A new assay yielding mechanistic information on the initiation reaction of Escherichia coli RNA polymerase has been developed. It was found to be useful in characterizing the promoters of bacteriophage DNA templates. The binding of the first two triphosphates in an RNA sequence was determined to be equilibrium ordered with ATP binding first followed by UTP on the λ promoters P₁ and P₂. The products resulting from phosophodiester bond formation, pppApU and PPi, dissociated rapidly in the absence of the other triphosphates required for RNA synthesis. The resulting steady state conversion of ATP and UTP into pppApU was the basis for the new assay. The rate-limiting step in the reaction was not precisely determined, but it was argued not to be entirely the release of product. The Zn²⁺ chelator, 1,10-phenanthroline, was partially characterized and found to be an uncompetitive inhibitor of ATP in the initiation reaction (Kᵢ = 100 µM). The unique advantage of this steady state assay is that several steps in the RNA initiation process are amplified kinetically and thus can be examined separately with techniques applicable to any other two-substrate, two-product enzyme reaction.

DNA-dependent RNA polymerase catalyzes the synthesis of all stable and messenger RNA in Escherichia coli. A recent volume (1) summarizes most aspects of the structure, mechanism, and regulation of this enzyme. The mechanism of RNA chain elongation has recently been thoroughly studied (2). However, the initiation process has been difficult to isolate for study because it is a unique event for each high molecular weight RNA molecule synthesized. We have recently described (3) an abortive initiation reaction catalyzed by RNA polymerase that, we argued, would allow an amplification of certain steps in the initiation reaction. This reaction was observed in cases where only the first two triphosphates corresponding to the beginning of an RNA transcript were present. The reaction was found to be promoter-specific, α-dependent, and rifampicin-resistant. In this paper, we present further evidence that the abortive initiation reaction can be used as a steady state assay of many steps in the normal initiation reaction. In the accompanying paper (4), we use this assay and other techniques to explain how rifampicin inhibits RNA synthesis.

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EXPERIMENTAL PROCEDURES

Materials—Unlabeled triphosphates and pancreatic DNase I (P-L Biochemicals). α-³²P-labeled UTP and CTP at 100 mCi/µmol (New England Nuclear); No. 3MM chromatography paper, 19 cm × 100 m (Whatman); E. coli alkaline phosphatase (Worthington, BAPC); AG 1-X2 anion exchange resin (Bio-Rad); Sephadex A-25 DEAE-anion exchange resin (Pharmacia); and 1,10-phenanthroline (Sigma) were purchased from the indicated sources. All other chemicals were reagent grade or better.

Methods—RNA polymerase was purified from E. coli B using the method of Burgess and Jendrisak (5). The specific activity on calf thymus DNA and poly[d(A-T)] .poly[d(A-T)] was 900 units/mg and 2100 units/mg, respectively, where 1 unit equals 1 nmol of UTP incorporated in 10 min. As judged by sodium dodecyl sulfate gel electrophoresis, the α content was about 60% stoichiometric (comparison to α₂ band). The above assay suggests that about 50% of the enzyme was active. In all instances where enzyme concentration is reported, we have based the values on protein concentration employing A₂₈₀, 6.3 (6).

Bacteriophage ACl857S7 was grown by temperature induction of the lysogen. Bacteriophage A8287 and T7 were grown by infection. The T7 lysate was precipitated with Carbobox 6000 (Fisher). The crude phage suspensions were first purified on a CaCl₂ step gradient and finally banded to equilibrium in CsCl in the Beckman 40 rotor at 33,000 rpm for 24 h. The DNA was extracted from phage with warm sodium dodecyl sulfate (65°C for 15 min); the phage proteins were precipitated with KCl and the resulting DNA solution was dialyzed exhaustively against 0.10 M NaCl, 0.01 M Tris-Cl, pH 8.1, 1.0 mM EDTA. The final storage solution was 0.01 M Tris-Cl, pH 8.0, 0.10 mM EDTA saturated with CHCl₃.

The Hae III restriction fragments were prepared by digesting 2 mg of intact DNA with 750 units of Hae III endonuclease (New England Biolabs) in 0.05 M NaCl, 6 mM MgCl₂, 10 mM Tris-Cl, pH 8.1, 1.0 mM dithiothreitol for 3 h. The DNA fragments were precipitated with 2 volumes of cold ethanol, dissolved in Buffer A (90 mM Tris, 90 mM boric acid, 2.5 mM EDTA), and separated on a 3.5% polyacrylamide gel (7). The DNA bands were localized by UV shadowing (8); slices of gel containing the fragments of interest were eluted by electrophoresis into a dialysis bag using 5 mM Tris, 2.5 mM acetic acid, pH 8.2. Ethanol precipitation of the eluted fragments followed by purification on Sephadex A-25 (washed with 0.1 M KCl, DNA eluted with 1.0 M KCl) and dialysis against 0.01 M Tris-Cl, pH 8.0, 0.10 mM EDTA, saturated with CHCl₃, completed the purification.

The template modifications employed to obtain the data in Table II were performed as follows: the A₂₂₅ Hae III digest was extracted twice with phenol:CHCl₃:isomylalcohol (50:48:2) and twice with diethyl ether. The solution was then dialyzed against 0.01 M Tris-Cl, pH 8, 0.10 mM EDTA; complete digestion was verified by acrylamide gel electrophoresis. Treatment of intact A₂₂₅ DNA with 10⁻⁴ µg/ml of pancreatic DNase I for 30 min yielded "nicked" DNA with an average single strand molecular weight of 50,000 as judged by sedimentation velocity in 0.90 M NaCl, 0.10 M NaOH according to the method of Studier (9). The ACl857 H strand was prepared by denaturing intact DNA (85°C for 10 min) and banding the H and L strands in the presence of poly[r(U,G)] in CaCl₂ following standard methods (10). The nicked λ-DNA and the λ-H strand were both dialyzed finally into 0.01 M Tris-Cl, pH 8, 0.10 mM EDTA.

Molar concentration of all DNA preparations were determined using an absorption coefficient of 6.5 mm⁻¹ DNA phosphorus at 260 nm. Spectra of restriction enzyme-generated DNA fragments were taken to verify the absence of other absorbing material. We have not yet solved the problem of determining the concentration of these
fragment preparations to better than about ±20%. Observed spectral ratios do not always conform to pure DNA samples. The work reported here, therefore, has used upper limits of the DNA fragment concentrations based on the above absorption coefficient.

The abortive initiation assay has been described (3). In this paper, several chromatographic separations correspond to final concentrations of the following components in 0.10 M Tris-Cl, pH 7.9 (0.04 M KCl; 0.1 M MgCl₂; 0.01 M CaCl₂); diethiothreitol (1 mM); ATP (0.5 mM); UTP or CTP (0.05 mM) with α-32P-labeled triphosphate added to a final specific activity of 50 to 250 cpm/pmol. The DNA template was 1 to 2 nm genome. RNA polymerase was 50 nM. The reactions were initiated in the addition of triphosphates following a 10-min preincubation of enzyme and template at 37°C. Aliquots were sampled onto 3MM paper chromatograms whose origins had been spotted with 0.10 mM EDTA. The chromatograms were developed with ascending chromatography in water:saturated ammonium sulfate:isopropyl alcohol (1:80:2) that was also 5 μM EDTA. This system (termed WASP) is an acronym for the paper chromatography solvent development previously outlined as the one developed by Markham and Smith (11). Because several chromatograms are often run for an individual experiment we designed a cylindrical Plexiglas cover for the 2-quart Mason jars onto which a paper chromatogram (29 × 19 cm) could be fastened with a rubber band. Each chromatogram was divided so as to accommodate three to five samples. The product peak was located on the dried chromatogram by counting 1-cm strips in a liquid scintillation counter without any scintillation fluid. The strips containing RNA chain elongation in reaction mixtures containing only two nucleotides were purified as follows: about 0.1 mmol of the triphosphate was loaded onto a column (1.25 × 11 cm) of AG 1-X2 (formate) anion exchange equilibrated with 0.5 mM ammonium formate, pHe 4.6. After extensive washing with the equilibrating buffer, the nucleotide was eluted with a 0.5 to 3.0 mM gradient (total volume = 300 ml) that had been adjusted to pH 7.4. 0.1% Triton X100 was added to the H₂O to a conductivity corresponding to 0.13 M. The triphosphate could subsequently be adsorbed to a column (2.1 × 20 cm) of DEAE-Sephadex, A-25 equilibrated with 0.15 M triethylammonium bicarbonate, pH 8.0. This column was washed with 0.15 M triethylammonium bicarbonate; the triphosphate was eluted with a 0.15 to 0.50 M gradient (total volume 300 ml). The peak fractions were taken to dryness in a rotary evaporator. The residue was dissolved in ethanol several times and redried until no triethylamine was detected by smell. The triphosphate was converted to the sodium salt by dissolving the triethylammonium salt in methanol and mixing with this with a cold 5% NaClO₃, in acetone solution (12). The flocculant precipitate was centrifuged and washed twice with cold acetone to remove excess NaClO₃. The precipitate was then washed twice with cold ether and centrifuged. The precipitate was cautiously dried under a vacuum, care being taken to avoid spattering of the gelatinous precipitate. The final product was weighed and dissolved in deionized water. Using standard spectral constants, the dried material corresponded to the disodium salt.

In the experiments reported previously (3), radioactivity was always observed at the origin of the WASP chromatograms. In the work reported here with purified triphosphates, we did not encounter this problem. Although we have not determined precisely the degree of cross-contamination still remaining in our triphosphates, we have found that commercially available purine monophosphates can be used without further purification because they contain no detectable contaminating triphosphates. An example of the purine monophosphate substitution strategy is shown in Table I for the hPa promoter. Although the starting sequence for this transcript is pppApApC (4), AMP can be used in the abortive initiation reaction to produce pApC. In the presence of ATP and CTP, however, pppApC is not detected. This result reproduces well the observed preference for an AAC start when the entire transcript is synthesized in vitro. The purine triphosphates have Rf values of: ATP = 0.40; GTP = 0.45 in the WASP system. We therefore have used only the labeled pyrimidine triphosphates. We have employed other chromatography systems (e.g. polyethyleneimine TLC plates in 1.5 M LiCl and paper electrophoresis) to separate the dinucleotides. Each of these methods has certain advantages (i.e. speed or resolution), but the WASP system is the most reproducible and versatile we have used. Because of this reproducibility and ease of quantitation the WASP system appears to be the method of choice when both accuracy and precision are required (e.g. kinetic studies). We have also listed in Table I the dihydrobiotinylated promoters that have been screened for these dinucleotide reactions. In many of these cases we have tested a promoter containing a dinucleotide fragment for the ability to template an incorrect dinucleotide synthesis. In all cases, the correct dinucleotide was produced at greater than 20 times the rate of the incorrect dinucleotides. We have also tested several restriction enzyme fragments from bacteriophage T7 DNA that do not contain promoters. These reactions similarly resulted in approximately background levels of dinucleotide production.

RESULTS

The original description of in vitro abortive initiation (3) was based on experiments that employed a single set of reaction conditions. To rule out the possibility that the phenomenon was due only to a particular set of reaction conditions, we have carried out experiments over a wider range of solution composition. The effect of KCl and MgCl₂ concentration on abortive initiation is shown in Figs. 1 and 2, respectively. The inhibitory effect of ionic strength on RNA chain initiation is known to be more pronounced than its effect on chain elongation (13). The salt dependence of pppApU synthesis shown in Fig. 1 corresponds to that typically seen in the initiation phase of RNA synthesis. Slightly greater reaction rates were obtained at the high salt concentrations by preincubating the enzyme and DNA together at 40 mM KCl.

With the corresponding monophosphate in which case all four pPpPy dinucleotides were resolved from the triphosphate substrate. Indeed, we have found that commercially available purine monophosphates can be used without further purification because they contain no detectable contaminating triphosphates. An example of the purine monophosphate substitution strategy is shown in Table I for the hPa promoter. Although the starting sequence for this transcript is pppApApC (4), AMP can be used in the abortive initiation reaction to produce pApC. In the presence of ATP and CTP, however, pppApC is not detected. This result reproduces well the observed preference for an AAC start when the entire transcript is synthesized in vitro. The purine triphosphates have Rf values of: ATP = 0.40; GTP = 0.45 in the WASP system. We therefore have used only the labeled pyrimidine triphosphates. We have employed other chromatography systems (e.g. polyethyleneimine TLC plates in 1.5 M LiCl and paper electrophoresis) to separate the dinucleotides. Each of these methods has certain advantages (i.e. speed or resolution), but the WASP system is the most reproducible and versatile we have used. Because of this reproducibility and ease of quantitation the WASP system appears to be the method of choice when both accuracy and precision are required (e.g. kinetic studies). We have also listed in Table I the dihydrobiotinylated promoters that have been screened for these dinucleotide reactions. In many of these cases we have tested a promoter containing a dinucleotide fragment for the ability to template an incorrect dinucleotide synthetic reaction. In all cases, the correct dinucleotide was produced at greater than 20 times the rate of the incorrect dinucleotides. We have also tested several restriction enzyme fragments from bacteriophage T7 DNA that do not contain promoters. These reactions similarly resulted in approximately background levels of dinucleotide production.
and then adding triphosphate and salt to initiate the reaction (data not shown). This effect varied with different promoters; we have not investigated it further. The requirement for MgCl₂ can also be met with MnCl₂ and CoCl₂ at 2 mM MgCl₂, the observed rates relative to MgCl₂ were 1.16 and 0.17, respectively. No detectable reaction was observed when CaCl₂ or ZnCl₂ were employed in the absence of MgCl₂. Although higher relative rates were obtained with MnCl₂, the use of this cation resulted in some incorrect initiation. When the dinucleotide formed in the presence of MnCl₂ was isolated and digested with alkaline phosphatase, 3²P, was released suggesting that pppApU or pppUpA had been formed. Whether this reaction occurred aberrantly at correct promoters or at other nonpromoter sites on the DNA was not determined. There was no significant effect on reaction rate when 2 mM spermine or spermidine was added at either 2.5 or 15 mM MgCl₂.

The effect of several treatments of the λDNA template on abortive initiation is shown in Table II. The enzyme concentration was rate-limiting in this experiment so that small differences in promoter binding could be accentuated. The DNA samples were purified following the enzymatic or physical treatment indicated as described under “Experimental Procedures.” Each reaction was run at 1 ± 0.1 nM DNA genome per min. The results have been normalized to units of turnover per genome per min. The AC1857 genome contains about 15% more DNA than λb2. There are additional promoters in this deleted region of λ (14). The small effect seen in this assay suggests that they do not begin with pppApU or that binding of RNA polymerase to these additional sites is not as strong as the binding at P₇ or P₉. The Hae III restriction enzyme cleaves λb₂ into about 50 pieces. None of the known λ promoters are affected by this cleavage (15). However, the concentration of DNA ends was about 50 times higher in this reaction than in those normally run with restriction fragments or intact DNA. This result demonstrates that end binding on DNA by RNA polymerase did not limit the promoter-specific reaction, nor did any extraneous pppApU synthesis occur as a result of the increase in free DNA ends. The digestion of λb₂ DNA with pancreatic DNase I resulted in nicked DNA with a weight average molecular weight of 93,000. For random nicking, this corresponds to a number average length of ~150 nucleotides. The observed inhibition of the pppApU synthetic rate proves that dinucleotides were not made at nicks in the DNA. In addition, this result indicates that nicks in the DNA compete with intact promoter regions for RNA polymerase or that the region defined by the promoter must be free of nicks in order for RNA polymerase to bind and synthesize dinucleotides. The latter interpretation is supported by the fact that a 10-fold higher enzyme concentration resulted in similar relative rates to those shown in Table II (data not shown).

The pppApU synthesized from the isolated H strand of λ cannot be due to intact promoters, but must have occurred from adventitiously folded secondary structure or looped regions in the DNA. The relevance of this reaction and the effect of rifampicin on it to assays done in crude extracts will be considered further in the accompanying paper (4).

We have used the abortive initiation assay to titrate active promoters on bacteriophage T7 and λDNA with RNA polymerase. The results of a titration with intact bacteriophage DNAs is shown in Fig. 3. T7 and λ each contain two known promoters that begin with pppApU. In each case, a plateau of synthesis occurred at high enzyme concentration. The plateau levels presumably reflect the sum of the intrinsic rates for the two promoters on each DNA template. The striking difference between the two genomes is seen to be the amount of enzyme

### Table II

<table>
<thead>
<tr>
<th>Template</th>
<th>pppApU synthesized (genomic/min)</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>λb2</td>
<td>11.4</td>
<td>100</td>
</tr>
<tr>
<td>AC1857</td>
<td>13.4</td>
<td>118</td>
</tr>
<tr>
<td>λb2 Hae III digest</td>
<td>13.7</td>
<td>120</td>
</tr>
<tr>
<td>λb2 (nicked)</td>
<td>5.6</td>
<td>49</td>
</tr>
<tr>
<td>λ (H strand)</td>
<td>4.9</td>
<td>43</td>
</tr>
</tbody>
</table>

*Nicked DNA had a M₀ = 93,000.

Fig. 1. The effect of KCl on pppApU synthesis. Each reaction solution contained in a total volume of 0.10 ml, 0.04 M Tris-Cl, pH 8.0; 0.08 M KCl; 0.01 M MgCl₂; 1 mM dithiothreitol; 0.5 mM ATP; 0.05 mM UTP (100 cpm/pmol). The template was 1 nM λb2 DNA.

Fig. 2. The effect of MgCl₂ on pppApU synthesis. The template was 2 nM λb2 DNA. Standard reaction conditions as described in Fig. 1 were employed except that the MgCl₂ concentration was varied as indicated.

Fig. 3. The effect of RNA polymerase concentration on pppApU synthesis. The standard conditions described in Fig. 1 were employed except that the RNA polymerase concentration was varied as indicated with T7 DNA (C, 2 nM) or λb2 DNA (G, 2 nM) as template.
required to saturate the promoter sites. In the case of T7 DNA, half-maximal activity was observed at a polymerase:genome molar ratio of about 10, whereas with λDNA a ratio of 50–75 was required to half-saturate the promoter sites. In each titration, the other promoter sites that were not assayed but that nevertheless bound polymerase and the bulk of nonspecific DNA are expected to compete for enzyme binding. This result suggests that there may be many sites on λDNA that bind RNA polymerase with high affinity that do not represent active promoters. If an independent estimate for the polymerase-promoter binding constant were available for each promoter, these data could be quantitatively evaluated. That the above interpretation is valid can be qualitatively demonstrated by repeating the titration on isolated promoter-containing restriction enzyme fragments.

As shown in Fig. 4, when RNA polymerase was added to the Hae 790 DNA fragment containing the AP₅ and P₅₆ promoters in the absence of the majority of nonspecific λDNA, a simple titration was obtained. In this experiment, considering the concentrations employed, a binding constant in excess of 10⁶ M⁻¹ can be estimated. However, the expected stoichiometry of one polymerase per promoter was not obtained. Approximately 2 enzyme molecules per fragment were added before activity was observed. Then, 5 more enzyme molecules per fragment were required to achieve equivalence. When corrections are made for σ content and enzyme specific activity, these results suggest at least two to three tight binding sites on this promoter-containing fragment. A resolution of the stoichiometric inconsistency is expected to follow from experiments with fully reconstituted holoenzyme and accurately determined promoter-containing DNA fragment concentrations.

The abortive initiation reaction offers the possibility of determining mechanistic features of the initiation phase of RNA synthesis because in contrast to normal RNA synthesis, this reaction is a simple two-substrate, two-product steady state reaction. As such, the variation of one substrate at fixed concentrations of the other can yield information regarding binary and ternary complexes of the enzyme bound to a promoter. The results of such an experiment are shown in double reciprocal form in Fig. 5. Variation of UTP at fixed concentrations of ATP resulted in a pattern that intersected at a point on the ordinate of Fig. 5A. A plot of the slopes and intercepts versus ATP⁻¹ is shown in Fig. 5B. The same data yielded a pattern that intersected to the left of the ordinate when ATP was the variable substrate and UTP was held constant, Fig. 5C. In this case, the slope replotted through the origin, Fig. 5D. The kinetic constants derived from the replots are given in the legend to Fig. 5.

The initial velocity equation that conforms to the kinetic results obtained is

\[ v = \frac{V(A)(B)}{KₐKₜ + Kₐ(A) + (A)(B)} \]

where \( v \) and \( V \) are the initial and maximal velocities, respectively; \( A \) corresponds to ATP, \( B \) corresponds to UTP, \( Kₐ \) is the dissociation constant for ATP, and \( Kₜ \) is the Michaelis constant for UTP. The \( Kₐ(B) \) term normally found in the denominator of sequential mechanism rate equations is missing here because as the UTP concentration approaches saturation, \( Kₜ \) approaches zero. The characteristic pattern seen in Fig. 5 and the above rate equation correspond to the equilibrium ordered addition mechanism. This means that all of the substrate-binding steps are at equilibrium in the steady state. It also means that ATP must bind first to the polymerase-promoter complex and that in so doing the kinetically significant binding site for UTP is created.

An indication of the relative affinity of the enzyme for the initiating purine nucleotide was obtained by varying the nucleotide concentration at a fixed concentration of UTP approximately equal to its Michaelis constant. From the above equation, it can be shown that under these conditions the apparent \( Kₜ \) will be one-half \( Kₐ \) and the apparent \( Vₘₐₓ \) will be one-half \( V \). Table III is a compilation of these apparent kinetic constants for the AP₅₆ promoter. The interpretation of these data in terms of equilibrium binding properties is not rigorous because there is no assurance that the Michaelis constant for UTP is identical for all of these nucleotides. Nevertheless, the decrease in apparent \( Kₜ \) as additional phosphates are added to the adenine moiety is an unambiguous trend.

The initiation of RNA chains has been shown to be more sensitive to the chelator 1,10-phenanthroline than is subsequent chain elongation (16). In Fig. 6, we show the results of an experiment in which 1,10-phenanthroline was used to inhibit the abortive initiation reaction. Only the maximal veloci-
TABLE III

Apparent kinetic constants for adenine nucleotides on WR

Standard assay conditions were employed except that UTP was 36 μM. Kinetic constants were determined from double reciprocal plots of the initial velocity data.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Km (μM)</th>
<th>Vmax (μmol/min·promoter⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.50</td>
<td>22.0</td>
</tr>
<tr>
<td>ADP</td>
<td>0.68</td>
<td>3.6</td>
</tr>
<tr>
<td>AMP</td>
<td>2.5</td>
<td>13.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>10.0</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Fig. 6. The effect of 1,10-phenanthroline on abortive initiation. Initial velocities are plotted versus ATP concentration in reciprocal form at the concentrations of 1,10-phenanthroline indicated (○); 0.25 mM (□); no inhibitor (○). The template was 1.67 mM λb2 DNA. Velocities are in micromolar per min. Other conditions were standard as indicated in Fig. 1.

The results are significantly affected. A replot of the intercepts versus 1,10-phenanthroline concentration was linear and yielded a Ki of 10 μM. We have also found that RNA polymerase was less sensitive to 1,10-phenanthroline when the inhibitor was added after initiation of RNA synthesis on poly[d(A-T)]·poly[d(A-T)], Ki ~ 2 mM (data not shown). The uncompetitive pattern obtained with this inhibitor demands that the rate equation written above for the initial velocity of abortive initiation be modified as follows:

\[
V = \frac{V(1)(B)}{K_cK_s + K_c(A) + (A)/B(1 + (I)/K_i)}
\]

Where the constants and concentration terms retain their previous meaning, but where (I) and Ki are inhibitor concentration and inhibitor dissociation constant, respectively. The mechanism of inhibition cannot be deduced from these data alone, but this experiment shows that an initiation-specific inhibitor can be studied using this assay with traditional steady state kinetic methods.

The reason we have been able to observe the steady state abortive initiation reaction is that in the presence of only two triphosphates, the stability of the initiating dinucleotide or trinucleotide is rather low. We therefore determined the effect of adding increasing concentrations of the fourth triphosphate to a reaction solution containing saturating concentrations of all the other triphosphates. The experiment was done using the λ P1 promoter; the starting sequence is pppApApCpGpA (4). The results are shown in Fig. 7. As the GTP concentration was increased, a sharp decrease in trinucleotide synthesis occurred that was mirrored by an increase in RNA synthesis. Nevertheless, even at the highest concentrations of GTP employed, pppApApC could be detected. This experiment suggests that RNA polymerase bound to the Pn promoter encounters considerable difficulty in achieving what could be termed a stable initiated complex. Additional work on other promoters will be required in order to judge whether this frequent abortive initiation is common to all promoters or whether it is promoter, sequence, or reaction conditions specific.

DISCUSSION

The steady state mechanism of abortive initiation can be represented by the following scheme in which C represents the RNA polymerase-promoter complex and the numbered arrows correspond to rate constants:

\[
\begin{align*}
\text{ATP} & \rightarrow \text{UTP} \\
\text{PP} & \rightarrow \text{PP, pppApU} \\
\end{align*}
\]

The ordered binding of ATP followed by UTP was shown by the initial velocity pattern in Fig. 5 from which we also concluded that both binding steps are at equilibrium in the steady state. The rate-limiting step(s) must be associated with chemical reaction or product release and must be much slower than either of the dissociation rates for ATP or UTP, Kₘ, and K₋₁, respectively. Although we have suggested above that PP, dissociates before pppApU, this conclusion is based only on the prejudice that such a sequence ordinarily occurs during stable initiation when pppApU does not dissociate. Additional mechanistic experiments will be required to determine whether or not product release is ordered.

The dissociation constant, Km, for ATP was found to be 1.8 mM. This is nearly 10 times higher than the dissociation constant determined by equilibrium dialysis and spectrophotometry (17, 18). In the studies cited, a template was not present, and although solution conditions were not identical, it seems clear that the binding of the purine triphosphate is a relatively unfavorable binding step during initiation. In contrast, the dissociation constant for UTP was 31 μM. This value
is comparable to the Michaelis constants measured for the chain elongation portion of RNA synthesis (2).

The large disparity between the binding affinities of the initiating purine triphosphate and subsequent triphosphates can be rationalized by considering the fact that RNA polymerase is unique among nucleic acid polymerases in that it does not require a primer to initiate synthesis. The stacking stabilization between primer and triphosphate that can occur in a DNA polymerase reaction must be supplied by the enzyme alone here. Once the weakly bound purine triphosphate has been positioned properly, the second triphosphate can bind to the complex and trap the first triphosphate such that it cannot dissociate without prior dissociation of the second triphosphate. The equilibrium-ordered mechanism for RNA chain initiation would seem to be an optimal one for enzymes catalyzing de novo chain synthesis.

The requirement for a high dissociation constant for the initiating triphosphate is in part dictated by the chemical equilibrium constant through the Haldane relationship. This apparently unfavorable aspect of the mechanism could also be relevant to in vivo control if the purine triphosphate concentrations were to vary in the range of 1 to 5 mM, or if other competitive nucleotides were present which could increase the apparent Michaelis constant for ATP or GTP. In passing, we note that the nearly absolute preference of this enzyme to begin RNA chains with ATP or GTP probably co-evolved with the tendency for these two nucleotides to be maintained at high concentration for energy transduction and protein synthesis.

The steady state kinetics of this enzyme have always resulted in nonlinear reciprocal plots when initiation was not experimentally separated from the elongation reaction (19, 20). For example, on poly[dA-T]-poly[dA-T], low concentrations of either ATP or UTP resulted in curved reciprocal plots. This is readily understood by considering the relationship of this pair during initiation. At low concentrations of ATP, initiation would become rate-determining for overall RNA synthesis. The reason is the high dissociation constant of ATP in initiation compared to its low Michaelis constant in elongation. The same behavior is predicted to result at low UTP concentrations according to our proposed mechanism. The reason here is that the apparent Michaelis constant for ATP is a very sensitive function of UTP concentration. Thus, although UTP does not have a high Michaelis constant, it can have a pronounced secondary effect on ATP binding with the result that initiation again becomes rate-limiting to synthesis as the UTP concentration is lowered.

A practical way to illustrate the relationship of ATP and UTP during initiation is as follows: to optimize the incorporation of $[^{32}P]ATP$ (or GTP) into an RNA chain, this model predicts that lowering the ATP or GTP concentration and increasing the concentration of the second triphosphate in an RNA sequence would be most efficacious. This effect has been seen qualitatively with the poly[d(A-T)]-poly[d(A-T)] template (20) and more recently on a tRNA promoter (21); but it should also be useful for current approaches to sequencing RNA from 5'-labeled ends (22). Two additional experimental advantages are suggested by these mechanistic results. First, the determination of the kinetic constants $k_i$ and $k_r$ is simultaneously a determination of the equilibrium dissociation constants of the triphosphate to the kinetically significant forms of promoter-bound RNA polymerase. This should be useful in studies of initiation specificity and fidelity. Second, the maximal velocity for this reaction can be determined by varying UTP at any ATP concentration sufficiently high to define the intercept of Fig. 5A accurately. This should allow a quantitative assay for RNA polymerase holoenzyme to be developed without the present ambiguities of measuring contributions from both initiation and elongation simultaneously.

The above discussion on the unique interdependence of ATP and UTP during initiation does not obtain during the chain elongation phase of RNA synthesis. Indeed, Rhodes and Chamberlin have shown that elongation proceeds with essentially independent triphosphate-binding steps (i.e. ping-pong initial velocity kinetics) (3). Presumably, the release of PPi and the obligatory translocation step are responsible for preventing kinetically significant interaction between the triphosphate binding events. We do not yet know whether the transition from sequential triphosphate binding to noninteractive binding occurs after the first translocation event or whether there is a gradual change during several incorporation events. Kinetic studies on promoters that allow abortive initiation of tri- and tetranucleotides should resolve this question.

Rhodes and Chamberlin have also suggested that the order of triphosphate binding is the opposite of that proposed here (23). Their conclusion was based on the pattern of rifampicin inhibition at different concentrations of ATP or UTP. We show in the accompanying paper that rifampicin inhibition of RNA initiation is more complicated than was once assumed. These recent results suggest that a re-evaluation of this inhibitor as a specific probe of initiation will be required. Although our interpretation of the initial velocity pattern, Fig. 5, is not entirely without ambiguity (24), we would argue strongly that it is the simplest explanation and that, in addition, the initiation mechanism proposed here explains previously inexplicable initial velocity data for this enzyme.

More importantly, perhaps, we would emphasize that the methods we have used to reach our conclusions on the order of addition of substrates are also the best available to test those conclusions and to find out more about the initiation reaction. In particular, product inhibition and substrate analog experiments should be useful in several mechanistic studies. The reverse reaction can be followed and an overall chemical equilibrium constant determined. In other words, by studying the abortive initiation pathway, we have been able to isolate for detailed study a previously inaccessible portion of the RNA synthesis pathway. In addition, the equilibrium-ordered addition of substrates allows an even simpler interpretation of steady state kinetic data than is found with most two-substrate, two-product enzyme mechanisms.

A detailed examination of individual reaction steps in initiation based on the abortive initiation assay will require some additional clarification of the rate-limiting step in this reaction. We know that in the normal course of synthesizing a long RNA molecule initiation is not aborted. If this process is of primary interest we must determine to what extent we have perturbed the normal initiation reaction by isolating it from subsequent translocation and elongation steps. Another way of stating this criticism would be: does the maximal velocity observed for the abortive initiation reaction under any set of conditions correspond to a step or steps on the normal initiation pathway or does it correspond to a step associated with the alternate pathway (e.g. release of pppApU)?

We have concluded that the abortive release of pppApU can only be partially rate-limiting in this reaction. Consider the steps with associated rate constants $k_i$ and $k_r$ in the above scheme. The release of PPi, $k_i$, also contains contributions from the unimolecular steps involved in interconversion of the central complexes and would be expected to occur in the initiation of any RNA whether subsequently aborted or not. The release of pppApU, $k_r$, is absolutely required in this assay.

2 W. R. McClure, unpublished results.
in order to maintain the steady state conversion of substrates to products. If this product release step were uniquely rate-determining, all of the inhibition observed by molecules such as 1,10-phenanthroline (Fig. 6) or rifampicin (4) would have to be interpreted as having resulted from a stabilization of the enzyme-pppApU complex. Similarly, the interpretation of the lower \( V_{\text{max}} \) values observed for adenosine or AMP would have to involve higher stability of the respective dinucleotides. Both of these hypothetical explanations seems improbable. It is, on the other hand, true that if any reaction conditions or additional molecules could stabilize the complex, they would also decrease pppApU synthesis. For example, without additional experiments we cannot conclude why high KCl concentrations (Fig. 1) or high MgCl\(_2\) concentrations (Fig. 2) inhibited the abortive initiation reaction. These dependences are typical for the initiation phase of RNA synthesis, but the inhibition could be due to decreased triphosphate binding, less affine association of polymerase with promoter or a stabilization of the enzyme-pppApU complex. The direct experiment in which the frequency of abortive starts was determined in the presence of all four triphosphates (Fig. 7) also showed that release of product (in this case pppApApC) was not entirely rate-limiting. Rather, it appears that once formed, a short initiating RNA chain is nearly as likely to dissociate as to translocate and to become incorporated into a full length transcript. Work currently in progress to quantitatively answer this question suggest that the probability of abortive or stable initiation is a delicate balance for several promoters on \( \lambda \) as well as on T7.

We have also found that the stability of an initiated oligonucleotide is not well correlated with chain length. Instead, the rule seems generally to be that a 3'-OH terminal purine (e.g. pppApUpG on AP\(_T\) or T7 A\(_3\)) is about 10 times more stable than a 3'-OH terminal pyrimidine (e.g. pppApUpC on AP\(_T\) or T7 A\(_3\)). This notion of a stabilizing effect by 3'-OH purine was also used to explain the stability of ApUpA versus UpApU on poly[d(A-T)]-poly[d(A-T)] (25). If the observed stabilization is due only to preferential translocation of 3'-OH purines into what was originally the purine-specific initiation site, then we speculate that abortive release of initiated oligonucleotides would most likely occur from the untranslocated complex.

The above considerations make reasonable the speculation that the 5'-terminal sequence of a transcript might also contribute to overall promoter efficiency (i.e. the fraction of initiation events that result in full length messages). The rate at which initiation occurs under our standard triphosphate concentrations can be estimated at about 0.1 to 0.2 s\(^{-1}\). If one or two oligonucleotides were to dissociate for each message that reached full length, the maximum productive initiation frequency would be about four to six per min. Other factors are certainly involved in determining productive initiation frequency, but the balance between abortive and productive initiation is an intrinsic part of this enzyme mechanism that could be used for control. Indeed, it would seem that the most useful definition of RNA chain initiation might be that number of triphosphate incorporation steps that results in a complex which ordinarily would no longer dissociate. In this view, initiation and its control would be a function of promoter, or transcription sequence, or both, as well as the presence or absence of activators or inhibitors.

The above discussion indicates that a final interpretation of the observed maximal velocities of the abortive initiation reaction cannot be made at this time. Indeed, if the proposed balance between abortive and productive initiation is a function both of the individual promoter under study and the reaction conditions employed, then the interpretation of these maximal velocities may well be different in each case. Under our standard assay conditions, however, the observed velocities for the T7 and \( \lambda \) promoters shown in Table I are in the range of 20 to 100/min/promoter. Smagowicz and Scheit (26) reported similar values on T7, although rifampicin was present in all of their reactions and individual promoters were not studied. The same work reported a \( K_v \) value much higher than that observed here on \( \lambda \)-DNA. The presence of rifampicin in those experiments precludes a direct comparison to our results, however. The possibility that promoter initiation efficiency could be related to higher rates of formation of the first phosphodiester bond or lower apparent Michaelis constants for initiating triphosphates will be tested with additional experiments on promoters that vary in their initiation frequency.

Another intrinsic control factor in overall RNA synthesis was demonstrated by the difference in the RNA polymerase titration behavior of intact T7 DNA versus intact \( \lambda \)-DNA. There are obviously a large number of sites on \( \lambda \)-DNA that bind RNA polymerase as tightly as do the known promoters, but at which RNA synthesis is not initiated. Multiple binding sites for RNA polymerase on \( \lambda \)-DNA has also been observed using the filter binding technique (27). In contrast, on T7 a plateauing of promoter occupancy occurred nearly at the expected stoichiometric ratio. T7 DNA is also transcribed about 10 times better in vitro than is \( \lambda \)-DNA at any RNA polymerase/DNA ratio. These observations suggest that RNA polymerase by stably binding to nonpromoter regions of \( \lambda \)-DNA may be impeding the transcription of other RNA polymers. Pronounced pauses in transcription have been observed in several laboratories (28–30). Although DNA sequence or product RNA secondary structure may cause some of the pauses, it is also possible that RNA polymerase could attenuate synthesis by direct interference within a transcription unit.

Even on the AP\(_R\) DNA fragment there appeared to be at least two other sites of approximately equal affinity to the polymerase (Fig. 4). One such site is the P\(_{156}\) promoter which is adjacent to P\(_{151}\) (31). However, the starting sequence of that transcript is also pppApU; if RNA polymerase were binding there, we would expect even more product than was observed. If discrete tight binding sites exist on \( \lambda \)-DNA it should be possible to locate them with a combination of titration experiments and additional restriction enzyme fractionation. The sequence of these putative tight-binding nonfunctional RNA polymerase binding sites could be very informative as to the DNA sequence requirements of active promoters.

The stoichiometric binding observed in the titration experiment of Fig. 4 also strongly suggest that \( \sigma \) subunit does not dissociate during the course of the preincubation or subsequent abortive initiation assay. We have previously shown that \( \sigma \) subunit is absolutely required for the promoter-specific synthesis of dinucleotides (3). If \( \sigma \) subunit were to prevent the enzyme to the promoter, we would predict that less than stoichiometric holoenzyme would be sufficient to yield the initiation seen in Fig. 4. Had \( \sigma \) release occurred on binding, the free subunit would have been able to associate with the core enzyme in this preparation. The resulting holoenzyme formed would then have been able to bind additional promoters, release \( \sigma \), etc. In other words, the "\( \sigma \) catalytic cycle" does not begin with promoter binding. Analogous arguments can be used to suggest that \( \sigma \) does not normally dissociate after the formation of the first or second phosphodiester bonds. A similar conclusion has been reached on the basis of the different rotational correlation times of free and bound \( \sigma \) during initiation on T7 DNA (32).
RNA polymerase has been shown to contain 2 tightly bound zinc atoms at least one of which may be involved in polynucleotide binding (33). The potent inhibition observed of abortive initiation in the presence of 1,10-phenanthroline may have resulted from binding to a zinc atom(s) that is accessible during initiation but that is protected by RNA product during chain elongation. Other experiments will be required to elucidate the role of zinc and the inhibition by 1,10-phenanthroline because D’Aurora et al. (34) have shown that the actual inhibitor of E. coli DNA polymerase I (another zinc-containing enzyme) was a 2:1 complex of 1,10-phenanthroline with adventitious cuprous ion (kept in the 1 oxidation state by thiol reagent). It will therefore be necessary to determine whether 1,10-phenanthroline itself or a more complex species is responsible for the observed inhibition of RNA polymerase. The important result here is that the addition of this inhibitor resulted in dramatic inhibition of the abortive initiation reaction and that this inhibition correlated with the previous observation of an initiation specific inhibition by l,lO-phenanthroline (16). Both 1,10-phenanthroline and rifampicin have similar effects on in vitro RNA synthesis. However, 1,10-phenanthroline is a dissociable inhibitor that affects only the maximal velocity of the abortive initiation reaction. We show in the following paper (4) that rifampicin, a very tight binding ligand, affects only the Michaelis constants in the abortive initiation reaction. Thus, two completely different mechanisms of inhibition lead to the same net result of inhibiting initiation of RNA synthesis.

The usefulness of the abortive initiation assay has recently been demonstrated by Cech et al. by using restriction enzyme fragments on T7 DNA. Additional mechanistic work on the PP, exchange reaction and the reverse reaction should now be facilitated by the steady state results reported here. Krakow and Fronk have demonstrated both of these experimental possibilities (35) but a thorough quantitative treatment in terms of discrete enzymatic steps has been lacking. It now appears straightforward to identify the individual steps in initiation and their velocities by employing these and many of the other techniques developed for standard two-substrate, two-product enzyme reaction. Preliminary experiments designed to measure the steps leading to the formation of the first phosphodiester bond are in progress. The rate of synthesis of promoter-specific dinucleotides seems not to be greatly different among about 10 promoters that have been screened. In addition, RNA polymerases from several sources (Salmonella typhimurium (36); Bacillus subtilis; and the T7-specific polymerase (37) are able to catalyze the abortive initiation reaction. If the inherent instability of initiating a nucleic acid chain de novo is a property of all RNA polymerases, the assay described here could be profitably applied toward a search for factors, or specific DNA sequences, or both, that will allow selective initiation of either prokaryotic or eukaryotic genes.

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A steady state assay for the RNA polymerase initiation reaction.
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