Mechanism of Ribonucleic Acid Chain Initiation. 1. A Non-Steady-State Study of Ribonucleic Acid Synthesis without Enzyme Turnover†

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ABSTRACT: A non-steady-state kinetic method has been developed to observe the initiation of long RNA chains by *Escherichia coli* RNA polymerase without the enzyme turnover. This method was used to determine the order of binding of the first two nucleotides to the enzyme in RNA synthesis with poly(dA-dT) as the template. It was shown that initiator [ATP, uridylyl(3′–5′)adenosine, or adenylyl(3′–5′)uridylyl-(3′–5′)adenosine] binds first to the enzyme–template complex, followed by UTP binding. The concentration dependence of UTP incorporation into the initiation complex suggests that more than one UTP molecule may bind to the enzyme–DNA complex during the initiation process. Comparison of the kinetic parameters derived from these studies with those obtained under steady-state conditions indicates that the steps involving binding of initiator or UTP during initiation cannot be rate limiting in the poly(dA-dT)-directed RNA synthesis. The non-steady-state technique also provides a method for active-site titration of RNA polymerase. The results show that only 36 ± 9% of the enzyme molecules are active in a RNA polymerase preparation of high purity and specific activity. In addition, the minimal length of poly(dA-dT) involved in RNA synthesis by one RNA polymerase molecule was estimated to be approximately 500 base pairs.

The initiation of RNA synthesis is an important control point in gene transcription; it regulates not only the amount of RNA synthesized but also the selectivity of the transcription. The study of in vitro RNA chain initiation and its control has been complicated by the unique nature of the process itself; initiation occurs only once for each high molecular weight RNA chain synthesized. Since RNA polymerase spends most of its time elongating RNA during the synthesis, it is not a simple matter to measure the rate of RNA chain initiation. An indirect method called "the rifampicin challenge technique" was devised by Mangel & Chamberlin (1974) to determine the rate constant of initiation based on the assumption that the bimolecular binding of rifampicin to RNA polymerase and RNA chain initiation are simple competitive processes. Unfortunately, it turned out that this assumption is invalid because the inactivation of RNA polymerase by rifampicin is not due to the bimolecular binding but rather is due to a conformational change of the enzyme following the binding (Yarbrough et al., 1976). Moreover, rifampicin does not inhibit the DNA-dependent formation of the first phosphodiester bond catalyzed by RNA polymerase (Johnston & McClure, 1976). Another method which can yield mechanistic information about the initiation process is to study the kinetics of "abortive initiation" (Johnston & McClure, 1976). This reaction is observed in cases where only the first two nucleoside triphosphates corresponding to the beginning of an RNA transcript are present. After the first report of abortive initiation, its steady-state kinetics were promptly studied (Smagowicz & Sheit, 1977; McClure et al., 1978). There is no evidence, however, to indicate that the abortive initiation and the normal (productive) initiation proceed along the same kinetic pathway. Thus, a new approach is needed to investigate the normal initiation phase of RNA synthesis.

One of the great difficulties in quantitative studies of RNA chain initiation is the occurrence of enzyme turnover under steady-state conditions leading to reinitiation of RNA chains. In the presence of enzyme turnover, the number of RNA chains initiated does not represent the number of active enzyme–DNA complexes in the system. Knowing the number of active enzyme–DNA complexes present in a system is essential for meaningful kinetic analysis. Such information has so far not been available for RNA polymerase.

In this paper, we report a non-steady-state technique which allows RNA synthesis to be studied in the absence of enzyme turnover using synthetic poly(dA-dT) as the template. This method was employed to determine the order of binding of the first two nucleotides to the enzyme–DNA complex during RNA chain initiation and to measure the number of active RNA polymerase molecules in the system. The application of this method to a rapid kinetic study of RNA chain initiation is described in the following paper (Shimamoto & Wu, 1980).

Materials and Methods

DNA and Nucleotides. Calf thymus DNA was obtained from Worthington. High molecular weight poly(dA-dT) was synthesized from dATP and dTTP by using the large fragment of *Escherichia coli* DNA polymerase I (from Boehringer Mannheim) which lacked the 3′-exonuclease activity (Setlow et al., 1972). The molar concentration of this DNA preparation was determined based on $\epsilon_{260} = 6.6 \times 10^3$ M^{-1} cm^{-1}. ATP and UTP purchased from Sigma Chemical Co. were further purified by DEAE-cellulose chromatography. Dinucleoside monophosphates were obtained from P-L Biochemicals or Sigma. Adenylyl(3′–5′)uridylyl(3′–5′)adenosine (ApUpA)[1] was synthesized from adenylyl(3′–5′)uridine (ApU) and ATP by RNA polymerase using poly(dA-dT) as the

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; ApU, adenylyl(3′–5′)uridine; UpA, uridylyl(3′–5′)adenosine; ApUpA, adenylyl(3′–5′)uridylyl(3′–5′)adenosine; UpApU, uridylyl(3′–5′)adenylyl-(3′–5′)uridine; pppApU, 5′-triphosphoadenylyl(3′–5′)uridine.

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template (Oen & Wu, 1978) and was purified on a DEAE-cellulose column.

Enzyme. DNA-dependent RNA polymerase was purified from *E. coli* K12 cells (3/4 log, Grain Processing Corp.) by the method of Burgess & Jendrisak (1975). The enzyme was 91–99% pure, and its content of σ subunit was about 70% as shown by $NaDodSO_4$–polyacrylamide gel electrophoresis. The concentration of protein was determined by amino acid analysis or by the method of Bücher (1974) which was standardized for RNA polymerase by amino acid analysis. The purified enzyme was stored in 0.05 M Tris-HCl (pH 7.9) containing 50% glycerol, 0.1 M KCl, and 0.2 mM dithiothreitol at −20 °C. Before use, the stock enzyme was diluted twofold by twofold successively to prevent inactivation (Gonzalez et al., 1977).

Enzyme Assay. RNA polymerase activity was assayed by the incorporation of ^{3}H-labeled ribonucleoside monophosphate into acid-insoluble material as described previously (Wu & Wu, 1973) with the following modifications. Glass filters were obtained from Enzo Biochemical (EGF-05-24). Assays in which poly(dA-dT) was the template were performed in the same buffer as that of the kinetic measurements described later. The specific activity of RNA polymerase determined by the standard assay was 1200 units/mg on calf thymus DNA and 3000 units/mg on poly(dA-dT) template. One unit of enzyme was defined as the amount which incorporates 1 nmol of ^{3}H-labeled nucleoside monophosphate in 10 min into acid-precipitable material.

Non-Steady-State Studies of RNA Synthesis. Three experimental systems (A, B, and C) were designed for non-steady-state studies of RNA synthesis (see Schemes I and II under Results). All solutions used in these studies contained 0.05 M Tris-HCl (pH 7.9), 0.1 M KCl, 0.01 M $MgCl_2$, and 0.2 mM dithiothreitol. All reactions were carried out at 37 °C. In system A, 1 volume of the solution (II) containing an initiator (ATP, UpA, or ApUpA) was added to 3 or 4 volumes of the solution (I) containing enzyme and DNA. After this mixture was incubated for 1 min, an equal volume of the solution (III) containing ATP, UTP and an appropriate amount of initiator was added and the elongation of RNA chains was allowed to occur for 2 min. The reaction was stopped by addition of 20 volumes of cold 5% trichloroacetic acid to 1 volume of the reaction mixture, and the final mixture was kept at 0 °C for at least 30 min before filtration on a glass-fiber filter. The filter was then dried and counted in a liquid scintillation counter. System B was similar to system A except that solution II contained UTP instead of the initiator, and initiator was added only in solution III together with ATP and UTP. In system C, solution I contained initiator, enzyme, and DNA, which were preincubated for 2 min at 37 °C. The other solutions were the same as those in system B except that initiator was deleted from solution III.

Analysis of Oligonucleotide Intermediates. For detection of oligonucleotide products which were too short to be precipitated by trichloroacetic acid, the filtrates from the acid precipitation mixtures were neutralized with ammonium bicarbonate and then lyophilized. The solid material obtained from lyophilization was washed with acetone to remove residual trichloroacetic acid and dissolved in a small volume of water. The sample was analyzed by descending chromatography on Whatman 3MM paper (45-mm wide) in the WASP solvent (Johnston & McClure, 1976). The distance traveled by the solvent front was approximately 1 m. In this solvent system, UpApU has an R_f value of 0.09 and longer oligonucleotides stay at the origin. Thus, the net incorporation of labeled UMP into acid-soluble oligonucleotides (longer than UpApU) in system C was estimated by subtracting the radioactive counts at the origin in experiment C1 from that at the origin in experiment C2. This subtraction could also eliminate the possibility that traces of labeled UTP might be adsorbed at the origin, thereby contributing to the radioactive counts. No radioactive peak was observed between the origin and the UpApU peak in these experiments.

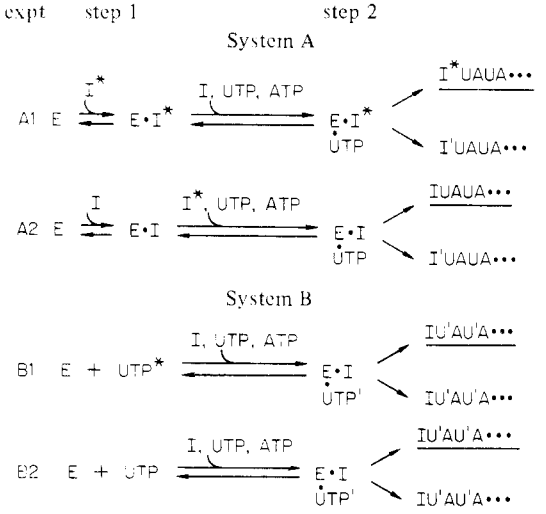
End-Addition Reaction Catalyzed by RNA Polymerase. In the presence of poly(dA-dT), RNA polymerase can catalyze not only the template-directed RNA synthesis but also the covalent attachment of UMP to the 3′-hydroxyl end of the DNA (Wickner et al., 1972; Nath & Hurwitz, 1974). We measured the end-addition reaction by incubating labeled UTP with the enzyme–poly(dA-dT) complex for 2 min at 37 °C and determining acid-precipitable radioactivity. By use of this method, it was found that with commercial preparations of poly(dA-dT) the [^{3}H]UMP incorporation due to end addition amounted to about 55–160% of the net [^{3}H]UMP incorporation due to poly(rA-rU) synthesis. Repetitive freezing and thawing of poly(dA-dT) led to increased end additions, sugesting that this reaction could occur at nicks in the DNA. To minimize the number of nicks, we synthesized high molecular weight poly(dA-dT) using the large fragment of DNA polymerase I (Setlow et al., 1972). With this relatively nick-free poly(dA-dT) template, the fraction of the end addition to the net UMP incorporation was about 3–4% under standard conditions in system C.

Results

Order of Substrate Binding in RNA Chain Initiation. Binding of at least two nucleoside triphosphate molecules to the enzyme–template complex is necessary for the formation of first phosphodiester bond in the initiation step of RNA synthesis. The first nucleotide at the 5′ end of the RNA chain (initiator) is almost always a purine nucleotide (Maitra & Hurwitz, 1965). Thus, in the poly(dA-dT)-directed RNA synthesis the initiator is ATP, which can also be replaced by AMP, ADP (Krakow & Fronk, 1969), UpA (So & Downey, 1970), or ApUpA (Oen & Wu, 1978). The kinetic mechanism for addition of the initiator and the second nucleotide, UTP, in the poly(rA-rU) synthesis may be ordered or random. For the ordered mechanism, either the initiator can bind first, followed by UTP, or vice versa.

Scheme I depicts two experimental systems (A and B) devised to determine the kinetic mechanisms of RNA chain initiation. System A measures the incorporation of radioactively labeled initiator into the initiation complexes, whereas system B measures the incorporation of labeled UMP into the same complexes. For simplicity, we present a case where the initiator binds first and no productive poly(dA-dT)–UTP–enzyme complex can be formed in its absence. Two experiments are described in system A. In experiment A1, radioactively labeled initiator was added to the enzyme–poly(dA-dT) complex and the mixture was incubated for 1 min at 37 °C (step 1). An equal volume of solution containing ATP, UTP, and unlabeled initiator was then added and incubated for 2 min to allow the elongation of RNA chains to occur (step 2). The concentration of either ATP or UTP was kept low (2.5 to ~8 μM) to minimize the turnover of enzyme molecules during the elongation step. The amount of unlabeled initiator added was identical with the labeled initiator present in step 1 so that the specific activity of the initiator became half in step 2 but its concentration remained constant throughout the experiment. The radioactive initiator molecules which bound to the enzyme–DNA complex in step 1 and subsequently

Scheme I: Two Non-Steady-State Kinetic Systems Designed to Test the Order of Substrate Binding in Initiation[a]



[a] The systems are presented based on the assumption of an ordered mechanism of the initiator binding followed by the substrate binding. E is the RNA polymerase–poly(dA-dT) complex and I is the initiator. The asterick denotes the radioactivity-labeled compound with high specific activity and the apostrophe denotes the labeled compound whose radioactivity is diluted with unlabeled nucleotide. The underline represents the product synthesized without enzyme turnover.

elongated in step 2 would retain higher specific activity after the dilution of radioactivity caused by addition of unlabeled initiator. The amount of initiator incorporated due to enzyme turnover could be measured by a control experiment (experiment A2) similar to experiment A1, except that unlabeled initiator was first incubated with the enzyme–DNA complex in step 1 and labeled initiator was added in step 2 together with UTP and ATP. In this control experiment, only unlabeled initiator would be incorporated in RNA synthesized without enzyme turnover (via step 1), whereas the initiator incorporated in step 2 would have the same specific activity as in the same step of experiment A1. Since a large excess (10^2 to $\sim 10^4$) of initiator relative to the enzyme–DNA complex was used in both experiments A1 and A2, the concentrations of free initiator (both labeled and unlabeled) in these two experiments were essentially identical. Thus, the net incorporation of radioactive initiator obtained by subtracting the incorporation in experiment A2 from that in experiment A1 would give the number of initiator molecules incorporated into RNA chains and, hence, the number of RNA chains which were synthesized without enzyme turnover. System B also consisted of a pair of experiments (experiments B1 and B2). In experiment B1, labeled UTP was first incubated with the enzyme–poly(dA-dT) complex (step 1). A solution containing a large excess of unlabeled UTP, a lower concentration of ATP, and an appropriate amount of initiator was then added to elongate the RNA chains (step 2). Experiment B2 was similar to experiment B1 except that unlabeled UTP was added in step 1 while labeled UTP was added in step 2. In both experiments the concentrations and specific activities of UTP were identical during the elongation period (step 2). Since it was assumed that no UTP–enzyme–DNA complex could be formed in the absence of initiator (Scheme I), one would expect that the amount of labeled UMP incorporation should be the same in both experiments B1 and B2; i.e., the difference between the unlabeled UMP incorporated into RNA products in these two experiments should be negligible.

Table I summarizes the criteria used to distinguish between the random and the two ordered mechanisms of initiation by

Table I: Criteria for Differentiation among Possible Kinetic Mechanisms of Initiation

mechanism	order of binding: first	order of binding: second	net incorpn of initiator or UMP: system A	net incorpn of initiator or UMP: system B
ordered[a]	initiator	UTP	yes	no
ordered	UTP	initiator	no	yes
random	initiator or UTP	initiator or UTP	yes	yes

[a] This case is shown in Scheme I.

Table II: Net Labeled-Nucleotide Incorporation in Systems A and B[a]

initiator	net incorpn: system A (pmol of initiator incorpd)	net incorpn: system B[b] (pmol of [^{3}H]UMP incorpd)
ATP	1.9	0.2
UpA	>1.2[c]	0.3
ApUpA	3.0	0.3

[a] In all experiments, the reaction volumes were 80 μL in step 1 and 160 μL in step 2. The concentrations of enzyme and poly(dA-dT) in step 1 were 105 μg/mL and 5×10^{-5} M (nucleotide), respectively. In system A, the concentration of initiator present in step 1 was 38 μM ATP, 50 μM UpA, or 10 μM ApUpA, while 125 μM ATP and 4 μM UTP were added in step 2. In system B, 100 μM UTP was present in step 1, while 6.3 mM UTP and 4 μM ATP (final concentrations) were added in step 2. The concentration of initiator added in step 2 was the same as that present in system A. [b] The net [^{3}H]UMP incorporation in system B was of the same order as that due to the end-addition reaction (0.2 pmol under similar conditions). [c] The incorporation of UpA was determined by its competition with labeled ATP as the initiator in system A. The reaction mixture was the same as that described above except that both UpA and [^{3}H]ATP were present. The net [^{3}H]AMP incorporation was 0.7 pmol as compared to 1.9 pmol in the absence of UpA. Thus, the net UpA incorporation was estimated to be at least 1.2 pmol.

experiments described in systems A and B. If the mechanism is ordered and the initiator binds first, system A would give a significant net labeled-initiator incorporation (experiment A1 > experiment A2) whereas the net labeled-UMP incorporation in system B would be negligible (experiment B1 ≃ experiment B2). The converse is true when the order of binding is UTP first and then the initiator. If there is no obligatory order of additions of initiator and UTP, both systems A and B would yield significant net incorporations. The experimental results obtained by using three different initiators (ATP, UpA, and ApUpA) are presented in Table II. All the results are consistent with an ordered mechanism in which initiator is the first substrate molecule bound. It should be mentioned that the above analysis is based on the assumption that the rate of dissociation of the enzyme–DNA–initiator complex (the reverse reaction in step 1) is *not* much faster than the rate of formation of the first phosphodiester bond (the forward reaction in step 2) which leads to the elongation of RNA chains. If it is very much faster, the enzyme–DNA–initiator complex will reequilibrate prior to the bond formation and the net labeled-initiator incorporation will be negligible since the specificity of the bound initiator will be the same as that in step 2 medium in both experiments A1 and A2. The observed positive net incorporations for all three initiators used indicate that the dissociation reaction in step 1 is significantly slower than the subsequent steps. This contention is further supported by the observation that the total concentration of

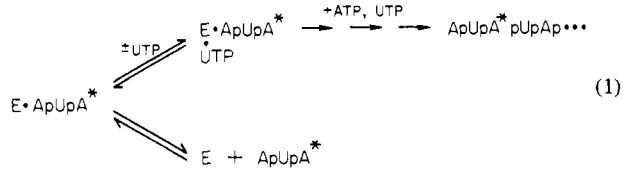
Table III: Maximum Net Incorporation of [^{3}H]ApUpA into RNA and Intrinsic Dissociation Constant of the [^{3}H]ApUpA-Enzyme-Poly(dA-dT) Complex in System A

substrate concn in step 2 (μM)			
ATP	UTP	n_{max} (pmol)	K_d (μM)
2.5	125	3.8 ± 0.3	3.8 ± 0.7
125	2.5	2.5 ± 0.1	3.9 ± 0.3

the active DNA–enzyme complex determined from the net labeled-initiator complex in system A is consistent with that obtained in system C (see below).

Active-Site Titration of RNA Polymerase. As described above, the net incorporation of labeled initiator in system A represents the number of RNA chains initiated without enzyme turnover. By increasing the concentration of the labeled initiator added to the enzyme–DNA complex, one can measure the maximum number of RNA chains initiated without enzyme turnover, which would provide a way to determine the number of enzyme–DNA complexes present in the system.

There are two possible fates for the labeled ApUpA–enzyme–DNA complex in system A: the labeled initiator could either dissociate from the complex or be incorporated into RNA chains in the presence of ATP and UTP as shown by eq 1. Thus, the net incorporation of labeled ApUpA into



(1)

RNA product is a function of the concentration of E·ApUpA* formed in step 1 and the efficiency of converting E·ApUpA* into RNA chains. This efficiency is dependent on the velocity of chain elongation and, hence, on the concentrations of UTP and ATP added in step 2. At a fixed set of UTP and ATP concentrations, the net incorporation of labeled ApUpA will only be proportional to the concentration of the E·ApUpA* complex. Under these conditions, the net labeled-ApUpA incorporation in system A (n) can be expressed assuming a simple bimolecular binding of ApUpA* to the enzyme–DNA complex:

$$n = \frac{n_{max}[\text{ApUpA*}]_0}{K_d + [\text{ApUpA*}]_0} \quad (2)$$

or

$$\frac{n}{[\text{ApUpA*}]_0} = \frac{1}{K_d}(n_{max} - n) \quad (3)$$

where n_{max} is the maximal net incorporation of ApUpA* into RNA product, $[\text{ApUpA*}]_0$ is the initial concentration of ApUpA* added to the enzyme–DNA complex in step 1 (which is approximately equal to the concentration of free ApUpA* because of a large excess of initiator), and K_d is the intrinsic dissociation constant of the ApUpA*–enzyme–DNA complex. Figure 1 shows the plot of $n/[\text{ApUpA*}]_0$ against n using the data obtained from system A. The linearity of the plot indicates that the experimental data are in accord with the relationship given by eq 3. If the binding of ApUpA* to the enzyme–DNA complex were cooperative or the initiator acted as an activator or inhibitor in the elongation process, deviation from the linearity would have been observed. The values of n_{max} and K_d determined from the slopes and intercepts of the plots are listed in Table III. With different sets of UTP and

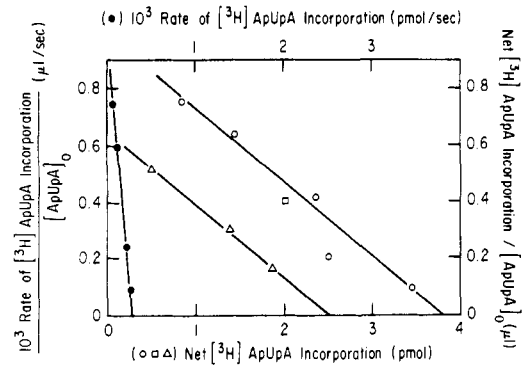
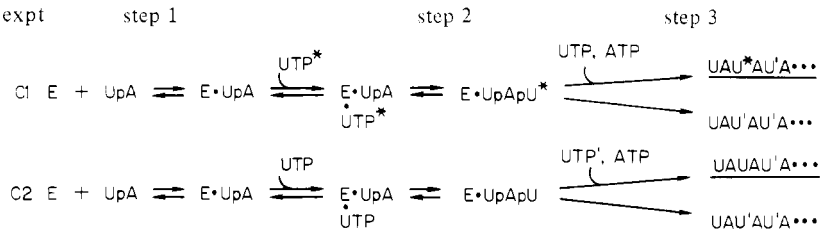


FIGURE 1: Dependence of the net incorporation of [^{3}H]ApUpA in system A on the concentration of ApUpA. The open symbols represent the ratio of the net incorporation of [^{3}H]ApUpA to the total concentration of ApUpA used in system A as described under Materials and Methods and under Results. Each point corresponds to the average value of data obtained from duplicated or triplicated sets of experiment A1 or experiment A2 in system A. The solid lines were obtained by a linear least-squares fit to the data points according to eq 3. (○) [UTP] = 125 μM and [ATP] = 2.5 μM; (□) [UTP] = 250 μM and [ATP] = 2.5 μM; (△) [UTP] = 2.5 μM and [ATP] = 125 μM. The closed symbols represent the rate of incorporation of [^{3}H]ApUpA under steady-state conditions as a function of the rate/[ApUpA] at [UTP] = 125 μM and [ATP] = 125 μM. The concentrations of the enzyme and poly(dA-dT) used were 175 μg/mL and 3×10^{-4} M (nucleotide) in step 1. The reaction volumes were 40 μL in step 1 and 80 μL in step 2. All the reaction mixtures contained 50 mM Tris·HCl (pH 7.9), 100 mM potassium chloride, 10 mM magnesium chloride, and 0.2 mM dithiothreitol. The error for the net [^{3}H]ApUpA incorporation was estimated to be about 5–10%.

ATP concentrations, the values of n_{max} vary whereas the values of K_d remain unchanged. This is expected since changes in the concentrations of UTP and ATP in step 2 would only alter the efficiency of converting E·ApUpA* into the RNA chain in this step but not the stability of the E·ApUpA* complex itself in step 1. As can be seen in Table III, the value of n_{max} is increased by increasing the concentration of UTP (but not ATP) which converts the E·ApUpA* complex into a probably more stable UTP·E·ApUpA* complex. The concentration of UTP at 125 μM is already saturating since the experimental data obtained at this concentration and those obtained when the UTP concentration was doubled to 250 μM fall approximately on the same straight line as shown in Figure 1. Therefore, the value of n_{max} determined at the UTP concentration of 125 μM should be equal to the number of active enzyme–DNA complexes which are capable of initiating and elongating RNA chains. The amount of active enzyme–DNA complex was calculated to be approximately 25% of the total amount of RNA polymerase added in these experiments. This value agrees well with that obtained from system C described below.

Incorporation of Second Nucleotide (UMP) in the Poly(rA-rU) Synthesis. In system A, we have measured the incorporation of initiator into RNA chains which were synthesized without enzyme turnover. Using a similar approach, one can study the incorporation of the second nucleotide, UMP, into RNA product. The experimental system devised for such a study is designated as system C (Scheme II). In this system, the experiment (experiment C1) can be divided into three steps: (1) preincubation of unlabeled initiator UpA with enzyme and poly(dA-dT) to form the E·UpA complex, (2) addition of labeled UTP to the E·UpA complex to allow the formation of the first phosphodiester bond, and (3) addition of UTP and ATP to elongate the RNA chains. Analogous to system A, only those labeled UTP molecules incorporated into E·UpApU* in step 2 and subsequently elongated without

Scheme II: Non-Steady-State Kinetic Experimental System to Determine the First UMP Incorporation into RNA Product (System C)[a]



[a] The notation is the same as that for Scheme I.

enzyme turnover in step 3 would retain high specific radioactivity; other labeled UMP moieties incorporated into RNA product in step 3 would have lower specific activity. The amount of labeled UMP incorporation in step 3 could be measured by a control experiment (experiment C2) in which unlabeled UTP was added in step 2 while labeled UTP was added afterward so that the total concentration and specific activity of the UTP in step 3 were the same as those in experiment C1. The difference in labeled UMP incorporation between experiment C1 and C2 would then represent the number of RNA chains synthesized without turnover of enzyme, which were elongated from the product formed in the reaction UpA + UTP* → UpApU*. By use of saturating concentrations of UTP and UpA, a value of $n_{max} = 3.59 \pm 0.27$ pmol was obtained from system C, in good agreement with a value of $n_{max} = 3.79 \pm 0.33$ pmol obtained from system A for the same preparation of enzyme. When UpA was replaced by ApUpA in system C, $n_{max} = 3.34 \pm 0.22$ pmol was obtained. Also, the number of active enzyme–DNA complexes determined by either system A or system C was dependent on the total amount of the enzyme present in these experiments but not on its concentration [(5×10^{-9})–(1×10^{-7}) M]. A question can be raised whether the labeled UpApU synthesized in step 2 can dissociate from the enzyme–DNA complex and later be incorporated in RNA product in step 3. This possibility, however, seems negligible, probably due to the low affinity of UpApU for the initiation site on the enzyme. The net labeled-UMP incorporation observed in system C was not changed by addition of excess labeled UpApU immediately before step 3 or by increasing the length of incubation of step 2.

Dependence of the Net UMP Incorporation on the Concentrations of UpA and UTP. Figure 2 shows the dependence of the net UMP incorporation in system C on the concentration of UpA at a saturating level of UTP (100 μM). The data were plotted according to an equation analogous to eq 3. Due to the significant contribution of labeled UMP incorporation in step 3, the error associated with the net UMP incorporation in experiments C1 and C2 became rather large. Although the experimental points in Figure 2 are somewhat scattered, no systematic deviation from a linear relationship seems to exist. By a linear least-squares analysis of the data, the values of $n_{max} = 2.9 \pm 0.2$ pmol and apparent $K_d = 12 \pm 2$ μM were obtained for UpA. Since the net UMP incorporation in system C is a measure of UpA binding to the enzyme–DNA complex as well as the formation of the first phosphodiester bond, the apparent K_d is not simply an intrinsic dissociation constant but rather represents the lower limit of the intrinsic dissociation constant. The observation that the apparent K_d for UpA is larger than that for ApUpA indicates that addition of AMP to the 5′-hydroxyl group of UpA stabilizes the initiator–enzyme–DNA complex.

The dependence of the net UMP incorporation on the concentration of UTP is shown in Figure 3. The plot of data

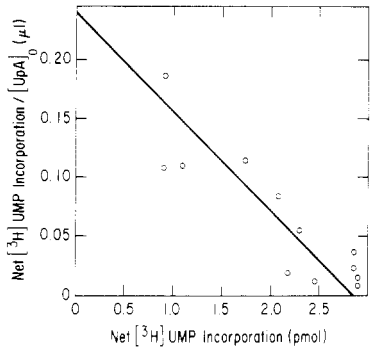


FIGURE 2: Dependence of the net [^{3}H]UMP incorporation in system C on the concentration of UpA. The concentrations at step 2 were [UTP] = 100 μM, [UpA] = 4–200 μM, [poly(dA-dT)] = 35 μM (nucleotide), and $[E]_0$ = 100 μg/mL. At step 3, [UTP] = 6.13 mM and [ATP] = 2–8 μM. A different preparation of enzyme was used in this experiment than that used in the experiments shown in Figure 1 and Table I. The solid line was obtained by a least-squares fit of the experimental data. The scattering of the data points was due partly to the errors caused by relatively large contributions of labeled UMP incorporation in step 3. For most UpA concentrations used in these experiments, the total radioactive counts in experiment C2 could be as high as 50% of those in experiment C1. The estimated error for the net [^{3}H]UMP incorporation was about 10%.

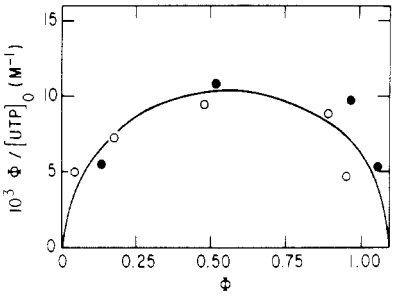


FIGURE 3: Dependence of the net [^{3}H]UMP incorporation in system C on the concentration of UTP. Two different preparations of the enzyme were used and [UpA] = 50 μM in step 2. Φ is the net [^{3}H]UMP incorporation divided by the amount of active enzyme–DNA complex determined in a separate experiment. The experimental conditions and the enzyme preparation were the same as those in Figure 1 with [UTP] = 125 μM and [ATP] = 2.5 μM (○) and in Figure 2 (●). The error for the net [^{3}H]UMP incorporation was estimated to be about 10%. The solid curve represents a best fit curve to the experimental data based on a two-site model according to the equation $\Phi = c[UTP]^2/(1 + a[UTP] + b[UTP]^2)$.

according to an equation analogous to eq 3 is not linear. The convex (upward) curve observed is characteristic of positively cooperative binding. This observation suggests that more than one UTP molecule may bind to the enzyme–DNA complex during the initiation step. In other words, UTP may bind to a site other than the substrate binding site on the enzyme, which facilitates the substrate binding or subsequent steps. This finding is not surprising in view of several reports of multiple nucleotide binding sites on *E. coli* RNA polymerase (Wu & Goldthwait, 1969; Slepneva et al., 1978).

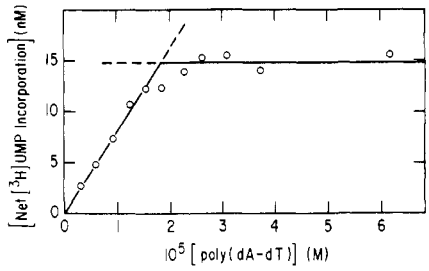


FIGURE 4: Dependence of the net [^{3}H]UMP incorporation in system C on the concentration of poly(dA-dT). The determination of net [^{3}H]UMP incorporation and the experimental conditions were the same as those in Figure 2 except that [UpA] = 50 μM and the poly(dA-dT) concentration was varied. The solid lines were obtained by a linear least-squares analysis of the data. The stoichiometric length of poly(dA-dT) was calculated from the slope of the best fit line. After a small correction due to incomplete saturation of UpA (81%), the inflection point corresponds to 508 base pairs per active enzyme.

Dependence of the Net UMP Incorporation on the Poly(dA-dT) Concentration. The net UMP incorporation in system C was also measured at a constant enzyme concentration and varying concentrations of poly(dA-dT). As shown in Figure 4, the net UMP incorporation increases linearly with increasing the poly(dA-dT) concentration and then levels off abruptly at the poly(dA-dT) concentration of about 2×10^{-5} M. This is a typical stoichiometric binding curve with a stoichiometric (inflection) point corresponding to approximately 500 base pairs per active enzyme–DNA complex as determined by the net [^{3}H]UMP incorporation, which presumably represents the minimal length of poly(dA-dT) necessary for RNA synthesis per active enzyme molecule. It is interesting to note that the actual enzyme–DNA ratio present at the stoichiometric point is about 1 enzyme molecule per 50 base pairs. Thus, the results shown in Figure 4 suggest that under these conditions either a large fraction of enzyme molecules do not bind poly(dA-dT) or they bind nonspecifically to the template but are inactive in chain initiation or both. If one assumes that all the enzyme molecules are active in binding poly(dA-dT), a question may arise as to how RNA synthesis can occur under conditions where RNA polymerase molecules are arranged almost shoulder to shoulder on the DNA template. For the incorporation of labeled UMP into the initiation complex as measured in steps 1 and 2 in system C, no translocation of enzyme on the DNA template is necessary. During the subsequent 2-min incubation in step 3, elongation of RNA chains may be possible due to rapid dissociation of nonspecifically bound RNA polymerase or by a slippage mechanism.

Discussion

The steady-state kinetics of the RNA polymerase reaction is complex and does not follow the simple Michaelis–Menten equation (Anthony et al., 1969; Downey & So, 1970). Previous investigations on the mechanism of RNA chain initiation by steady-state kinetics have not been very satisfactory due to oversimplified assumptions. By means of the so-called rifampicin challenge assay, Rhodes, & Chamberlin (1975) [see also Krakow et al. (1976)] concluded that RNA chain initiation on T7 DNA follows a kinetic mechanism in which the second nucleoside triphosphate associates rapidly with the T7 DNA–enzyme complex, followed by the binding of the initiating nucleotide which is rate limiting for the initiation process. Noting the pitfalls of the rifampicin challenge assay, Smagowicz & Scheit (1977) carried out a steady-state kinetic analysis of T7 DNA-dependent formation of pppApU from ATP and UTP catalyzed by RNA polymerase (abortive initiation) in the presence of rifampicin. Their results suggested that the order of nucleotide triphosphate binding is the opposite of that proposed by Rhodes and Chamberlin. The mechanism in which ATP binds first followed by UTP in the pppApU synthesis was subsequently confirmed by McClure et al. (1978) on the λ promoters P_l and P_r in the absence of rifampicin. Nevertheless, one is still left with some doubt whether the binding sequence in these abortive initiations is also applicable to the normal, productive initiation.

In the present study, the order of binding of the first two nucleotides in RNA synthesis on poly(dA-dT) template was determined by a non-steady-state approach. This approach resembles a pulse–chase experiment and provides a way to study an individual step such as initiation in the multistep RNA polymerase reaction. Moreover, it allows determination of the substrate binding sequence in a complex system where Michaelis–Menten kinetics is not applicable. The order of substrate binding derived from this non-steady-state kinetic analysis is consistent with the result obtained by the steady-state kinetic studies of abortive initiation, suggesting that abortive initiation may be part of the normal initiation process. In addition to the reaction sequence described above, the approach that we have developed also provides a way to determine the amount of active enzyme–DNA complex. This is based on the fact that the net incorporation of initiator or subsequent nucleotide into acid-precipitable product as measured by these non-steady-state methods represents the number of RNA chains synthesized without enzyme turnover. Since a stoichiometric saturation was seen in the formation of active enzyme–DNA complex (Figure 4), the number of active enzyme–DNA complexes determined should be equal to the number of active RNA polymerase molecules unless there exist extremely tight, nonproductive enzyme binding sites on the poly(dA-dT) template. It should be noted that the efficiency of trapping acid-precipitable material on glass-fiber filters could be less than 100%. Furthermore, small acid-soluble oligonucleotides might be released from the enzyme–template complex as the product of abortive initiation. To check these possibilities, the filtrates obtained after acid precipitation in experiments C1 and C2 at estimated substrate concentrations were subjected to analysis for oligonucleotides by paper chromatography. The amount of oligonucltides longer than UpApU in these filtrates was estimated to be 6 ± 5 pmol as compared to the net UMP incorporation of 13.4 pmol into the acid-precipitable product. If this value is included in the calculation of the number of active enzyme–DNA complexes, the fraction of active enzyme molecules becomes $36 \pm 9\%$ rather than 25% as indicated before. This calculation indicates that 55–73% of RNA polymerase molecules in an enzyme preparation are inactive. The existence of such a large fraction of inactive RNA polymerase molecules and the possible implications have, so far, not been seriously considered in many in vitro gene transcription studies.

Another piece of information derived from these non-steady-state studies is the apparent K_d of initiator or substrate. These parameters can be obtained by studying the concentration dependence of net nucleotide incorporation. It is difficult to compare these parameters with those reported elsewhere since they may vary depending on the template used and other experimental conditions. For example, we have determined that the apparent K_d for UpA in the initiation of RNA synthesis on poly(dA-dT) template is 12 ± 2 μM. This is roughly comparable to the K_i (10–50 μM) for the UpA inhibition of [γ-^{32}P]ATP incorporation into RNA chains using T4 DNA as the template (Hoffman & Niyogi, 1973).

For comparison of the incorporation of [^{3}H]ApUpA into RNA chain under steady-state and non-steady-state conditions, steady-state experiments similar to those described in system A but with enzyme turnover were performed with lower concentrations (1/20 of those used in system A) of RNA polymerase and poly(dA-dT) and higher concentrations of both of ATP and UTP (125 μM). The time course of the incorporation into acid-precipitable RNA product observed was linear up to 30–60 min. The data obtained (Figure 1) were analyzed by a typical equation for Michaelis–Menten kinetics:

$$v = \frac{V_{max}[\text{ApUpA*}]}{K_m + [\text{ApUpA*}]} \quad (4)$$

The best fit values of K_m and V_{max} obtained were 0.26 ± 0.02 μM and 2.7 × 10^{-4} pmol/s, respectively. The value of K_m observed for ApUpA is 1 order of magnitude smaller than the value of K_d given in Table II, indicating that UTP binding which occurs immediately after ApUpA binding cannot be the rate-determining step in the poly(dA-dT)-directed RNA synthesis. It should be noted that the ATP present in these experiments could compete with ApUpA in initiating RNA synthesis. This competition, however, might not be very significant under our experimental conditions because the value of K_d for ApUpA was not altered by increasing the ATP concentration in system A (Table III). From the value of V_{max} and the concentration of active enzyme–DNA complex added to the reaction mixture, the value of k_{cat} was calculated to be 1.4 × 10^{-3} s^{-1} according to the relation $V_{max} = k_{cat}[E]_0$. This value is too small to indicate whether the initiation or elongation step is rate determining in RNA synthesis. At saturating concentrations of initiator and UTP, we have observed that the initiation of the poly(dA-dT)-directed RNA synthesis occurs in less than 0.5 s [see Shimamoto & Wu (1980)]. The rate constant for the elongation step in a similar system was estimated to be about 40 s^{-1} [Rhodes & Chamberlin (1974) [see also Krakow et al. (1976)]]. Thus, it appears that the rate-determining step in RNA synthesis under steady-state conditions is neither the initiation nor the elongation step but probably the product release or the enzyme liberation from the DNA template.

The poly(dA-dT)–RNA polymerase complex formed at a large excess of enzyme was observed to have one enzyme molecule per every 60 base pairs by electron microscopy (Williams, 1977). The length of DNA covered by RNA polymerase holoenzyme was estimated to be about 45–65 base pairs by protection against nuclease digestion (Ptashne et al., 1976). We have found, however, that the minimal length of poly(dA-dT) required for active RNA synthesis appears to be approximately 500 base pairs per active RNA polymerase molecule. Whether all 500 base pairs of DNA are actually needed for RNA synthesis is not clear. It is conceivable that the formation of an active enzyme–DNA complex at one site may induce a conformational change of the DNA some distance away from this site so as to prevent the formation of another such complex. Alternatively, part of poly(dA-dT) molecule may not be able to act as template for active synthesis due to some unique tertiary structure or due to occupation by inactive RNA polymerase molecules. Further investigations are necessary to clarify all these possibilities.

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