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Mechanism of Ribonucleic Acid Chain Initiation. Molecular Pulse-Labeling Study of Ribonucleic Acid Synthesis on T7 Deoxyribonucleic Acid Template†

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ABSTRACT: The mechanism of the productive initiation of RNA synthesis in vitro by *Escherichia coli* RNA polymerase holoenzyme was investigated by using DNA from the T7 deletion mutant ΔD111 as a template under conditions such that RNA chains are initiated exclusively from the A1 promoter. Kinetic studies by a non-steady-state method revealed that the binding of the first two nucleoside triphosphates, corresponding to the 5′-terminal and penultimate nucleotides of the RNA transcript, to the enzyme–promoter complex during the productive initiation is rapid and ordered, namely, ATP binds first followed by UTP. The same mechanism was also observed for productive initiation at the D promoter. The time course of the incorporation of the first four 5′-terminal nucleotides into the RNA synthesized from the A1 promoter was studied by a fast kinetic technique. Under our experimental conditions, the times for the half-maximal incorporation of ATP, UMP, CMP, and GMP, each at an initial concentration of 0.1 mM, were 0.03 (or less), 0.16, 0.35, and 0.40 s, respectively. These incorporations were completed within 1 s and maintained for up to 90 s at a constant level which was dependent on the initial nucleotide concentration. The half-saturation concentrations for ATP, UTP, CTP, and GTP were 80, 88, 20, and 7 μM, respectively. At saturating nucleotide concentrations, the level of incorporation was approximately equal to the promoter concentration. We have found that productive initiation at the A1 promoter is activated by the third nucleotide, CTP, and the fourth nucleotide, GTP, the former being a more potent activator than the latter. The observation that mono- and diphosphates of these two nucleotides are also activators of the productive initiation suggests that the activation is not due to the phosphodiester bond formation. Kinetic analysis indicates that the activator binds to a regulatory site on the enzyme–promoter complex and exerts its action at a step after the formation of the first phosphodiester bond. Furthermore, GTP inhibits the abortive initiation at the A1 promoter. A minimal mechanism of RNA chain initiation consistent with all our results is proposed. RNA polymerase binds to a promoter site to form an open-promoter complex. The binding of the first two nucleotides to this binary complex is rapid and ordered. A phosphodiester bond is then formed to yield a dinucleotide, with the release of pyrophosphate. In the presence of a regulatory nucleotide, corresponding to the third or fourth nucleotide, the translocation of the enzyme along the DNA template is facilitated to form a productive initiation complex which is ready for elongation of an RNA chain. In the absence of the regulatory nucleotide, the dinucleotide is released from the enzyme–DNA complex, and the initiation is aborted. Discrimination between the productive and abortive initiation pathways by a regulatory nucleotide may play an important role in the control of specific RNA synthesis and in the enhancement of the fidelity of transcriptional initiation.

Gene transcription by bacterial RNA polymerase can be divided into several steps (Goldthwait et al., 1970; Chamberlin, 1976): (a) binding of enzyme to a promoter on the DNA template, (b) initiation of RNA synthesis, (c) elongation of RNA chains, and (d) termination of RNA synthesis. Although a wealth of information has been accumulated in the past decade about prokaryotic gene transcription, we are still far from understanding the molecular mechanisms by which RNA polymerase carries out this complicated series of reactions.

The initiation of RNA synthesis involves the binding of both the 5′-terminal and the penultimate nucleoside triphosphates to the RNA polymerase–promoter complex as well as the formation of the first phosphodiesterase bond (Goldthwait et al., 1970). Earlier studies on initiation have indicated that the 5′-terminal nucleoside triphosphate is primarily a purine nucleotide (Maitra & Hurwitz, 1967) and that purine nucleotides are required at relatively higher concentrations for initiation than for elongation (Anthony et al., 1969). Thus initiation can be distinguished from elongation by the purine nucleotide requirement and by the relatively high apparent K_m derived from steady-state kinetic analysis (Goldthwait et

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al., 1970). This notion was supported by the demonstration by physical methods of an initiation site on the enzyme which preferentially binds purine nucleotides with a dissociation constant higher than that of the second nucletide site (elongation or polymerization site) which has an equal affinity for all nucleoside triphosphates (Wu & Goldthwait, 1969a,b). Detailed analysis of the mechanism of the initiation, however, was difficult because of the complexity and speed of the reaction.

An indirect method for analysis of the initiation reaction, called the "rifampicin-challenge" assay, was devised by Chamberlin's group (Mangel & Chamberlin, 1974; Rhodes & Chamberlin, 1974). In this assay, nucleoside triphosphates and the inhibitor rifampicin are added simultaneously to the enzyme–DNA complex. The rate of initiation is then calculated by the number of RNA chains synthesized under these conditions and the independently determined bimolecular rate constant of rifampicin binding to the enzyme–DNA complex. The rifampicin-challenge assay is based on the assumption that the bimolecular binding of rifampicin to RNA polymerase and the initiation reaction are two simple competitive reactions. It turns out that this assumption is an oversimplification because the inactivation of RNA polymerase by rifampicin is not caused by the bimolecular binding but rather is due to a conformational change of the enzyme following the binding (Yarbrough et al., 1976). Moreover, the rifampicin-challenge assay was further questioned (Johnston & McClure, 1976) by the finding that rifampicin does not inhibit the formation of the first phosphodiester bond in the abortive initiation catalyzed by RNA polymerase. Abortive initiation involves the catalytic synthesis of a dinucleoside tetraphosphate in the presence of only the first two nucleoside triphosphates at the 5′ terminus of an RNA transcript. After abortive initiation was first reported, its steady-state kinetics were promptly studied by using poly(dA-dT) (Oen & Wu, 1978; Hansen & McClure, 1979) and T7 DNA (Oen et al., 1979; McClure et al., 1978; Smagowicz & Scheit, 1977, 1978; Cech et al., 1980) as templates in order to yield information about the mechanism of RNA chain initiation. Nevertheless, from a mechanistic point of view, abortive initiation may be merely an alternative pathway that is ordinarily not followed in the presence of all four nucleoside triphosphates. There is no evidence, however, to indicate that abortive initiation and productive initiation proceed along the same kinetic pathway.

Another method used to study RNA chain initiation is the measurement of the incorporation of γ-^{32}P-labeled ATP or GTP into RNA products (Maitra & Hurwitz, 1967). Nierman & Chamberlin (1979, 1980a,b) have recently studied directly the productive initiations of RNA chains from the A1 and A2 promoters on T7 DNA by following the rates of [γ-^{32}P]ATP or [γ-^{32}P]GTP incorporation in the presence of low concentrations of all four nucleoside triphosphates. However, since RNA polymerase can catalyze reactions beyond the initiation step in the presence of all four nucleoside triphosphates even at low concentrations, it is not clear whether the kinetics of [γ-^{32}P]ATP or [γ-^{32}P]GTP incorporation actually represents the initiation step or some steps which take place later in RNA synthesis (e.g., subsequent translocation or phosphodiester bond formation). In order to clarify this point, it is essential to resolve the initiation from other elementary steps of the RNA polymerase reaction on the time axis by using fast kinetic techniques.

We have developed a method to determine the order of binding of the first two nucleoside triphosphates in RNA synthesis by non-steady-state kinetic analysis (Shimamoto & Wu, 1980a) and to perform a real time measurement of product initiation by fast kinetic techniques (Shimamoto & Wu, 1980b). The principle of this method is analogous to a pulse-labeling experiment in which a radioactive nucleotide is incorporated for short but varying time intervals into the initiation complex (enzyme–promoter–dinucleoside tetraphosphate complex), which is then elongated into an RNA chain by addition of all four unlabeled nucleoside triphosphates. This method was applied to the study of the kinetic mechanism of RNA chain initiation on a synthetic poly(dA-dT) template. The results revealed that the order of substrate binding in the initiation reaction is ATP first, followed by UTP. These binding steps are faster than those which may be attributed to the formation of the first phosphodiester bond and the subsequent translocation. Furthermore, the kinetic data obtained indicated that in addition to being a substrate, UTP can bind to a regulatory site on the enzyme–template complex to activate the productive initiation.

In this paper, we report the application of this molecular pulse-labeling method to the investigation of productive initiation on native T7 DNA template. The kinetic mechanism thus obtained is compared with that of the productive initiation on synthetic poly(dA-dT) template. In addition, we propose a possible kinetic mechanism of RNA chain initiation by which the specific RNA synthesis and the fidelity of chain initiation are modulated by a regulatory nucleotide through a discrimination between the abortive and productive initiation pathways.

Materials and Methods

RNA Polymerase. RNA polymerase was purified from *E. coli* K12 cells (Grain Processing Corp.) according to the procedure of Burgess & Jendrisak (1975). The enzyme isolated was more than 95% pure, and its σ subunit content was about 70%, as shown by $NaDodSO_4$–polyacrylamide gel electrophoresis. This enzyme preparation was then chromatographed on phosphocellulose in the presence of 50% glycerol (Gonzalez et al., 1977) to isolate polymerase holoenzyme containing a full equivalent of σ subunit. RNA polymerase activity was assayed by the incorporation of ^{3}H-labeled ribonucleoside triphosphate into acid-insoluble material as described previously (Wu & Wu, 1973). The specific activity of RNA polymerase determined by the standard assay using calf thymus DNA as template was 1200 units/mg of protein, where 1 unit of enzyme was defined as the amount of enzyme which incorporates 1 nmol of ^{3}H-labeled nucleotide in 20 min at 37 °C.

DNA and Nucleotides. T7 ΔD111 DNA was prepared as described by Hillel & Wu (1978) except that the phage was grown in *E. coli* C at 37 °C in the medium of Fraser & Jerrel (1953) in a microfermentor and infection was carried out at A_{550} = 5 with a multiplicity of infection of 0.3. The ΔD111-deletion T7 phage strain was kindly provided by F. W. Studier of Brookhaven National Laboratory.

Ultrapure nucleoside triphosphates were purchased from ICN Pharmaceuticals, Inc. ATP and UTP from Sigma were also used after further purification by DEAE-cellulose chromatography. ^{3}H-Labeled nucleotides were obtained from Schwarz/Mann or New England Nuclear, and their purity was checked by PEI-F cellulose thin-layer chromatography (J. T. Baker Chemical Co.) before use.

Manual Mixing Experimentrs. Non-steady-state kinetic analysis to determine the order of binding of the first two substrates in RNA synthesis (see Scheme I) was carried out with manual mixing. In a typical experiment, a solution containing enzyme, DNA, and unlabeled first nucleotide

(initiator) was preincubated at 37 °C for 2 min and then mixed with an equal volume of solution containing labeled second nucleotide to form an initiation complex, i.e., the enzyme–promoter–dinucleoside tetraphosphate complex (step 1). After incubation for 1.5 min at the same temperature, an equal volume of solution containing all four unlabeled nucleotides was added to elongate the dinucleoside tetraphosphate into an RNA chain (step 2). The unlabeled second nucleotide added was in large excess (by more than 2 orders of magnitude) to dilute out the labeled second nucleotide present. The elongation reaction was allowed to proceed for 2 min at 37 °C. The RNA synthesized was precipitated with 10% trichloroacetic acid for 30 min at 0 °C and then filtered on a glass-fiber filter. The filter was dried and counted in a liquid scintillation system. In a control experiment, unlabeled rather than labeled second nucleotide was present in step 1, while labeled second nucleotide with the same final specific activity as in the original experiment was added in step 2. The amount of acid-precipitable radioactivity measured in the control experiment was used to correct for the amount of labeled nucleotide incorporation taking place during the elongation step. All solutions used in these studies contained 50 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10 mM $MgCl_2$, and 0.2 mM dithiothreitol (buffer A). All reactions were carried out at 37 °C.

Fast Kinetic Experiments. A multimixing apparatus (see Figure 3) was used for rapid kinetic measurements of the incorporation of individual nucleotides into RNA initiated at a specific promoter. The construction of the apparatus and the experimental details have been described in a previous report (Shimamoto & Wu, 1980b). In a typical experiment, the RNA polymerase–promoter complex, in the absence or presence of some nucleotides, was rapidly mixed with a labeled nucleotide at the first mixing jet. The product of this first reaction was "aged" for various time intervals by passage through a delay line and then mixed at the second mixing jet with other unlabeled nucleoside triphosphates to elongate RNA chains. The reaction time of the first reaction was therefore the time elapsed during the flow of reaction mixture from the first mixing jet to the second. The nitrogen pressure used to drive the plunger of the sample syringes and the length of the delay line were adjusted to allow for the expected reaction time. The dead time of this system was determined to be 30 ms. Reduction of this dead time can be achieved by increasing the flow speed but results in DNA shearing which causes a significant end addition reaction (Nath & Hurwitz, 1974) at nicks and breaks. The final reaction mixture was collected in a thermostated collecting syringe, transferred to a test tube, and incubated for 2 min at 37 °C. The RNA formed was precipitated by adding 10% cold trichloroacetic acid and allowing the mixture to stand at 0 °C for 0.5–2 h before it was filtered through a glass-fiber filter. The filter was then dried, and the radioactivity incorporated into acid-precipitable RNA was determined by liquid scintillation. The buffer system used in the rapid mixing experiments was identical with that used in the manual mixing experiments. When the RNA polymerase concentration used was less than 5 μg/mL, bovine serum albumin (50 μg/mL) was added to the reaction mixture to prevent adsorption of the enzyme to the syringe wall and test tubes.

Abortive Initiation Reaction. The procedure used for these reactions was a modification of that described by Oen et al. (1979). Fifty microliters of solution I, containing specified concentrations of RNA polymerase and ΔD111 DNA in buffer A, was preincubated at 37 °C for 2 min before mixing with 50 μL of solution II, containing in the same buffer 0.2 mM each of initiator (ATP or CpA) and [^{3}H]UTP (500–1500 cpm/pmol) with or without 0.2 mM GTP. The resultant mixture was incubated for another 30 min at 37 °C. The reactions were stopped by chilling the mixture on ice and adding EDTA to a final concentration of 50 mM.

For analysis of the pppApU or CpApU formed, 10-μL aliquots of the reaction mixtures were streaked onto 0.5-in.-wide strips of Whatman 3MM paper, which were developed by ascending chromatography with WASP solvent (Johnston & McClure, 1976). The chromatograms were then air-dried, cut into 1-cm strips, and counted in Econofluor (New England Nuclear) in a liquid scintillation counter.

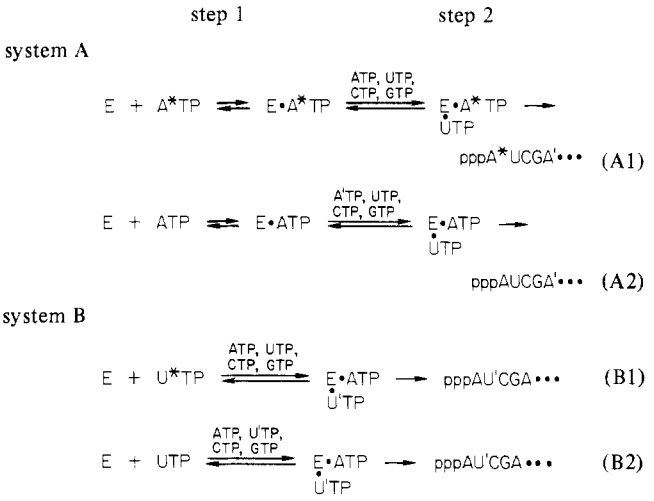
Electrophoretic Analysis of RNA Transcripts. RNA transcripts were prepared under conditions similar to those employed for the initiation reactions. RNA polymerase (5.5 or 55 nM), ΔD111 DNA (2.3 nM), and 0.1 mM each of ATP, CTP, GTP, and [^{3}H]UTP were incubated in buffer A for 30 min at 37 °C. The reaction was stopped by adding a solution containing 0.1 M EDTA, 0.1% $NaDodSO_4$, 27 mM Tris, 27 mM boric acid, 0.1% bromphenol blue, and 30% glycerol. A portion of the reaction mixture was subjected to electrophoresis on 2% acrylamide–0.5% agarose slab gels as described by Peacock & Dingman (1968). The gel was cut into 0.5-cm slices which were dried onto Whatman 3MM paper and counted by liquid scintillation.

Results

Order of Binding of the First Two Nucleotides in the Initiation of RNA Synthesis. Studies on the kinetic mechanism of RNA chain initiation may be hindered by the complexity of the promoter structure of DNA template because an unambiguous interpretation of the kinetic data requires that all RNA chains initiate synchronously at a single promoter on the template. To overcome this difficulty, we have carried out studies using as a template ΔD111 DNA isolated from a deletion mutant of T7 phage, which contains only one major promter A1, and three minor promoters, C, D, and E (Studier, 1975). The 5′-terminal sequence for the RNA synthesized from the A1 promoter is pppApUpCpGpA (Kramer et al., 1974; Siebenlist, 1979) and that from the C promoter is pppApCpApGpG (McConnell, 1979). From the results of single-step addition reactions with T7 DNA as template, D promoter transcript is suggested to start with pppGpUpU (Oen et al., 1979; Cech et al., 1980). The sequence of RNA arising from the E promoter is not known, but this promoter is transcribed only at salt concentrations lower than those used in this study (Stahl & Chamberlin, 1977) and thus is not considered here.

There are three possible kinetic mechanisms (two ordered and one random) by which the first two nucleoside triphosphates may bind to the promoter–enzyme complex during the initiation of RNA synthesis. For example, in the RNA chain initiation at A1 promoter, either ATP may bind first, or UTP may bind first, or both ATP and UTP may have random access to the promoter–enzyme complex. These three possibilities can be distinguished by a non-steady-state method developed recently (Shimamoto & Wu, 1980a). This method measures the incorporation of the first or the second nucleotide into the initiation complex, which is subsequently elongated into an RNA chain without enzyme turnover or reinitiation. Scheme I illustrates the reactions that would take place for the ordered mechanism in which ATP binds first. In experiment A1 (system A), labeled ATP is first incubated with the promoter–enzyme complex (step 1), and then the initiation and elongation of RNA chains are allowed to occur by addition of the other three nucleoside triphosphates (step 2). At the

Scheme I: Experimental Systems To Determine the Order of Substrate Binding during Initiation at the A1 Promoter[a]



[a] The systems presented are based on the assumption of an ordered mechanism in which ATP binds first and UTP binds next in RNA chain initiation at the A1 promoter. E is the RNA polymerase holoenzyme–T7 DNA (ΔD111) complex. The asterisk denotes labeled nucleotide with high specific activity, and the prime denotes labeled nucleotide whose specific activity has been reduced by unlabeled nucleotide. The RNAs illustrated represent the products which are synthesized in the first round of catalysis without any exchange of labeled nucleotides in the RNA with those in the bulk solution. For determination of the order of binding of the first two nucleotides (GTP and UTP) during RNA chain initiation at the D promoter, labeled ATP in system A is replaced with labeled GTP.

same time, a large excess of unlabeled ATP is also added to minimize the incorporation of labeled AMP or ATP due to the elongation or reinitiation. Even with such precautions, there will still be a finite amount of labeled AMP incorporation in step 2. This can be quantitated by control experiment A2 in which unlabeled ATP is incubated with the enzyme–promoter complex in step 1, while labeled ATP is added in step 2 at the same concentration and specific activity used in experiment A1. Thus the difference obtained by subtracting the radioactive nucleotide incorporation into RNA product in experiment A2 from that in experiment A1 is denoted as the "the net labeled nucleotide incorporation" and represents the amount of first ATP incorporated into RNA chains (without reinitiation). If labeled UTP is incubated first with the promoter–enzyme complex and then all four unlabeled nucleoside triphosphates are added (system B), labeled UMP incorporation will occur only during the elongation step, because no E·UTP complex will be formed according to the ordered mechanism assumed here. Therefore, the net labeled nucleotide incorporation will be finite in system A whereas it will approach zero in system B. On the contrary, if the mechanism is ordered with UTP binding first, the net labeled nucleotide incorporation in system B will be finite while that in system A will approach zero. Both systems A and B will yield finite net labeled nucleotide incorporations in the random access mechanism (Shimamoto & Wu, 1980a). The experimental results shown in Table I are in accord with the ordered mechanism in which ATP binds first, followed by UTP, to the A1 promoter–enzyme complex during the initiation of RNA chain.

In a similar manner, the order of binding of the first two nucleotides (GTP and UTP) in RNA chain initiation at the D promoter was determined by replacing labeled ATP with labeled GTP in system A. We found that a significant net labeled GTP incorporation in system A was observed only at

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

expt	system	[T7 DNA] (nM)	[RNA polymerase] (nM)	labeled nucleotide in step 1	net labeled nucleotide incorporation (nM)
1	A	2.3	5.5	ATP	1.9
2	B	2.3	5.5	UTP	0.2
3	A	2.3	5.5	GTP	0.2
4	A	2.3	11	GTP	0.4
5	A	4.6	22	GTP	2.4
6	B	4.6	22	UTP	0.4

[a] The concentration of ΔD111 DNA was determined by optical absorption at 260 nm by using a value of $\epsilon_{1\%} = 200$ and a mol wt of DNA of 2.4×10^7. The concentration of RNA polymerase holoenzyme was calculated by using a mol wt of 5×10^5. The DNA–enzyme complex was preincubated for 2 min at 37 °C. In all experiments, the reaction volumes were 120 and 420 μL for step 1 and step 2, respectively. In step 1, the concentration of labeled nucleoside triphosphate added was 100 μM. After incubation for 1.5 min at 37 °C, 6.1 mM of the same but unlabeled nucleotide was added to the reaction mixture together with 100 μM each of the other three nucleoside triphosphates. Elongation of the RNA chains (step 2) was allowed to occur for 2 min at 37 °C. The reaction was stopped by addition of 20 volumes of cold 10% trichloroacetic acid to 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. The net labeled nucleotide incorporation was calculated as described in the text.

high enzyme and DNA concentrations with a large excess of enzyme over DNA and that the net labeled UMP incorporation in system B was neglibible (Table I). Thus, while transcription of the D promoter occurs only at higher concentrations of RNA polymerase and DNA, with a larger enzyme to DNA ratio than those needed for transcription of the A1 promoter, the mechanisms of initiation of RNA synthesis at these two promoters are identical. The order of nucleotide binding is in accord with the 5′ sequence of the RNA transcript.

The absence of transcription of the D promoter at lower enzyme to DNA ratio was confirmed by electrophoretic analysis of RNA products on acrylamide–agarose (2%–0.5%) gels. When 2.3 nM ΔD111 DNA and 5.5 nM RNA polymerase were incubated with all four nucleoside triphosphates (each at 0.1 mM and UTP labeled with tritium) for 30 min at 37 °C, an RNA species corresponding to the A1 transcript (mol wt 2.25×10^4) was the major product. RNA products equivalent to the D and C transcripts were seen only when the concentration of RNA polymerase was increased to 55 nM. These observations were similar to those made previously by Stahl & Chamberlin (1977).

Selective Initiations at the A1 and D Promoters As Determined by the Incorporation of Second Nucleotide. Since both A1 and D promoters require UTP as the second substrate, the selective initiation at these two promoter sites can also be studied by measuring the net incorporation of labeled UMP into the productive initiation complex as illustrated in Scheme II. In step 1, the RNA polymerase–ΔD111 DNA complex and labeled UTP are incubated with CpA, the initiator for the A1 promoter (system C), or with GTP, the initiator for the D promoter (system D), to form CpApU* or pppGpU*pU*, respectively. In step 2, an excess amount of unlabeled UTP as well as other necessary nucleoside triphosphates is then added to elongate RNA chains. It is possible that the formation of the first phosphodiester bond may be followed by a translocation or a conformational change of the enzyme, thereby producing several intermediates in step 1. The in-

Scheme II: Experimental Systems To Determine the Selective Initiation at the Promoters A1 (Systems C) and D (System D)[a]

step 1 step 2

system C

$$E + CpA + U^{*}TP \longrightarrow E{\cdot}CAU^{*} \xrightarrow{UTP,\ ATP,\ CTP,\ GTP} CAU^{*}{\cdot}{\cdot}U'{\cdot}{\cdot} \quad (C1)$$

$$E + CpA + UTP \longrightarrow E{\cdot}CAU \xrightarrow{U'TP,\ ATP,\ CTP,\ GTP} CAU{\cdot}{\cdot}U'{\cdot}{\cdot} \quad (C2)$$

system D

$$E + GTP + 2U^{*}TP \longrightarrow E{\cdot}GU^{*}U^{*} \xrightarrow{UTP,\ ATP,\ CTP,\ GTP} pppGU^{*}U^{*}{\cdot}{\cdot}U'{\cdot}{\cdot} \quad (D1)$$

$$E + GTP + 2UTP \longrightarrow E{\cdot}GUU \xrightarrow{U'TP,\ ATP,\ CTP,\ GTP} pppGUU{\cdot}{\cdot}U'{\cdot}{\cdot} \quad (D2)$$

[a] E in system C represents the RNA polymerase–A1 promoter complex whereas in system D it represents the RNA polymerase–D promoter complex. In system C, CpA is used as an initiator instead of the natural initiator ATP. The asterisks and primes have the same meaning as described in Scheme I, Only RNAs synthesized in the first round and without exchange of their labeled nucleotides are shown in the scheme.

termediates which are ready for immediate elongation in step 2 are defined as the initiation complexes. The amount of initiation complexes formed can be determined by the net labeled UMP incorporation in system C (experiments C1 and C2) or in system D (experiments D1 and D2).

Figure 1 shows the dependence of the formation of initiation complex (normalized for the amount of A1 promoter) on the enzyme concentration when both CpA and GTP are present as initiators. In these experiments, the concentrations of the A1 and D promoters were kept constant at 2.3 and 6.9 nM, respectively. In both cases, biphasic curves were observed, with the inflection points occurring at the same molar ratio of enzyme of DNA (2.4). The slope of the curve following the inflection point is steeper for the higher DNA concentration than that for the lower DNA concentration. These curves are consistent with the observations described above in that the A1 promoter is transcribed mainly at the lower DNA concentration and the D promoter is transcribed only at the higher DNA concentration with a high enzyme to DNA ratio (Table I). When the DNA concentration is low (2.3 nM), the curve almost levels off after the inflection point with the molar ratio of enzyme to DNA = 2.4. This apparent stoichiometric point suggests that only 40% of the enzyme molecules are capable of initiating RNA chains at the A1 promoter, which represents the fraction of active RNA polymerase molecules in our enzyme preparation. A similar value was reported previously for the fraction of active RNA polymerase molecules in the poly(dA-dT) system (Shimamoto & Wu, 1980a).

When the concentration of DNA was increased from 2.3 to 6.9 nM at an enzyme to DNA ratio ≤2.4 (Figure 1), the number of initiation complexes formed decreased to about half, indicating that the formation of initiation complexes was affected by the absolute enzyme or DNA concentration. It has been reported (Smagowicz & Scheit, 1978; McClure et al., 1978) that the abortive initiation on the wild-type T7 DNA shows a positive cooperativity with respect to the enzyme concentration. We have also observed a similar effect for the abortive initiation with ΔD111 DNA template (data not shown). Thus the decrease in the productive initiation observed here could be due to the enhancement of abortive initiation at higher enzyme concentrations. At high DNA concentration (6.9 nM) and high enzyme to DNA ratio (>2.4), an increase

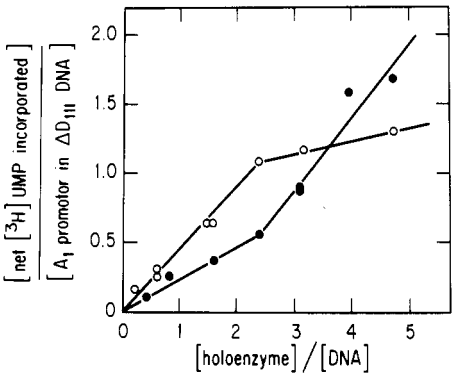


FIGURE 1: Dependence of the net [^{3}H]UMP incorporation on the concentration of RNA polymerase in systems C and D (Scheme II). Varying concentrations of RNA polymerase were preincubated with a fixed concentration of ΔD111 DNA, either at 2.3 nM (○) or at 6.9 nM (●). Initiators for both the A1 promoter, CpA (100 μM), and the D promoter, GTP (100 μM), were added with the second nucleotide, [^{3}H]UTP (100 μM), to the enzyme–DNA complex (step 1); 50 μM (final concentration) each of ATP, GTP, and CTP and 6.1 mM (final concentration) of UTP were then added to elongate RNA chains (step 2). Other experimental conditions were the same as those described in Table I. The net [^{3}H]UMP incorporation, normalized for the amount of A1 promoter, is plotted against the molar ratio of RNA polymerase holoenzyme to ΔD111 DNA.

in the net [^{3}H]UMP incorporation was observed. This clearly is due to transcription of the D promoter as indicated by the appearance of D transcript under such conditions in the electrophoretic analysis described before.

On the basis of the above results, it is possible to construct a transcription system at lower DNA concentrations (2.3 nM) where only the A1 promoter is functioning on ΔD111 DNA. Under these conditions, RNA polymerase forms a tight open-promoter complex only with the A1 promoter (apparent dissociation constant $< 10^{-9}$ M) but not with the minor promoters (apparent dissociation constant $> 10^{-9}$ M). The concentration of the initiation complexes thus formed is almost equal to the concentration of A1 promoter present in the system.

Activation of Productive Initiation by the Third and Fourth Nucleotides. In the initiation studies with poly(dA-dT) template (Shimamoto & Wu, 1980a), we observed a positive cooperativity in the dependence of productive initiation on the concentration of the second nucleotide UTP, suggesting that an additional UTP molecule binds to a regulatory site on the enzyme–DNA complex and acts as an activator. No such cooperativity was observed in the dependence of productive initiation of the A1 promoter on the concentration of UTP as evidenced by the linear Edie-type plot (Figure 2). A similar analysis of the A1 promoter system indicates that the dependence of the net [^{3}H]UMP incorporation on the first nucleotide ATP is also noncooperative (data not shown). However, we have found that nucleotides corresponding to those located at the third or fourth position in the 5′-terminal RNA sequence can serve as activators of productive initiation. In the experimental system of Figure 1, both CpA and GTP were incubated with the DNA–enzyme complex and [^{3}H]UTP in step 1. If GTP was eliminated from this step, a substantial decrease in the net [^{3}H]UMP incorporation was observed even at low enzyme and DNA concentrations. Table II shows that the presence of 50–100 μM GTP in step 1 resulted in 2–5-fold increase in the net [^{3}H]UMP incorporation as compared to that in the absence of GTP. This increase in the net [^{3}H]UMP incorporation in the presence of GTP was not due to initiation at the D promoter because (a) the amount of the net [^{3}H]-UMP incorporation (2.1 nM) was almost equal to that of the

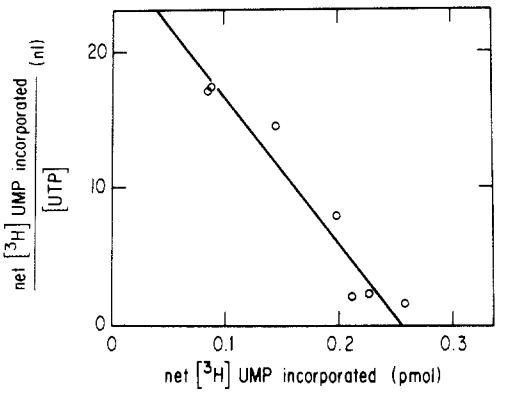


FIGURE 2: Dependence of the net [^{3}H]UMP incorporation in system C on the concentration of [^{3}H]UTP in step 1. The concentrations of ΔD111 DNA and RNA polymerase homoenzyme were 6.9 and 11 nM, respectively. Other experimental conditions were the same as in Figure 1 except that in step 1 CpA was the only initiator present (GTP was omitted) and the concentration of [^{3}H]UTP was varied. The net [^{3}H]UMP incorporation measured, n, was analyzed by the Edie-type equation (Shimamoto & Wu, 1980a), $n/[UTP^*] = (n_{max} - n)/K_d$, where n_{max} is the maximal net incorporation of [^{3}H]UMP into the RNA product, [UTP*] is the concentration of [^{3}H]UTP added to the enzyme–DNA complex in step 1, and K_d is the apparent dissociation constant of the initiation complex. The solid line was obtained by a least-squares analysis of the data, and the apparent dissociation constant calculated was 9.4 μM. The estimated error for the net [^{3}H]UMP incorporation was about 5–10%.

Table II: Activation of the Net [^{3}H]UMP Incorporation in System C[a]

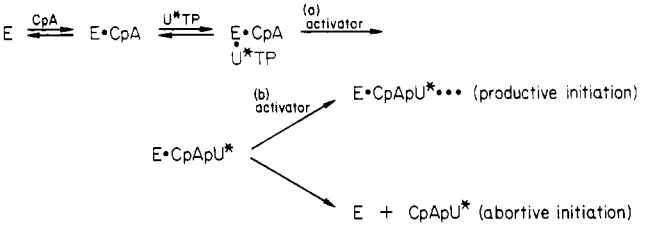
activator added in step 1	concentration (μM)	(net [^{3}H]UMP incorporation with activator)/(net [^{3}H]UMP incorporation without activator)
GTP	50	2.5
GTP	100	4.7
GDP	100	2.5
GMP	100	1.7
CDP	100	2.6
CMP	100	1.8
ATP[b]	100	1.0

[a] In step 1, [DNA] = 2.3 nM, [haloenzyme] = 5.5 nM, and CpA was replaced by 100 μM ATP. [b] CpA (100 μM) was used as the initiator in step 1.

net [^{3}H]ATP incorporation (1.9 nM) and (b) essentially no net [^{3}H]GTP incorporation was detected under these experimental conditions (Table I, experiment 3). The effect of the third nucleotide, CTP, on the initiation at A1 promoter could not be examined by the manual mixing experiments because CTP could react covalently with pppApU to form pppApUpC. However, we found that CDP and CMP, which did not incorporate into the RNA product, could activate productive initiation at the A1 promoter. GDP and GMP were also activators, and the order of the magnitudes of activation was GTP > CDP > GDP > CMP > GMP. These results suggest that the thrid and fourth nucleotides are activators of the productive initiation and that the nucleotide base may be the essential structure responsible for the activation. The observation that UTP is an activator of productive initiation in the transcription of poly(dA-dT) template may be explained by the fact that it is the fourth as well as the second substrate. No activation of the productive initiation by ATP was observed when CpA was used as the initiator.

Mechanism of Activation by the Third and Fourth Nucleotides. Scheme III shows a plausible kinetic pathway of RNA chain initiation at the A1 promoter. After formation of the first phosphodiester bond between the initiator CpA and

Scheme III: Two Possible Mechanisms (a and b) for the Activation of the Initiation at the A1 Promoter[a]



[a] E represents the RNA polymerase–A1 promoter complex.

Table III: Inhibition of Abortive Initiation at the A1 Promoter by GTP[a]

initiator	RNA polymerase (nM)	ΔD111 DNA (nM)	CpApU* or pppApU* released (μM) −GTP	CpApU* or pppApU* released (μM) +GTP	inhibition by GTP (%)
ATP	2.5	2.3	3.8	2.3	39
CpA	2.5	6.9	0.6	0.4	33
CpA	7.5	6.9	3.7	2.9	22

[a] The procedures for the abortive initiation assay and the analysis of CpApU and pppApU are described under Materials and Methods.

the second nucleotide UTP, the CpApU formed can either be released from the enzyme–promoter complex (abortive initiation) or be elongated into an RNA chain (productive initiation). The assumption that both productive and abortive initiation are derived from a common intermediate is based on our previous finding that the order of the first two substrates to the enzyme–template complex in the productive initiation [Shimamoto & Wu (1980) and results described above] is identical with that in the abortive initiation (Smagowicz & Sheit, 1977, 1978; McClure & Cech, 1978; McClure et al., 1978). There are two possible mechanisms by which the third or fourth nucleotide may activate the productive initiation. The activator may either stimulate the formation of the first phosphodiester bond (mechanism a) or act at a later step in the kinetic pathway, leading to productive initiation (mechanism b). These two mechanisms can be distinguished by the effect of the third or fourth nucleotide on abortive initiation: the abortive initiation will be enhanced in mechanism a whereas it will be inhibited or unaltered in mechanism b. Our results showed that GTP exerted a significant inhibition of the abortive initiation at the A1 promoter by using either ATP or CpA as the initiator (Table III), which clearly favors mechanism b.

Rapid Kinetic Studies of the Incorporation of Individual Nucleotides into RNA. In the manual mixing experiments described in systems A and C, the formation of the initiation complexes was allowed to proceed for 0.5–2.0 min during which time a stationary state was attained (step 1). Since the net labeled nucleotide incorporation measures how much initiation complexes are formed during step 1, one can study the kinetics of the formation of initiation complexes by varying the duration of this step by using a multimixing apparatus. This type of experiment has been carried out successfully for studying the kinetics of incorporation of the second nucleotide into RNA synthesized on the poly(dA–dT) template (Shimamoto & Wu, 1980b).

Figure 3 illustrates the experimental design of the rapid kinetic study of the incorporations of individual nucleotides into RNA synthesized from the A1 promoter on ΔD111 DNA template. In system a, the preformed A1 promoter–RNA

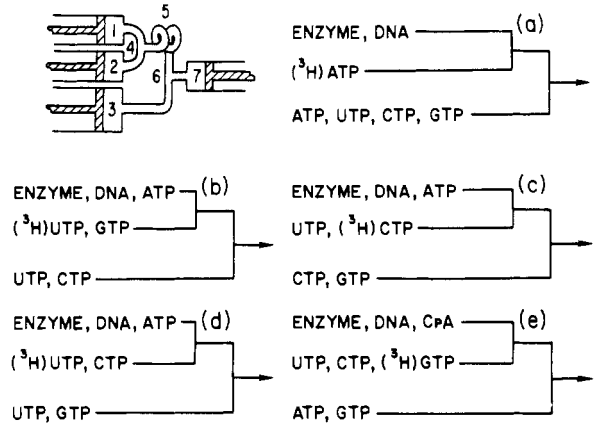


FIGURE 3: Multimixing apparatus and rapid kinetic experiments to measure the time course of individual nucleotide incorporation into RNA initiated at the A1 promoter. A simplified illustration of the rapid mixing apparatus is given at the upper left corner. Essential parts of the apparatus are three sample syringes (1, 2, and 3), two mixing jets (4 and 6), a delay line (5), a collecting syringe (7). The sequence of mixing and other experimental procedures are described in the text and under Materials and Methods. Experimental systems a–e show the contents of syringes 1, 2, and 3 connected by the direct flow lines. These systems were designed to measure the kinetics of the incorporation of the first four nucleotides, ATP (a), UMP (b and d), CMP (c), and GMP (e), into RNA initiated at the A1 promoter. System a is equivalent to experiment A1 shown in Scheme I. System b is equivalent to experiment C1 shown in Scheme II except that the initiator CpA is replaced by ATP. Control experiments equivalent to experiments A2 and C2, which are not shown in this figure, were also performed to determine the net labeled nucleotide incorporations. Systems b and d measure the net incorporation of the second nucleotide, [³H]UMP, in the presence of GTP and CTP, respectively.

polymerase complex (in syringe 1) was first mixed (at mixing jet 4) within a few milliseconds with labeled first nucleotide, [³H]ATP (in syringe 2). The reaction mixture was allowed to stand in the delay line (5) for varying time periods which are denoted as the reaction times. The mixture was then rapidly mixed (at mixing jet 6) with a large excess of unlabeled ATP plus the three other nucleoside triphosphates (in syringe 3) to elongate RNA products, which were collected (in collecting syringe 7) and precipitated by addition of cold trichloroacetic acid. A plot of the net [³H]ATP incorporation into acid-precipitable RNA product vs. the reaction time is shown in Figure 4 (curve a). After the dead time of the multimixing apparatus (approximately 30 ms), a constant level of [³H]ATP incorporation was observed over the whole range of reaction times examined up to 90 s. This time course indicates a rapid ATP binding to the promoter–enzyme complex, which is completed in less than 30 ms. From the dead time and the concentration of [³H]ATP used in this experiment, the lower limit of the second-order rate constant for ATP binding was estimated to be about 10^5 M^{-1} s^{-1}.

In studying the kinetics of the second nucleotide incorporation (system b, Figure 3), the fourth nucleotide, GTP (100 μM), was added as an activator together with ATP and [³H]UTP in the first mixing to produce a sufficient amplitide of the [³H]UMP incorporation. The time course observed (curve b, Figure 4) indicates a time lag and can be analyzed by a two-exponential approximation having one negative term and one positive term with relaxation times of 110 and 120 ms, respectively. A similar time course with two shorter relaxation times (43 and 98 ms) was prevously reported for the incorporation of the second nucleotide in the poly(dA–dT)-dependent RNA synthesis (Shimamoto & Wu, 1980b). In the same manner, the kinetics of the incorporation of the third (CMP) and fourth (GMP) nucleotides were also studied (Figure 4, curves c and e). In the experiment to determine

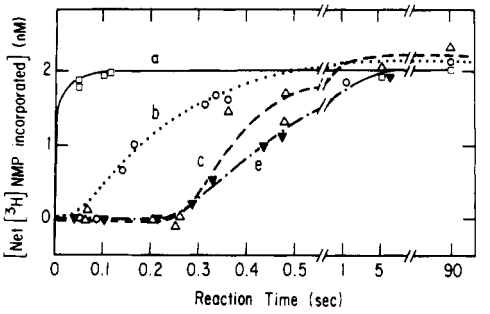


FIGURE 4: Time courses of the net incorporation of the first four nucleotides into RNA initiated the A1 promoter. Curves a, b, c, and e show the time courses of net labeled ATP (□), UMP (○), CMP (△), and GMP (▼) incorporations, respectively, as measured in the corresponding experimental systems (a, b, c, and e) given in Figure 3. In all experiments, the concentration of RNA polymerase was 5.5 nM, and the concentration of ΔD111 DNA was 2.3 nM. The concentrations of all nucleotides present in the first mixture, including the labeled nucleotide, were 100 μM. In the second mixing, the concentrations of all unlabeled nucleotides were 50 μM except that the one identical with the labeled nucleotide used in step 1 of the same experiment was 6.1 mM. Curve b was obtained by a least-squares fit of the data to a two-exponential equation (Shimamoto & Wu, 1980b), with two relaxation times, 110 and 120 ms. Other curves were drawn arbitrarily.

incorporation of the fourth nucleotide, CpA was used as the initiator to avoid the complication that might be caused by the presence of ATP which is also the fifth nucleotide. Comparison of these four time courses reveals a temporal sequence of nucleotide incorporations characterized by an increased time lag in each step in the order A < U < C < G. The times for the half-maximal incorporations of ATP, UMP, CMP, and GMP were 0.03 (or less), 0.16, 0.35, and 0.40 s, respectively. This is in accord with the transcription sequence of the A1 promoter. It should be noted, however, that a strict comparison between the time course of the fourth nucleotide (curve e) and the other three curves (a, b, and c) is not possible because the use of different initiators (CpA vs. ATP) could affect the time course of incorporation.

Activation of [³H]UMP Incorporation by CTP. As described before, the incorporation of the second nucleotide into RNA synthesized at the A1 promoter was stimulated by the presence of the fourth nucleotide, GTP. The stimulation by the third nucleotide, CTP, could not be examined by manual mixing experiments because of the possibility of a covalent bond formation between pppApU and CTP. This complication can be avoided by rapid kinetic studies. A multimixing experiment (system d in Figure 3) in which CTP is present during the formation of pppApU* in the first mixing was designed for this purpose. The result is shown in Figure 5. Comparison of the time courses of [³H]UMP incorporation in the presence of CTP (curve d) with that in the presence of GTP (curve b) indicates that the former rises earlier than the latter. At the reaction time of 0.1 s, the net [³H]UMP incorporation was about 1 nM in the presence of CTP but was less than 0.3 nM in the presence of GTP. Thus, the third nucleotide, CTP, is a more potent activator of the productive initiation than the fourth nucleotide, GTP.

We showed earlier that GTP activates the productive initiation at a step after the formation of the first phosphodiester bond (mechanism b, Scheme III). To test whether CTP activates the same step as GTP does, we have examined the concentration dependence of the rapidly increasing phase of the net [³H]UMP incorporation in the presence of CTP (curve d). When the concentration of [³H]UTP in this experiment was decreased by an order of magnitude to 10 μM, the amount of the noncovalent E·ATP·UTP complex formed and thereby

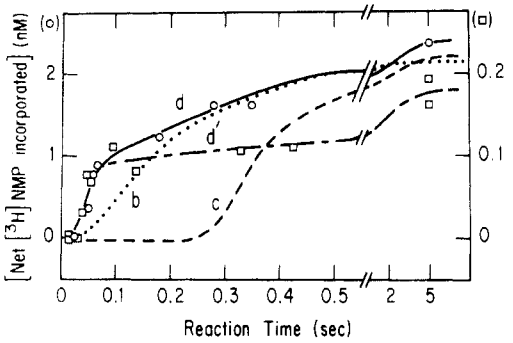


FIGURE 5: Activation of the net [^{3}H]UMP incorporation by the third nucleotide, CTP. The experimental system used to measure the effect of CTP on the net [^{3}H]UMP incorporation (○) (curve d) was described in Figure 3 (system d). The experimental conditions for system d were the same as those for system c (Figure 4) except that 100 μM of labeled UTP (instead of CTP) was present during the first mixing and that, in the second mixing, [ATP] = [UTP] = [GTP] = 50 μM and [CTP] = 6.1 mM. Curve d' (□) also shows the time course of the net [^{3}H]UMP incorporation in the presence of CTP, but the concentration of [^{3}H]UTP in the first mixing was decreased to 10 μM. Other conditions were identical with those of system d. The lines connecting the data points were drawn arbitrarily. For comparison, curves b (···) and c (- - -) from Figure 4 are also included in this figure.

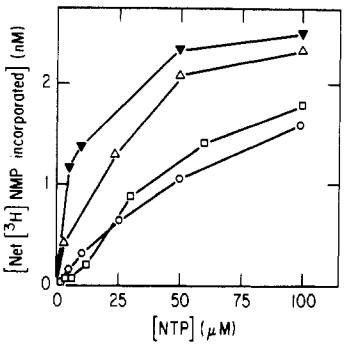


FIGURE 6: Concentration dependence of the net incorporation of the first four nucleotides into RNA synthesized at the A1 promoter. The experimental systems for measuring the net incorporation of the first four nucleotides, ATP (□), UMP (○), CMP (△), and GMP (▼), were shown in Figure 3 except that the solutions were mixed manually. The concentration of labeled nucleotide in syringe 2 was varied as indicated on the abscissa. The concentrations of other nucleotides, RNA polymerase, and ΔD111 DNA were the same as described in Figure 4. The incubation time after the first mixing (reaction time) was 90 s at 37 °C. After the second mixing, the reaction mixture was further incubated for 2 min at 37 °C to elongate RNA chains before acid precipitation and determination of radioactivity incorporation. Control experiments (analogous to experiment A2 in system a, Scheme I) were performed in each system to give the net labeled nucleotide incorporations. At saturating nucleotide concentrations, the maximal labeled nucleotide incorporation was approximately equal to the amount of A1 promoter present in the system. The half-maximal incorporations of ATP, UMP, CMP, and GMP were estimated to be 80, 88, 20, and 7 μM, respectively, from the corresponding concentration dependence curves with the same calculating method as in Figure 2.

the rate of the step that immediately followed would be reduced. Thus if the step immediately following the UTP binding is the step activated by CTP (mechanism a, Scheme III), the activation should be offset by a reduction of the UTP concentration, and, hence, the early rise in the time course of [^{3}H]UMP incorporation would be retarded. The result (Figure 5, (curve d') clearly demonstrates that the early rise was not altered. Therefore, the activation step is not the step immediately following the UTP binding but is one which occurs later. This conclusion indicates that the mechanism of activation for CTP is the same as that drawn earlier for GTP.

It can be seen in Figure 3 that systems c and d are essentially identical except for the labeled nucleotide. In the first mixing, CTP is labeled in system c whereas UTP is labeled in system d. Thus curve d in Figure 5 reflects the time course of the incorporation of the second nucleotide under the same experimental conditions. The time lag observed in curve c relative to curve d can, therefore, be attributed to steps occurring between the second and the third nucleotide incorporations, which should include at least a translocation step. From the length of the time lag, it can be estimated that these steps are completed within about 0.2 s.

Concentration Dependence of the Nucleotide Incorporation. In Figure 4, all four kinetic curves approach a constant maximal level of approximately 2 nM between the reaction times of 5 and 90 s. The same level of the net labeled nucleotide incorporation was observed at 90 s when similar experiments were performed by manual mixing (e.g., experiment 1, Table I). We have thus carried out manual mixing experiments to examine the dependence of the incorporation of each of the first four nucleotides on the concentration of labeled nucleotide in syringe 2 in the four experimental systems (a, b, c, and e) described in Figure 3. The results (Figure 6) indicate that while the concentration dependence curves of the first two nucleotides (ATP and UTP) were similar, those of the third (CTP) and fourth nucleotides (GTP) were distinctly different. The concentrations for the half-maximal incorporations of ATP, UMP, CMP, and GMP can be estimated from these curves to about 80, 88, 20, and 7 μM, respectively. These apparent dissociation constants may indicate that the affinity of individual nucleotides for the promoter–enzyme complex increases with increasing length of oligonucleotide synthesized (except for the first two nucleotides). This may also explain the result that the apparent dissociation constant of UTP was decreased to 9.4 μM when the longer initiator CpA was used instead of ATP. However, this interpretation may be an oversimplification because the concentration of nucleotide required is also dependent on other complex steps (such as catalytic processes) as well as on nucleotide binding.

Discussion

Initiation of RNA synthesis is a complex and rapid process, and kinetic studies of this process require a careful interpretation. Because the enzyme–promoter complex does not act catalytically in productive initiation, steady-state kinetic analysis cannot be used in this case. In this paper, we have determined by non-steady-state kinetic analysis that binding of the first two nucleotides during the initiation is rapid and ordered. Using a fast kinetic method, we have also observed directly the kinetics of incorporation of the first four nucleotides into the 5′-terminal region of RNA synthesized at the A1 promoter. These methods also allow us to detect the activation of the productive initiation by the binding of either the third or fourth nucleotide to a regulatory site on the enzyme–promoter complex. Earlier attempts by Chamberlin's group (Mangel & Chamberlin, 1974; Rhodes & Chamberlin, 1974) to study these problems using the rifampicin-challenge assay led to the conclusions that RNA chain initiation occurs very rapidly and that the binding of the 5′-terminal nucleotide to the enzyme appears to be rate limiting for the initiation reaction. This is in conflict with our finding that the binding of both the first and second nucleotides are rapid as compared to the subsequent catalytic step. Although one may question the validity of the assumptions used in the rifampicin-challenge assay (see introduction), Nierman & Chamberlin (1979, 1980a,b) have claimed recently that they have verified the major qualitative conclusions of their rifampicin-challenge

experiments by direct kinetic analysis of the incorporation of γ-^{32}P-labeled nucleotide triphosphate into RNA products. In these latter studies, [γ-^{32}P]ATP or [γ-^{32}P]GTP was first incubated with the enzyme–promoter complex in the presence of low concentration (2–10 μM) of all four nucleoside triphosphates. At a given time (10–200 s), the number of RNA chains which had been initiated was measured by the addition of an excess of ATP or GTP (together with CTP and UTP) to block further incorporation of labeled nucleotide, followed by a determination of the amount of radioactivity incorporated into acid-insoluble materials. Since the initiation process is extremely rapid, as shown in our fast kinetic studies, it is possible that in the presence of all four nucleoside triphosphates, even at low concentrations, the RNA polymerase reaction could have proceeded beyond the initiation step. From the data of Nierman & Chamberlin (1979), the second-order rate constant for ATP binding to the enzyme–A1 promoter complex can be calculated to be approximately 10^4 M^{-1} s^{-1} at saturating UTP concentrations. That this value is at least an order of magnitude smaller than the same rate constant determined from our rapid kinetic studies suggests that what they observed might be some slower process(es) occurring after the initiation step in the productive pathway. Moreover, the agreement between the results obtained from the γ-^{32}P-labeled nucleotide incorporation and those obtained from the rifampicin-challenge experiments may be fortuitous because rifampicin is known to inhibit some steps following the first phosphodiester bond formation (McClure & Cech, 1978).

In our non-steady-state kinetic studies on the order of substate binding during RNA chain initiation, it was assumed that the rate of dissociation of labeled ATP (in systems A) or labeled UTP (in system B) after its binding to the enzyme–promoter complex was not much faster than the rate of the subsequent steps in the initiation pathway, so that a significant fraction of the E·[^{3}H]ATP complex formed was converted to the productive initiation complex. This assumption was obviously justified in system A since a positive net [^{3}H]ATP incorporation was obtained (Table I). The situation in system B was less clear. One may argue that the E·[^{3}H]UTP complex could have formed but that the bound [^{3}H]UTP molecules rapidly dissociated from this complex, resulting in the small net [^{3}H]UTP incorporation observed. Thus the mechanism of substrate binding might be random rather than ordered. However, in a random access mechanism in which UTP and ATP dissociate very rapidly from the enzyme–promoter complex, the net labeled nucleotide incorporation would approach zero in both systems A and B, which is clearly inconsistent with the experimental results. Thus our conclusion of an ordered mechanism for binding of the first two nucleotides is still valid in this case.

After binding of the first and second substrates to the enzyme–promoter complex, a phosphodiester bond is formed between these two nucleotides with a concomitant release of pyrophosphate. The dinucleotide formed can either revert to the original nucleoside triphosphates in the presence of pyrophosphate (pyrophosphorolysis), be released from the enzyme–promoter complex (abortive initiation), or be elongated into an RNA chain (productive initiation). Only those enzyme–promoter–dinucleotide complexes which undergo chain elongation (productive initiation complexes) are quantitated by the net labeled nucleotide incorporations. At saturating substrate concentrations, the net labeled nucleotide incorporation measured for all nucleotides was approximately equal to the promoter concentration (Figures 4 and 5), indicating that most of the dinucleotides formed were elongated into RNA chains and that the pyrophosphorolysis and the abortive initiation reactions were negligible under these conditions. This is not true at low substrate concentrations (Figure 5) or at high enzyme and DNA concentrations (Figure 1). Although pyrophosphate seems to be almost irreversibly released after the phosphodiester bond formation (Maitra & Hurwitz, 1967), abortive initiation is enhanced by high concentrations of enzyme and DNA (McClure et al., 1978; Smagowicz & Scheit, 1978). The mechanism of such an enchancement is not clear.

It is interesting to compare the kinetic mechanisms of RNA chain initiation occurring on native T7 DNA template with that occurring on synthetic poly(dA–dT) template. T7 DNA is a linear double-stranded DNA whereas poly(dA–dT) contains many hairpin or snapback structures (Davidson et al, 1965). While there are limited numbers of promoter sites on T7 DNA, RNA synthesis can be initiated almost all over the poly(dA–dT) template. Transcription originated at a promoter site is usually unidirectional. On the other hand, transcription of poly(dA/dT) proceeds in both directions along the template. In spite of these differences, the kinetic mechanisms of initiation on the poly(dA–dT) template and at the A1 promoter on T7 DNA have revealed some surprising similarities. In both cases, the binding of the first two substrates to the enzyme–template complex is rapid and follows the same ordered mechanism. The biphasic time courses for net [^{3}H]UMP incorporation are also similar, though with different relaxation times. These similarities suggest that poly(dA-dT) may contain local structures resembling native promoters which RNA polymerase can recognize. It is not unreasonable for one to assume that the flexibility of poly(dA-dT) may increase the occurrence of promoter-like structures.

A significant difference, however, was observed for the dependence of the net labeled nucleotide incorporation on the concentration of UTP. In the poly(dA-dT) system, there exists a strong positive cooperativity of UTP binding, suggesting that more than one UTP molecule may bind to the enzyme–DNA complex during the initiation process (Shimamoto & Wu, 1980a). UTP may bind to a site other than the substrate binding site on the enzyme and thus activate the productive initiation. No cooperative binding of UTP was detected in the T7 DNA system. Instead, the net [^{3}H]UMP incorporation was activated by CTP and GTP. This apparent difference can be resolved by considering the nucleotide sequence of the RNA transcripts. The activators CTP and GTP correspond to the third and fourth nucleotides, respectively, on the 5′ sequence of RNA synthesized from the A1 promoter. On the other hand, the positive cooperativity of UTP binding observed in the poly(dA-dT) system may be explained by its dual roles as a substrate (second nucleotide) and as an activator (fourth nucleotide). Thus it seems that the activation of the productive initiation by the third or fourth nucleotide according to the RNA sequence is a common feature of transcription on both native and synthetic DNA templates. The generality of this type of regulatory mechanism for other promoters is currently under examination.

The molecular mechanism of such an activation is not known. The finding that mono- and diphosphate derivatives of cytidine and guanosine are also activators like their corresponding nucleoside triphosphates suggests that the activators need not be covalently bound to the enzyme–template complex and that the base moiety of the nucleotide may play an important role in the activation. It has been shown (Saucier & Wang, 1972) that binding of RNA polymerase to a double-stranded DNA induces a local unwinding of the DNA, a process which may lead to the formation of so-called

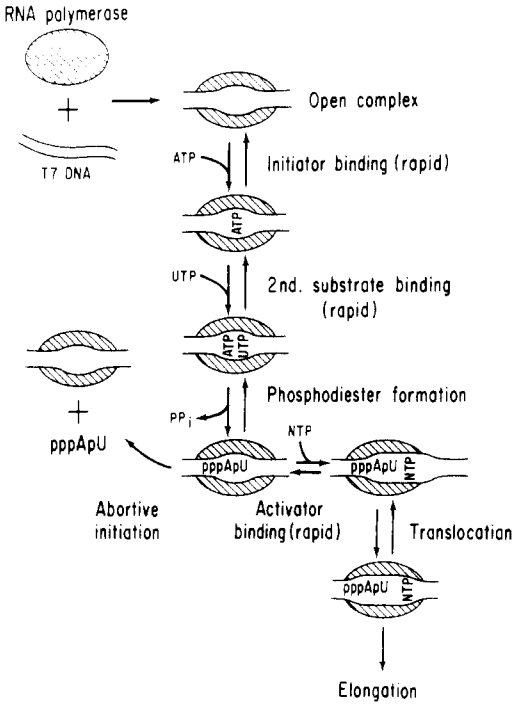


FIGURE 7: Kinetic mechanism of RNA chain initiation. Both the productive and the abortive initiation pathways are illustrated.

"open"-promoter complex. Maintenance and propagation of a locally melted DNA loop are necessary for RNA polymerase to translocate along the template and to copy the subsequent DNA sequence. It seems plausible that after the formation of the first phosphodiester bond, the third or fourth nucleotide may help to stabilize the locally melted DNA loop by hydrogen bonding to its complementary base in the single-stranded DNA region of the loop, thereby facilitating the subsequent translocation and phosphodiester bond formation.

A minimal kinetic mechanism of RNA chain initiation consistent with all the results presented in this paper is shown in Figure 7. RNA polymerase binds to a promoter site on the DNA template to form an open-promoter complex. The binding of the first two nucleotides to the enzyme–promoter complex is rapid and ordered. A phosphodiester bond is then formed to yield a dinucleotide with the release of a pyrophosphate. The enzyme–DNA–dinucleotide complex can bind an activator nucleotide, corresponding to the third or fourth nucleotide on the transcriptional sequence, which facilities the translocation. The posttranslocation intermediate is the productive initiation complex which is ready for elongation of the RNA chain. In the absence of the proper activator, the dinucleotide is released from the enzyme–DNA complex, and the initiation is aborted. In this way, the third or fourth nucleotide acts as a discriminator between the two initiation pathways by stimulating productive initiation while inhibiting abortive initiation. In the presence of a discriminator and at high substrate concentrations, the majority of enzyme–promoter complexes proceeds along the productive initiation pathway. The above mechanism is in agreement with one of the two possible mechanisms that we proposed previously for the initiation of RNA chain on the poly(dA-dT) template (Shimamoto & Wu, 1980b). Although this mechanism is qualitatively in accord with our experimental observations, the data presented here are, unfortunately, insufficient to make a detailed quantitative evaluation of the kinetic parameters in this model.

What is the functional significance of a regulatory nucleotide which can discriminate between the abortive and productive initiation pathways? Two possible roles can be envisioned for the regulatory nucleotide in the control of RNA synthesis. When the concentration of the third or fourth nucleotide (specified by the DNA sequence) is low, abortive initiation becomes the dominant pathway and productive initiation can scarcely take place. Thus by modulating a particular nucleotide concentration in the cell, the quantity of a specific RNA transcript may be regulated at the stage of initiation. Furthermore, the ability of RNA polymerase to discriminate between the abortive and productive initiation pathways may provide an efficient way to ensure the fidelity of the transcription initiation. If the correct regulatory nucleotide is not bound to the enzyme-promoter complex after the formation of the first phosphodiester bond, abortive initiation becomes the predominant pathway and specific RNA synthesis is inhibited. Such a mechanism does not shut off the specific RNA synthesis completely but reduces its rates in proportion to the rates of abortive as opposed to productive initiation. It is conceivable that in this way RNA polymerase may use abortive initiation as a safety valve to prevent an error in RNA chain initiation during gene transcription.

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Differential Induction by Cadmium of a Low-Complexity Ribonucleic Acid Class in Cadmium-Resistant and Cadmium-Sensitive Mammalian Cells†

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ABSTRACT: The Chinese hamster ovary (CHO) cell line and the subline Cd^{r}20F4 have been used to compare cadmium-induced ribonucleic acid (RNA) synthesis in cadmium-sensitive and cadmium-resistant cells, respectively. Gel electrophoresis of the cell-free translation products directed by polyadenylated [poly(A+)] messenger RNA (mRNA) from cadmium-induced Cd^{r}20F4 cells revealed four low molecular weight species (M_r 7000–21 000), including metallothionein, whose synthesis was not detected after translation of either cadmium-induced or uninduced CHO cell poly(A+) mRNA. At least two of these species were also detected after translation of an abundant 400-nucleotide (NT) RNA class purified from the cadmium-induced Cd^{r}20F4 cell RNA. Molecular hybridization of complementary deoxyribonucleic acid (cDNA) complementary to this abundant, cadmium-induced 400-NT RNA fraction indicates that the cadmium-induced RNA class possesses a total kinetic complexity of about 2000 NT's. At least half of these inducible sequences are also represented constitutively in less abundant RNA classes of both uninduced CHO and Cd^{r}20F4 cells. Induction of Cd^{r}20F4 cells with cadmium increases the cellular concentration of the 2000-NT-complexity RNA class to a level at least 2×10^3-fold greater than its constitutive level in uninduced Cd^{r}20F4 cells. Induction of CHO cells with cadmium increases the cellular concentration of a subset of the sequences in the 2000-NT-complexity class, but only to a level 100-fold over the constitutive level in uninduced CHO cells. The remainder of these sequences belongs to the least abundant CHO cell poly(A+) RNA class.

The Chinese hamster ovary (CHO) cell subline Cd^{r}20F4 is a stable, cadmium-resistant variant derived from the CHO line. It is distinguished from the parental CHO line by a 100-fold higher cadmium toxic threshold, a 50-fold or greater cadmium-inducible metallothionein synthesis rate, and a 50-fold or greater level of cadmium-inducible translatable metallothionein messenger ribonucleic acid (mRNA)[1] (Enger et al., 1981; Hildebrand et al., 1979).

Although the mechanism of cellular cadmium detoxification has not been established, metal-induced synthesis of cadmium-binding metallothioneins is thought to play an important role [Enger et al., 1980; Hildebrand et al., 1979; reviewed by Kägi & Nordberg (1979)]. Thus, the differences in both metallothionein synthesis and cadmium sensitivity between these two cell lines provide means for studying the molecular events involved in cellular metal detoxification.

In this context, we recently reported that the poly(A+) RNA from cadmium-induced Cd^{r}20F4 cells contains a highly abundant RNA class not present in the poly(A+) RNA from uninduced CHO cells (Walters et al., 1980). In this paper, we report the results of a more detailed examination of this cadmium-inducible RNA class. We describe (1) the cell-free translation products directed by the poly(A+) RNA from cadmium-induced and uninduced Cd^{r}20F4 and CHO cells, (2) the isolation of cDNA sequences enriched for sequences complementary to a cadmium-induced abundant poly(A+)

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; poly(A+), polyadenylated; $NaDodSO_4$, sodium dodecyl sulfate; cDNA, complementary deoxyribonucleic acid; NT, nucleotide; tRNA, transfer RNA; EDTA, ethylenediaminetetraacetic acid; HAP, hydroxylapatite.

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