present assay (Stage II) is thus more than adequate for completion of all T4 DNA-RNA polymerase complexes initiated during Stage I. The 30-min incubation period for protein synthesis is likewise adequate to provide for the 15-min period of initiation which has been arbitrarily set at 3 min on the basis of experiments with ATP when used as the only initiating agent. In this particular case optimal initiation occurred at this time.
Deoxynucleotide Kinase Messenger RNA

Effect of ATP Concentration on Initiation of Deoxynucleotide Kinase Messenger RNA - The rate of kinase mRNA initiation as a function of ATP concentration during Stage I is shown in Fig. 1. When ATP alone is present during the initiation period for kinase mRNA, a sigmoid response is observed with increasing ATP levels until about 50% of the total potential amount of message is initiated at saturating concentrations of ATP as compared to vessels containing all four nucleoside triphosphates during this period.

When UTP at a concentration of 0.4 mM is also present during the initiation period and the requirement for ATP again is examined, nearly 100% of the total potential amount of kinase mRNA is initiated at saturation levels of ATP, and the sigmoid type response, previously noted with subsaturating levels of ATP, is eliminated. We previously reported (1) that UTP alone is not able to initiate kinase mRNA; thus, the effect of UTP in combination with ATP is a cooperative rather than an additive one.

Effect of Oligoribonucleotides of Adenine on Initiation of Deoxynucleotide Kinase Messenger RNA - In our previous report of the requirements of nucleotides for the initiation of the messenger RNAs for several phage-specific enzymes (1), only the initiation of deoxynucleotide kinase mRNA occurred in the presence of ATP alone. The other enzymes required at least two nucleoside triphosphates for a significant reaction. The former effect suggested the possibility that the first few nucleotides in the initiation region for kinase mRNA may consist of a string of adenine bases. Since both Downey et al. (2, 3) and Hoffman and Niyogi (4) have attempted to characterize the initiation sites in the promoter regions of T4 DNA by using oligoribonucleotides for the stimulation of general RNA synthesis, we examined the effect of oligoribonucleotides of adenine on the initiation of kinase mRNA synthesis by the four-step procedure described under "Experimental Procedures."

As a result of previous investigations (1) our choice of oligoribonucleotides containing principally adenine and uracil was indicated by the effects of ATP and UTP in combination on the initiation of kinase mRNA synthesis. Dinucleoside monophosphates and oligonucleotides containing other bases (e.g., GpC, CmG, ApG, and GpA) have been tested but are not effective as initiators.

Fig. 2 demonstrates that oligomers of adenine are able to initiate the synthesis of kinase mRNA. There is a progressive increase in the initiation caused by the use of the di-, tri-, and tetranucleotides. Maximal stimulation of kinase mRNA syn-

RESULTS

Effect of ATP Concentration on Initiation of Deoxynucleotide Kinase Messenger RNA - The capacity of oligoribonucleotides of adenine to stimulate initiation of the synthesis of kinase mRNA was tested. The concentration of all oligonucleotides was 0.4 mM. In Experiment 2 (Curves B, C, and D) the oligonucleotides (0.25 mM) were incubated in the presence of a saturating concentration (0.4 mM) of ATP; and in Experiment 3 (Curves C, D, and E), the oligonucleotides (0.25 mM) were incubated in the presence of a saturating concentration (0.4 mM) of UTP. The level of initiation found with ATP alone at saturating (0.4 mM) and subsaturating (0.025 mM) concentrations is indicated by △ and ∆, respectively.

Fig. 1. The effect of ATP concentration on the initiation of the synthesis of deoxynucleotide kinase mRNA. The initiation procedure and assay of kinase mRNA are described under "Experimental Procedures." The concentration of ATP was varied from 0 to 0.4 mM during the initiation phase and then adjusted to 0.4 mM following the addition of rifampicin (● — ●). In experiments with vessels that contained UTP, the level was maintained at 0.4 mM and the concentration of ATP varied (O — O). The background radioactivity, determined by omitting nucleoside triphosphates during the initiation step, was subtracted from the values reported in each experiment.

Fig. 2. The effect of the size of oligoribonucleotides of adenine alone or in combination with ATP or UTP on the initiation of deoxynucleotide kinase mRNA. The initiation procedure and assay of kinase mRNA are described under "Experimental Procedures." In Experiment 1 (Curves A, □ — ■) the capacity of oligoribonucleotides of adenine to stimulate initiation of the synthesis of kinase mRNA was tested. The concentration of all oligonucleotides was 0.4 mM. In Experiment 2 (Curves B, □ — ■) the oligonucleotides (0.25 mM) were incubated in the presence of a saturating concentration (0.4 mM) of ATP; and in Experiment 3 (Curves C, □ — ■), the oligonucleotides (0.25 mM) were incubated in the presence of a saturating concentration (0.4 mM) of UTP. The level of initiation found with ATP alone at saturating (0.4 mM) and subsaturating (0.025 mM) concentrations is indicated by △ and ∆, respectively.
thesis, equivalent to that with saturation concentrations of ATP, occurred with the tetranucleotide. However, there was a rapid decrease in effectiveness of polynucleotides of a size greater than this length. We propose that the ability of ATP, when present alone, to initiate kinase mRNA synthesis results from the capacity of RNA polymers to catalyze the synthesis of polyadenylates from this substrate. The synthesis of these homopolymers has been shown in our experiments.

Effect of Oligoribonucleotides of Adenine in Combination with Nucleoside Triphosphates—Since both the di- and trioligoribonucleotides of adenine could initiate kinase mRNA but to a level somewhat lower than that observed with the tetraoligoribonucleotide or with saturating amounts of ATP, we attempted to determine whether extension, in vitro, of these smaller compounds would increase the level of initiation. When oligoribonucleotides of adenine were incubated in combination with subsaturating levels (0.025 mM) of ATP, both the products of the reaction of the di- and tri-compounds were able to initiate the synthesis of kinase mRNA to levels equivalent to that observed with the tetraoligoribonucleotide of adenine or with saturating amounts of ATP (Fig. 2). This effect strongly indicated that an extension of the di- and trioligomers to possibly the tetramer had occurred with a significant increase in the capacity of the product to stimulate the initiation process as compared to that observed with any of the reactants alone. In support of this hypothesis reaction of oligoribonucleotides of adenine larger than the tetramer with subsaturating concentrations of ATP resulted in no significant increase in the initiation rate when compared to the stimulation observed with unreacted substrate.

When UTP at 0.4 mM is present in the incubation mixture during initiation, nearly 100% of the potential kinase mRNA is initiated in the presence of the di- and trioligoribonucleotides, whereas close to 80% of maximal initiation is observed with the tetranucleotide as cosubstrate (Fig. 2). Initiation by oligoribonucleotides containing greater than four adenine bases again showed no significant increase in stimulation after reaction with UTP, but exhibited the same lowered or nonreactivity of the longer adenine polynucleotides once more. Thus, like the combination of ATP and UTP, combinations of the di- to tetranucleotides of adenine with UTP result in the highest levels of kinase mRNA initiation.

Effect of Concentration of Adenine Oligonucleotides on Initiation of Deoxynucleotide Kinase mRNA—The rate of kinase mRNA initiation as a function of adenine oligoribonucleotide concentration is shown in Fig. 3A. The curves for the three oligoribonucleotides studied are not sigmoid as is the case when ATP is the sole initiating nucleotide. The relative $S_{0.5}$ values for the di-, tri-, and tetraoligomers decrease from 110 to 27 to 12 $\mu M$, respectively. The value of 12 $\mu M$ observed with the tetranucleotide is significantly lower than the 60 $\mu M$ value observed for ATP in the presence of saturating concentrations of UTP, a condition in which a sigmoid curve is also not obtained. The shift from sigmoid to hyperbolic response has been observed by others when nucleoside triphosphates at low concentration are supplemented with dinucleoside phosphates during the initiation period (3).

Utilization of Uridine Homologues for Initiation of Deoxynucleotide Kinase mRNA; Effect of Concentration—When UTP alone is present during the initiation period of RNA synthesis, little or no kinase mRNA is observed (1). In contrast, oligoribonucleotides of uridine are able to initiate the synthesis of kinase mRNA to a level comparable to that observed with adenine oligomers. Fig. 3B shows that when the requirements of the di-, tri-, and tetraoligoribonucleotides of uridine are examined, kinase mRNA is initiated to a level equal to that when saturating amounts of ATP or the tetraligomer of adenine are used. As in the case of the adenine compounds, oligomers of uridine do not give rise to a sigmoid response at subsaturating levels of initiator compounds. A determination of $S_{0.5}$ values of the tri- and tetraoligomers of uridine calculates to be 10 $\mu M$, a value equivalent to that observed with the tetraoligoribonucleotide of adenine. Diuridine monophosphate gives a value of 40 $\mu M$, which lies between the values of the di- and trioligoribonucleotides of adenine of 27 and 110 $\mu M$, respectively.

Effect of Hetero-oligoribonucleotides on Initiation of Deoxynucleotide Kinase Messenger RNA—Since oligoribonucleotides of adenine yield significant levels of initiation that may be enhanced by the presence of UTP, it was of interest to examine hetero-oligomers of adenine and uridine in combination with UTP. Table I gives data from two typical experiments in which the effect of ApU, ApApApU, and ApUpU with and without saturating levels of UTP was examined. A minimal response is observed with the dioligomer ApU when present alone during the initiation period. The presence of UTP (0.4 mM) in combination with this compound enhances significantly the level of kinase mRNA initiation. When the tetraoligoribonucleotide ApApApA is examined, the level of initiation greatly exceeds that of ApU, but in the presence of UTP the increase, although substantial, is not as great as that observed where ApU is augmented with UTP. When ApApU is tested, the most significant initiation of any oligonucleotide tested is observed, but upon addition of UTP little or no further stimulation results. The effect of the addition of UTP...
phosphates are having their effects on the initiation of the synthesis of a labeled Nucleoside Phosphates into RNA

ApApApU + UTP could a significant initiation of the synthesis of mRNA for the hydroxymethylase and the (Y- and β-glucosyltransferases. The sequences can initiate kinase mRNA, we examined their ability to initiate other mRNA species, specifically those for dCMP.

nucleotides are thus specific for kinase mRNA within the terminal positions in mRNA, one might anticipate demonstrating the incorporation of 32P-labeled nucleoside phosphates into RNA. For this purpose we have prepared several oligonucleotides in the series of ApA to ApApApA or UpA to Up-UpUpU and of ApUpU labeled in position 5' with 32P by transphosphorylation with [γ-32P]ATP.

UpApA or UpApUpU of specific activity 15 to 18 Ci/mm was incubated under the conditions outlined under "Methods" but with 10 times (0.5 ml) the reaction volume. After 3 min rifampicin was added to the solution, which remained at 25° for 1 min and thereafter cooled to 0°. Then the four nucleoside triphosphates for RNA synthesis were added and incubation was continued for an additional 10 min at 25°. Transfer RNA (100 μg/ml) was added as a carrier. The RNA was then precipitated in 5% trichloroacetic acid containing imidazole and pyrophosphate. The precipitates were collected by centrifugation and dissolved in a solution of 0.5 M sodium bicarbonate containing 8 M urea. Blue dextran was then added as a marker for determination of the void volume, and the samples were applied to columns (0.9 x 39 cm) of Sephadex G-25 equilibrated with the bicarbonate/urea solution. The samples were eluted with a flow rate of 0.066 ml/min, conditions that permitted excellent separation of RNA and the radioactive nucleoside phosphates. Aliquots of 10 μl were removed for direct radioactive measurement; the absorbance of each fraction was also determined at 260 nm. Residual radioactivity of the nucleoside phosphates appeared in vessels 44 to 60. However, no radioactivity was found in the 10-μl aliquots of the samples eluted coincident with the tRNA carrier or blue dextran (data not shown).

In an extension of the above experiments (Table II, Experiments 1 to 4) we have shown that the dinucleoside phosphate, ApApA, is an effective initiator. In contrast, ApApU not only did not serve as an initiator of kinase mRNA synthesis, but surprisingly inhibited the usual stimulation afforded by saturation levels of ATP (Table II, Experiments 5 to 7).

Initiation of Other T4-specific mRNAs with Oligonucleotides – In our past study (1) on the characteristics of initiation of several T4-specific mRNAs we concluded that the initiation sites of each of several messages consist of a different initiation region on the template sequence. Since specific oligonucleotide sequences can initiate kinase mRNA, we examined their ability to initiate other mRNA species, specifically those for dCMP hydroxymethylase and the α- and β-glucosyltransferases. The combination of initiating nucleotides tested were: ApU, ApApA, and ApUpU with and without UTP. In no instance could a significant initiation of the synthesis of mRNA for the latter three enzymes be achieved (data not shown). These nucleotides are thus specific for kinase mRNA within the scope of messengers tested.

Unsuccessful Attempts to Demonstrate Incorporation of 32P-labeled Nucleoside Phosphates into RNA – If nucleoside phosphates are having their effects on the initiation of the synthesis of kinase mRNA by competing with ATP or GTP for 5'-terminal positions in mRNA, one might anticipate demonstrating the incorporation of 32P-labeled nucleoside phosphates into RNA. For this purpose we have prepared several oligonucleotides in the series of ApA to ApApApA or UpA to Up-UpUpU and of ApUpU labeled in position 5' with 32P by transphosphorylation with [γ-32P]ATP.

32P- and 32P-labeled RNA and tRNA could be used as a marker for determination of the void volume, and the samples were applied to columns (0.9 x 39 cm) of Sephadex G-25 equilibrated with the bicarbonate/urea solution. The samples were eluted with a flow rate of 0.066 ml/min, conditions that permitted excellent separation of RNA and the radioactive nucleoside phosphates. Aliquots of 10 μl were removed for direct radioactive measurement; the absorbance of each fraction was also determined at 260 nm. Residual radioactivity of the nucleoside phosphates appeared in vessels 44 to 60. However, no radioactivity was found in the 10-μl aliquots of the samples eluted coincident with the tRNA carrier or blue dextran (data not shown). Then, in order to determine the total radioactivity of each sample, the acid-precipitable material was measured after addition of further tRNA to alternate tubes. Between tubes 16 and 34 it was difficult to distinguish any difference in the small amount of radioactivity present in the precipitates from the tubes to which tRNA had and had not been added. Many of these samples, however, contained tRNA from the elution of the chromatogram. However, between tube 41 and 56 the samples precipitated in the presence of added tRNA did contain radioactivity that could only have been present by entrainment of residual radioactive substrate nucleotides.

In a control experiment in which 14C-labeled RNA (prepared by incubation of T4 DNA, RNA polymerase, and 14C-labeled
nucleoside triphosphates) was chromatographed under the same conditions as described above, 83% of the radioactivity was eluted in the vessels containing blue dextran and the remaining 17% in the following two vessels. If we add the total counts in the blue dextran-containing tubes, we can account for approximately 460 counts out of a total 3,600 cpm (or 0.21 pmol) that might have been present had 1 pmol of kinase mRNA been formed per pmol of T4 DNA. Even these few counts are suspect, since in fractions in which no RNA could have been present there were on an average 200 cpm/fraction.

Since there was the possibility that the terminal 5' radioactive phosphate might have been lost for one reason or another, the experiment was repeated with a nucleoside phosphate with an internally labeled phosphorus, i.e. ApApA62pA (data not shown). The product was subjected to homochromatography and the radioactivity remaining in the RNA at the origin was measured by autoradiography. No significant differences were observed in the density of this area of x-ray film when compared to that of controls in which DNA was omitted from the incubation or rifampicin was added at zero time.

DISCUSSION

As presently understood, a promoter site (10) on a DNA segment is comprised of approximately 40 base pairs that may combine with RNA polymerase (21) and thus be protected from digestion by nucleases. Within this segment of DNA (10) there is a point at which active transcription in vivo starts. Within the promoter region another section of the DNA comprising about 7 A-T rich base pairs is a nontranscribed site for the oriented and stable attachment of RNA polymerase. Between this segment and the actual initiation site there are approximately five or six base pairs.

In possibly one of the most definitive studies on the role of dinucleoside monophosphates as initiators of RNA synthesis Kupper et al. (22) have shown that the dinucleoside phosphates, CpG, GpC, and CpC may be incorporated into the mRNA transcribed from the complement of the tRNA5' promoter of the φ80psu DNA segment, TATGATGCCGCCTCC. The point of initiation in vivo starts with GCCCTC as indicated by the arrow. However, in vitro, CpC and CpG can stimulate RNA synthesis and be incorporated into RNA in the underlined region even though they precede the normal point of message formation. Kupper et al. (22) have pointed out that the bases corresponding to the first seven bases of the longer sequence shown above constitute a nontranscribed binding site for RNA polymerase that is found with some variation in other promoter sites (8).

Theoretically, nucleoside triphosphates or nucleoside phosphates could serve in one or more steps of the initiation process. This would include serving either as primers at the site for the actual beginning of transcription in vivo or in the melting of DNA at the RNA polymerase binding site. In the first case one might expect the nucleoside phosphates to be incorporated; in the second case one would not, since the RNA polymerase binding site is not usually transcribed.

In the present studies in vitro we could not detect incorporation into RNA of highly labeled nucleoside phosphates specific for kinase mRNA initiation. Although the sensitivity of our experiments should have been great enough to detect one kinase transcript/molecule of T4 DNA present in the incubation, we cannot categorically rule out that only a fraction of our system was responding to stimulation and that the degree of incorporation of radioactive compounds was too low for detection. If this were the case, we would expect that the sequence of nucleotides on the transcribed strand of DNA at the point of initiation in vivo would consist of a segment of four thymine bases either preceded or followed by a sequence of from two to four adenines. With this model one could rationalize the role of the active nucleoside phosphates, ApApApA, UpUpUpUp, UpAp, and ApUp, as serving as specific primers for the formation of kinase mRNA.

However, if our inability to detect incorporation of radioactive nucleoside phosphates is valid, then we would want to consider an alternate explanation in which they might have an effect on initiation without undergoing reaction and incorporation. It is possible that the RNA polymerase binding site of the kinase gene (and possibly of all quasimate, delayed-early genes) is composed of a sequence of four thymines flanked on one side by an adenine and on the other by two adenines. In the presence of the complementary strand, a double stranded segment of DNA would have the following composition:

\[
\begin{align*}
5' & \quad U-A & A-U-U & 3' \\
3' & \quad A-A & A-A & 5' \\
3' & \quad T-A & T-A & 3' \\
3' & \quad U-U & U-U & 5'
\end{align*}
\]

One can thus foresee that separation of strands for binding of RNA polymerase at its binding site might be achieved by hydrogen bonding of ApApApA to one strand or of UpUpUpUp to the other. Both ApUpU and UpA could hydrogen bond to either strand. According to this point of view, the function of the active nucleoside phosphates would be the melting or separation of the base pairs at the RNA polymerase binding site to aid in the formation of a stable DNA-enzyme complex.

The distribution of nucleotides as shown was suggested by the fact that, although UTP itself is unable to cause initiation, it plays a supplementary role in promoting the effectiveness of ApU and ApApU probably by addition of one more uridine at the 3'-terminal side. The fact that UTP does not further enhance the effectiveness of ApUpU would suggest that two uridines are sufficient.

The initiation of kinase gene transcription by ATP alone could result from the formation of polyadenylates, which then undergo reaction at the RNA polymerase recognition site for the kinase gene. RNA polymerase has the capacity of forming homopolymers from only one nucleoside triphosphate, namely ATP (23). We have confirmed that under the conditions of our incubation for initiation polyadenylates of a variety of lengths are formed.

Finally, we have found that none of the oligonucleotides effective for the initiation of kinase mRNA was active for the other three mRNAs tested. Kinase mRNA is an example of the quasi-late, delayed-early type, whereas α- and β-glucosyltransferase mRNAs are delayed-early and dCMP hydroxymethylase an immediate-early variety of phage-specific mRNA (24). The last three are believed to be initiated at early promoters, whereas kinase mRNA originates at a separate site, a P4 promoter, which becomes available only 1.5 to 2.0 min after infection (24). The unwinding protein, the product of gene 32, is also a member of this latter category (25) and provides a means of testing whether members of this subgroup of "early" mRNA have the same or different effectors during the initiation of synthesis of these specific mRNAs.

Previous investigators have reported that formation of a phosphodiester bond, whether from nucleoside triphosphates themselves (26) or from a nucleoside triphosphate and a diri-
bonucleoside phosphate (2-4, 7, 8, 22) is a requirement for initiation of general RNA synthesis. A striking feature of the experiments reported in the present communication is the finding that oligonucleotides alone can serve as initiators of kinase mRNA synthesis. Whether this observation reflects a variation in experimental procedure or a basic difference in the initiation properties of \( P_d \) and \( P_r \) promoter sites remains to be determined.

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P J Natale and J M Buchanan

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