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Complex RNA chain elongation kinetics by wheat germ RNA polymerase II

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ABSTRACT

Kinetics of RNA chain elongation catalyzed by wheat germ RNA polymerase II have been studied using various synthetic DNA templates in the presence of excess dinucleotide monophosphate primers. With single- or double-stranded homopolymer templates, the double reciprocal plots 1/(velocity) as a function of 1/(nucleotide substrate) exhibit positive, negative or no curvature. With poly(dAT) as template, the mechanism of nucleoside monophosphate incorporation into RNA is not the ping-pong kinetic mechanism which was derived for E.coli RNA polymerase (6). Noncomplementary nucleoside triphosphates inhibit RNA transcription allosterically. Cordycepin triphosphate behaves as ATP, and not only inhibits AMP incorporation but also that of UMP and GMP on appropriate templates. The reason for this complex kinetic behavior is not yet understood. Possibilities are raised that there are several nucleoside triphosphate binding sites on wheat germ RNA polymerase II, that additional nucleoside triphosphate dependent enzymatic activities are required for reaction to occur or that the Km value for incorporation of a given nucleoside monophosphate into RNA is dependent on the length of the RNA chain and/or the nucleotide sequence surrounding the complementary base on the DNA template.

INTRODUCTION

The basic features of the transcription reaction can be summarized as (reviewed in 1): (i) enzyme binding to DNA template and localized melting of the helix; (ii) initiation corresponding to the formation of the first phosphodiester bond: a purine nucleoside triphosphate at the 5' end of the RNA synthesized is usually required; (iii) elongation involving the sequential incorporation of nucleoside monophosphates; and (iv) termination of the RNA chain. It is generally felt that the control of RNA synthesis in eucaryotic cells probably lies in the initial steps of transcription (RNA polymerase: DNA recognition process, initiation of RNA synthesis) rather than in other steps of the polymerization reaction. However, recent results illustrate the possible importance of other steps in eucaryotic transcription. Thus Dauphinais (2) suggested that, rDNA transcription during lymphocyte activation might be controlled at the level of the elongation reaction. Studies conducted with plant RNA polymerases II also suggest that the elongation-translocation

properties of the enzymes could depend on the nucleotide sequence (and/or conformation) of the DNA sequence being transcribed. Thus pausing and non processivity of plant RNA polymerases II in RNA synthesis have been observed with both natural (3) and synthetic DNA templates (4). Similar observations have been reported during the in vitro transcription of the yeast alcohol deshydrogenase I gene by yeast RNA polymerase II (5). Relatively little is known, however, about the actual mechanism of enzymatic polymerization catalyzed by eucaryotic RNA polymerases. For E. coli RNA polymerase, for which the results are best documented, Rhodes and Chamberlin (6) proposed that the enzyme is kinetically characterized in the elongation step by a single binding site for the nucleoside triphosphate substrates. By applying steady-state kinetics, these authors have determined the Ks values for the nucleotide substrates on a number of synthetic templates. In their studies, differences in the Ks values were not large, and did not depend on the DNA base sequence. Low efficiency competitive inhibition of the elongation reaction is observed with high concentrations of noncomplementary nucleotides, which is attributed to a general affinity of the polymerase in the enzyme/RNA/DNA ternary complex for nucleoside triphosphates. Therefore, all ternary complexes have equal affinity for noncomplementary nucleoside triphosphates. From the kinetic study using these complexes, a simple ping-pong kinetic model was derived and was shown to fit the data obtained with alternating copolymer templates (6).

Although information concerning the mechanism of interaction of eucaryotic RNA polymerases with nucleoside triphosphates is scarse, recent results on RNA polymerases from higher plant cells (soybean, parsley and wheat germ) revealed that these enzymes could be allosterically regulated (7-9). These studies suggested that the enzymes contain from two to five ligand sites. In addition, Grossmann and Seitz (7-8) showed that nucleoside triphosphates in excess of the divalent cations acted as allosteric inhibitors of enzyme activity. These few results tend to support the contention that nucleoside triphosphates and divalent cations may act as low molecular weight regulators of transcription in eucaryotic cells. In this context, these interesting properties may reveal hiterto unconsidered mechanisms for regulation of transcription.

From the above concepts, and in view of the fundamental importance of nucleoside triphosphates in controlling the activity of RNA polymerases, we have undertaken a kinetic study of the RNA chain elongation reaction catalyzed by wheat germ RNA polymerase II. In this initial study, the reaction mechanism was investigated by means of steady-state kinetics. The results obtained using various synthetic templates are compared to those reported for E. coli RNA polymerase (see for instance 1 and 10).

MATERIALS AND METHODS

Reagents

Nucleoside triphosphates, nucleoside monophosphates, cordycepin triphosphate and the dinucleoside monophosphates were purchased from Sigma. ^{3}H-ATP, ^{3}H-CTP, ^{3}H-GTP and ^{3}H-UTP (25, 27, 16 and 16.2 Ci/mmol, respectively) were from ICN. Synthetic polymers were from PL Biochemicals; they were dissolved in 50 mM Tris-HCl buffer, pH 7.8, except poly(dC) which was dissolved in 50 mM Tris-HCl buffer, pH 8.9. All buffer components were reagent grade.

RNA polymerase

Wheat germ RNA polymerase IIA was purified by the method of Jendrisak and Burgess (11), as modified by Job et al. (9). The specific activity of the enzyme was 300 units/mg and 6000 units/mg, using denatured calf thymus DNA and poly(dC) as template, respectively (9). Protein determinations were effected according to Bradford (12). Enzyme concentration was calculated assuming a molecular weight of 650 000 (13).

Transcription assays

Unless otherwise noted in the legends of figures and tables, the reaction mixtures contained 0.25 nM enzyme, 5 µg/ml DNA, 0.125 mg/ml of a dinucleotide complementary to DNA, 1 µM ^{3}H-labelled nucleoside triphosphate substrates complementary to DNA and 1.5 mM MnCl2. For inhibition studies, they also contained unlabelled nucleotide derivatives as indicated in the legends of figures. In all assays, other components were 64 mM Tris-HCl buffer pH 7.8, 12.5% (v/v) glycerol, 12.5 mM 2-mercaptoethanol, 5 mM α-thioglycerol, 0.05 mM EDTA, 0.05% (v/v) Triton X100, 1.1 mM DTT and 1.5 mM NaF. Reactions were usually started by addition of nucleotide substrates, after 10-15 minutes preincubation of all other components at 0°C. The template and dinucleotide primer concentrations at which maximal enzymatic activity occured were determined by titration studies. Activity increased linearly as a function of enzyme concentration. Final volumes were 30 µl, and assays were usually incubated for 30 minutes at 35°C (incorporation rates were linear for up to 60 minutes). For activity measurements, reaction mixtures were processed using three different methods. First, 30 µl of reaction mixtures were adsorbed on Whatman GF/C filters and washed four times with an ice-cold solution containing 5% (w/v) trichloroacetic acid and 0.04 M sodium pyrophosphate, with moderate stirring. After two ethanol washings, the filters were dried under a heat lamp and counted for radioactivity in 5 ml of toluene-PPO-POPOP liquid scintillation cocktail (9). Second, 30 µl of same reaction mixtures were spotted onto DEAE-cellulose paper disks (DE-81 from Whatman) and processed

as recommended by Lewis and Burgess (14): paper disks were washed five times with 5% (w/v) sodium phosphate with moderate stirring; after two ethanol washings, DE-81 disks were dried under a heat lamp and heated for 30 minutes at 96°C in 1 ml of 5% trichloroacetic acid. Following centrifugation, 0.7 ml of the supernatants were counted for radioactivity in vials containing 8 ml of Phase Combining System (Amersham). Third, 10 µl of the reaction mixtures were spotted onto PEI-cellulose sheet (20 x 20 cm) from Macherey-Nagel; RNA synthesized were separated from unreacted substrates by ascending chromatography with 1.0 M LiCl, according to Randerath and Randerath (15). The sheets were dried, then sprayed with EN3HANCE spray from NEN, and autoradiographed for 36 hours at -70°C, using Fuji RX films. Spots containing labelled RNA were cut, then heated for 30 minutes at 96°C in 1 ml of 5% trichloroacetic acid. After centrifugation, 0.7 ml of the supernatants were counted for radioactivity in vials containing 8 ml of Phase Combining System. Assays were run in triplicate.

RESULTS

Quantitation of synthesized RNA

Since we have previously shown that RNA synthesis catalyzed by wheat germ RNA polymerase II, using poly(dAT) as template, occurs non processively (4), it was of necessity to ensure that all RNA molecules synthesized in the experimental conditions of this study, especially the shortest chains, could be detected. Therefore, three different methods were compared for their efficiency to quantitate RNA synthesis. In the first one, GF/C filters were used. The second method is based upon utilization of DE-81 paper disks, which have been shown to retain RNA molecules of trinucleotide size or larger (14). In the third method unreacted substrates are separated from synthesized RNA by thin layer chromatography on PEI-cellulose sheets (15); in most conditions two labelled spots were visualized after autoradiography, one migrating with RF values close to those reported by Randerath and Randerath (15) and corresponded to the labelled nucleoside triphosphate, the second one, which remained at the origin of the chromatogram was identified as synthesized oligonucleotides, since its presence on the autoradiograms was absolutely dependent on the presence of a DNA template in the reaction mixtures. Furthermore this spot did not appear when α-amanitin or heparin was included in the reaction mixture. When labelled ATP is used as a substrate in the transcription assay, an additional minor spot, accounting for 3-6% of the input nucleotide could be detected on the autoradiograms. From its RF value this spot can be tentati-

vely assigned to be ADP (15). Studies are in progress to further characterize this activity.

The comparison of the three above methods was done under 21 different experimental conditions, including all the DNA templates used in this study, at either low (1 μM) or high (100 μM) nucleoside triphosphate substrate concentration. In these experimental conditions, incorporation of labelled substrates into RNA ranged from 120 cpm to 317000 cpm. The following linear relationships were obtained:

cpm (30 μl reaction mixtures on GF/C filters) = (1.47 ± 0.045) x cpm (30 μl reaction mixtures on DE-81 after solubilization and hydrolysis) + (5536 ± 2900); correlation coefficient r^2 = 0.983.

cpm (30 μl reaction mixtures on GF/C filters) = (4.86 ± 0.08) x cpm (10 μl reaction mixtures on PEI sheets after solubilization and hydrolysis) + (3648 ± 1597); correlation coefficient r^2 = 0.995.

The correlations obtained indicate that all three methods give similar results. However, the method using the GF/C filters was prefered because of its simplicity, and since higher signal together with lower background were systematically observed. Furthermore, in contrast to the methods using DE-81 paper disks or PEI sheets, hydrolysis and solubilization of the ^{3}H-labelled RNA before they are counted were not necessary. In all cases the base composition of the RNA products was that expected from the base composition of the DNA templates, using the corresponding radioactive nucleoside triphosphate substrates. Radioactive noncomplementary nucleoside triphosphates were not incorporated into RNA, at least to the limits of detection of the methods used, i.e. less than 1/1000 of the input nucleotides.

Priming with dinucleotides

In the studies presented by Rhodes and Chamberlin (6) the reaction of RNA chain elongation was investigated by employing ternary complexes containing E.coli RNA polymerase, a DNA template and product RNA. The stability of these complexes was such that they could be isolated by passage through a gel exclusion column. When stored at 4°C, they lost elongation activity slowly, with one-half of the activity being lost in about one week. Unfortunately, the same strategy cannot be used with wheat germ RNA polymerase II. Recent results (4) indicate that the ternary complexes are much less stable when formed with the wheat germ enzyme than with the bacterial enzyme: with poly(dAT) as template, we were unable to recover any active ternary complex after passage through a Bio-Gel P2 column. Furthermore, RNA synthesis using this template was completely abolished in the presence of heparin (not shown). These observations

Table 1

Enhancement of the rate of RNA synthesis in the presence of dinucleotide primers.

Assays were performed as descibed under Materials and Methods, in the absence or in the presence of the appropriate dinucleotide primers. Nucleoside triphosphate substrate concentrations were 2μM.

template	substrate	primer	rate enhancement
poly(dA)	UTP	UpU	14.7
poly(dA)-poly(dT)	UTP	UpU	1.2
poly(dA)-poly(dT)	ATP	ApA	2.6
poly(dAC)-poly(dGT)	GTP + UTP	GpU	1.8
poly(dAC)-poly(dGT)	GTP + UTP	UpG	1.5
poly(dAC)-poly(dGT)	CTP + ATP	ApC	1.5
poly(dAC)-poly(dGT)	CTP + ATP	CpA	1.9
poly(dAT)	ATP + UTP	ApU	1.3
poly(dAT)	ATP + UTP	UpA	1.5
poly(dC)	GTP	GpG	16.3
poly(dC)-poly(dG)	CTP	CpC	0
poly(dC)-poly(dG)	GTP	GpG	3.1
poly(dGC)	CTP + GTP	CpG	1.1
poly(dGC)	CTP + GTP	GpC	1.8
poly(dG)	CTP	CpC	0
poly(dT)	ATP	ApA	>100

are in agreement with the results of Ackerman et al. (16), and it appears that ternary transcription complexes formed with eucaryotic RNA polymerase II are fragile and can be easily disrupted upon dilution. A different strategy, based on utilization of dinucleotide primers was therefore employed. It is well known that procaryotic as well as eucaryotic RNA polymerases can use dinucleotide primers to initiate RNA synthesis (3, 17-22). With E.coli RNA polymerase and wheat germ RNA polymerase II the primer is incorporated into RNA (20). The enzymes can also use dinucleotide primers to catalyze DNA dependent trinucleotide synthesis in the presence of a nucleoside triphosphate (3, 17, 23). The specific activity of the plant RNA polymerase in this transcription assay compares very well with that of enzymes from yeast or E.coli (3). Therefore, in the presence of excess of these primers, kinetic limitations due to the initiation step in the overall transcription reaction are overcome, and the initiation step is bypassed (24). The results in Table 1 show that dinucleotide primers markedly enhance the observed transcription rates on various synthetic DNA templates. For the series of homopolymers, poly(dA), poly(dC) and poly(dT), the reactions are almost exclusively dependent on the presence of the appropriate primers. Poly(dG) as well as the purine strand

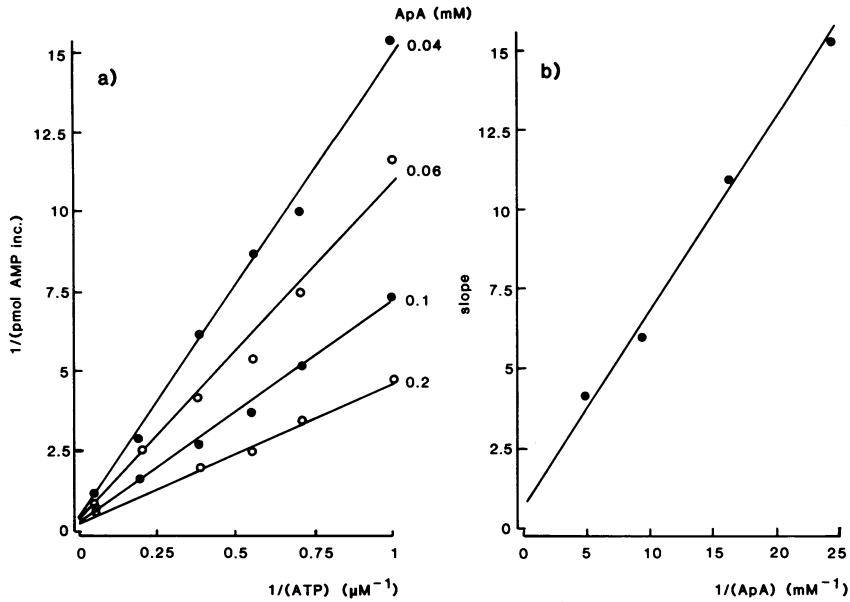

FIGURE 1: Initial velocity pattern for transcription of poly(dT) by wheat germ RNA polymerase II in the presence of ApA.
a) reciprocal velocities plotted as a function of 1/ATP concentration at the fixed ApA concentrations indicated. All conditions for transcription assays are given under Materials and Methods.
b) replot of the slopes of a).

of poly(dC)-poly(dG) are not transcribed, even in the presence of excess CpC. In order to investigate the kinetic mechanism which could account for the dinucleotide primer utilization in the reaction, rate measurements were performed by considering a pair of substrates, i.e. the nucleoside triphosphates and the primer. The results obtained for the transcription of poly(dT), in the presence of ApA and ATP are shown in Figure 1. Titration experiments ensured that the reaction media are saturated with DNA and that the reaction rates are proportional to enzyme concentration (not shown). An intersecting pattern is found when 1/v is plotted versus 1/(ATP) at various ApA concentrations in the presence of saturating poly(dT). Furthermore, a replot of these data in the form slopes versus reciprocal concentration of ApA is linear (Figure 1,b). Thus, the reaction of poly(A) formation corresponds to a sequential mechanism, in which both dinucleotide primer and triphosphate substrates must bind to the enzyme active site before reaction occurs.

Nucleoside triphosphate substrate kinetics

The reaction rates were measured as a function of nucleoside triphosphate

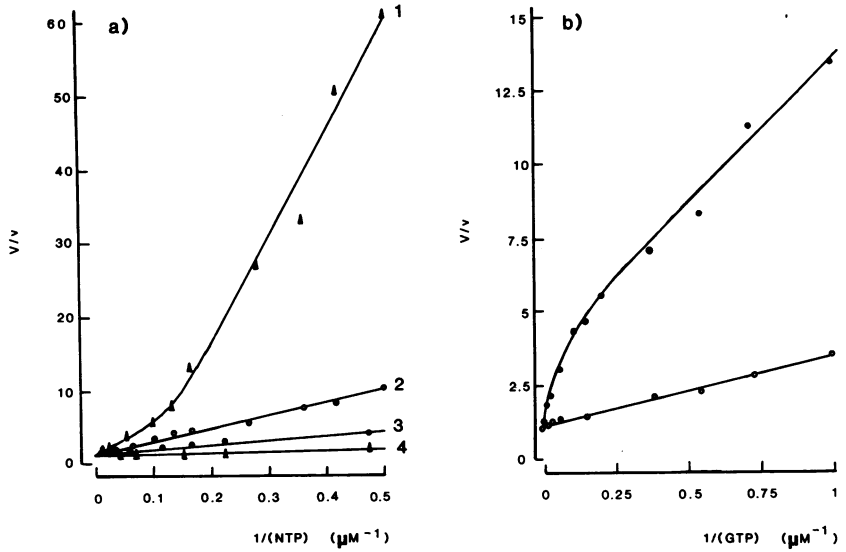

FIGURE 2: Initial velocity patterns for transcription of synthetic templates by wheat germ RNA polymerase II in the presence of dinucleotide primers.
All conditions for transcription assays are given under Materials and Methods. The data are plotted as normalized reciprocal velocities as a function of reciprocal concentration of complementary nucleoside triphosphate substrates. Template-dinucleotide primer pairs are:
a) poly(dA)-poly(dT) + ApA (1), poly(dT) + ApA (2), poly(dA)-poly(dT) + UpU (3), poly(dA) + UpU (4).
b) poly(dC) + GpG (1), poly(dC)-poly(dG) + GpG (2).

substrate concentrations for a large number of the combinations (template and primer) as listed in Table 1. No reaction occured in the absence of DNA template. The results obtained for the transcription of single- and double-stranded homopolymers are compiled in Figure 2. The results are expressed as V/v versus 1/(NTP), where V is the maximal velocity corresponding to a given template-primer pair. As outlined above, these measurements are carried out under saturating concentrations of both template and primer. Various types of plots are obtained, exhibiting positive, negative or no curvature. It should be noted that linearity is obtained for the transcription of poly(dT) but not for the same strand in poly(dA)-poly(dT) and that the converse is true when considering poly(dG) and poly(dG)-poly(dC).

Figure 3 shows the results obtained when an alternating purine-pyrimidine polymer such as poly(dAT) is used as template. In these experiments, UTP concentration is varied at fixed concentrations of ATP. In the substrate

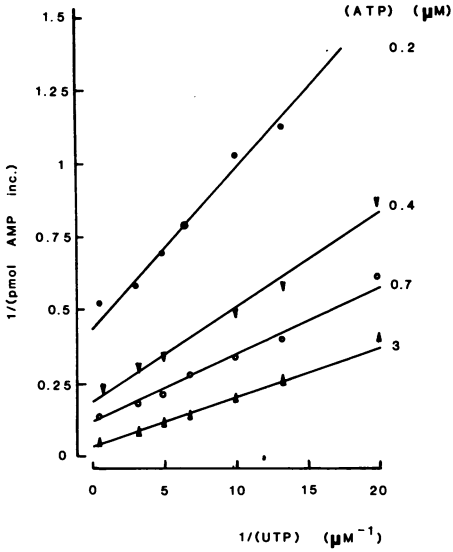

FIGURE 3: Initial velocity pattern for transcription of poly(dAT) by wheat germ RNA polymerase II in the presence of UpA. All conditions for transcription assays are given under Materials and Methods. Reciprocal velocities are plotted as a function of 1/UTP concentration at the fixed concentrations of ATP indicated.

concentration range shown linear plots are obtained which are not parallel. Furthermore, in this substrate concentration range, a replot of slopes in Figure 3 versus reciprocal concentration of ATP is linear (not shown). For higher substrate concentrations than those indicated, complex non linear plots are observed, probably arising from inhibition reactions between the two nucleotide substrates (see later, Figure 4,h). The combined results of Figures 2 and 3 indicate that the elongation mechanism in the case of wheat germ RNA polymerase II is complex. The non parallel plots obtained in the study of the transcription of poly(dAT) give no indication of the occurence of a ping-pong mechanism as shown for E. coli RNA polymerase (6) and for replication of same template by E. coli DNA polymerase I under steady-state conditions (25).

Maximal transcription rates, as well as apparent Km values for nucleoside triphosphate substrates were determined for a number of template and primer combinations. The numerical values are listed in Table 2. As suggested by Rhodes and Chamberlin (6), the Km values were calculated taking into account the base composition of the template. However, interpretation of these values is difficult due to the curvature of some of the rate profiles: noticeable differences for the utilization of the substrates by the enzyme are seen. Apparently, they depend on the nature of both the substrate and the template-primer pair considered. The maximal rate values are found to vary considerably for the template-primer pairs studied. Some of them are in agreement with previously reported data for which the dinucleotide primers were not used (9, 26).

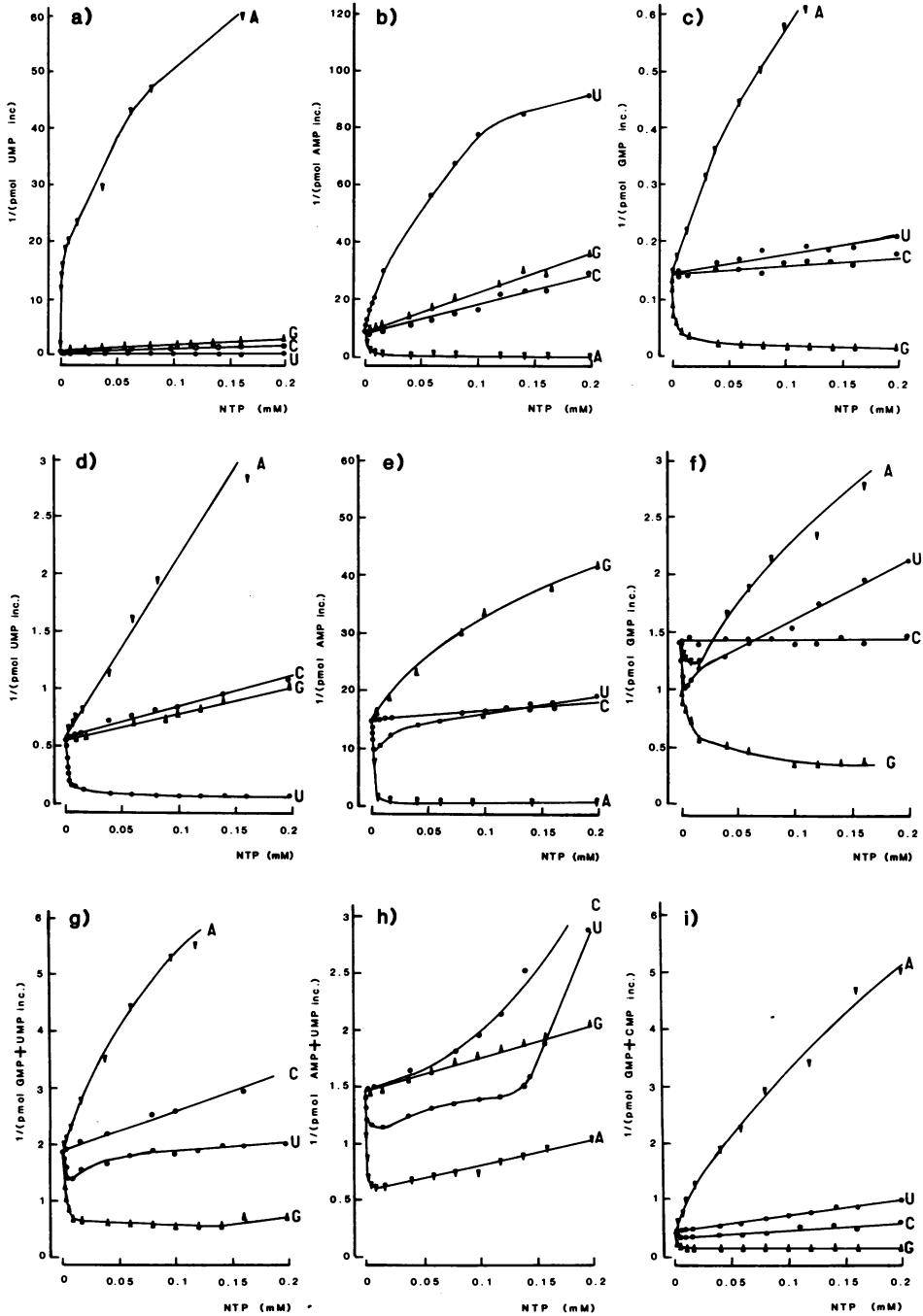
a)
1/(pmol UMP inc)
NTP (mM)
A
G
C
U
b)
1/(pmol AMP inc.)
NTP (mM)
U
G
C
A
c)
1/(pmol GMP inc.)
NTP (mM)
A
U
C
G
d)
1/(pmol UMP inc.)
NTP (mM)
A
C
G
U
e)
1/(pmol AMP inc.)
NTP (mM)
G
U
C
A
f)
1/(pmol GMP inc.)
NTP (mM)
A
U
C
G
g)
1/(pmol GMP+UMP inc.)
NTP (mM)
A
C
U
G
h)
1/(pmol AMP+UMP inc.)
NTP (mM)
C
U
G
A
i)
1/(pmol GMP+CMP inc.)
NTP (mM)
A
U
C
G

Table 2

Apparent Km values and maximal velocities (pmol substrate incorporated) for the transcription of synthetic templates by wheat germ RNA polymerase II, in the presence of appropriate dinucleotide primers.

* Values estimated from the linear parts of the curved plots of 1/v vs. 1/(NTP), i.e. in the high substrate concentration range. Vmax values are expressed as pmol of substrate incorporated into RNA for reaction mixtures as described under Materials and Methods.

template	primer	apparent Km (μM) for				Vmax
		ATP	GTP	CTP	UTP	(pmol)
poly(dA)	UpU				2.8	0.92
poly(dA)-poly(dT)	UpU				5.6	4.25
poly(dA)-poly(dT)	ApA	>40*				3.25
poly(dAC)-poly(dGT)	GpU		7.2		5	1.95
poly(dAC)-poly(dGT)	CpA	n.d.		25		0.22
poly(dAC)-poly(dGT)	CpA + GpU	1.6		3.6		n.d.
poly(dAT)	UpA	17			2.9	12.6
poly(dC)	GpG		>20*			38.5
poly(dC)-poly(dG)	CpC					0
poly(dC)-poly(dG)	GpG		2			1.5
poly(dG)	CpC					0
poly(dGC)	GpC		5	1.6		8.8
poly(dT)	ApA	19				4.5

Nucleotide inhibition kinetics

As shown by Rhodes and Chamberlin (6), the study of inhibition of chain elongation by nucleoside triphosphates and derivatives provides a powerful tool for elucidation of the reaction mechanism. Experiments were thus conducted in which noncomplementary nucleoside triphosphates are used as inhibitors in the reactions of transcription of various synthetic DNA templates in the presence of the appropriate dinucleotide primers. The results obtained are presented in Figure 4. The rate measurements are plotted in the form 1/v versus (NTP), since in this way linear plots as a function of noncomplementary NTP concent-

FIGURE 4: Initial velocity patterns for transcription of synthetic templates by wheat germ RNA polymerase II in the presence of complementary dinucleotide primers. Effect of complementary and noncomplementary nucleoside triphosphates.
All conditions for transcription assays are given under Materials and Methods. As indicated in this section, the concentration of complementary 3H-labelled nucleoside triphosphate substrates was fixed at 1 μM and the variable unlabelled nucleoside triphosphates were ATP (A), CTP (C), GTP (G) and UTP (U). Data are plotted as reciprocal velocities as a function of nucleoside triphosphate concentration. Template-dinucleotide primer pairs are:
a) poly(dA) + UpU, b) poly(dT) + ApA, c) poly(dC) + GpG, d) poly(dA)-poly(dT) + UpU, e) poly(dA)-poly(dT) + ApA, f) poly(dC)-poly(dG) + GpG, g) poly(dAC)-poly(dGT) + GpU, h) poly(dAT) + UpA, i) poly(dGC) + GpC.

rations are obtained for a one-sited enzyme (6). In all the experiments, the concentrations of correct ^{3}H-labelled nucleoside triphosphate substrates are fixed at 1 μM and the concentrations of noncomplementary unlabelled NTP are varied in the range 0-0.2 mM. Care was taken to avoid excessively high nucleotide concentrations which could give rise to complications due to limitation in the available quantity of the divalent cation. In fact, similar results are obtained at 1 mM Mn^{2+} and at 1.5 mM Mn^{2+}, suggesting that such an effect does not occur during analysis. For comparison, the rate measurements obtained by varying the concentrations of correct complementary nucleotide substrates are also included in this Figure. From the results corresponding to the single- and double-stranded homopolymer series, it is clearly evident that a given nucleotide substrate can never be an inhibitor of its own incorporation, at least in the concentration range studied.

In some cases, ^{3}H-labelled noncomplementary nucleoside triphosphates were introduced in the reaction medium, in the presence of 1 μM unlabelled correct substrates. No detectable radioactivity is found associated with the RNA synthesized, suggesting that the inhibitory effect of noncomplementary NTP is not due to misincorporation of these molecules into RNA, at least to the limit of less than 1/1000 (not shown). These results also indicate that there is no appreciable cross contamination of the nucleoside triphosphates. As is apparent from the plots in Figure 4, noncomplementary NTP behave differently, depending on the template and primer used. In many cases, non linear plots are obtained, sometimes exhibiting mixed curvatures. Activation, albeit rather small, is even noted in three cases. Thus very low concentrations of UTP slightly activate the transcription of the pyrimidine strands of both poly(dA)-poly(dT) and poly(dG)-poly(dC) in the presence of appropriate primers and substrates (Figure 4 e, f). Similarly, slight activation of the transcription of the pyrimidine strand of poly(dG)-poly(dC) occurs for low concentrations of ATP in the presence of saturating GpG and 1 μM GTP (Figure 4 f). One explanation could be that UTP contains small amounts of ATP and GTP, and ATP small amounts of GTP. This possibility could be eliminated since the activation behavior is not encountered in the case of transcription of the above corresponding single-stranded homopolymers (Figure 4, b and c). Again, as previously indicated, no RNA is detected after incubation of the RNA polymerase with DNA template-dinucleotide pairs and the noncomplementary nucleoside triphosphates. It should be noted that UTP strongly inhibits the transcription of poly(dT) in the presence of saturating ApA and 1 μM ATP (Figure 4 b), whereas only rather slight inhibition is observed for the same strand in poly(dT)-poly(dA) under the same experimental conditions

(Figure 4 e). In one case, incorporation of a purine substrate is inhibited by a pyrimidine triphosphate (Figure 4 b), and in two cases incorporation of a pyrimidine substrate is inhibited by a purine triphosphate (Figure 4 a and d). From inspection of the data in Figure 4, no obvious correlation is emerging between the nature of the template-primer pair and the nucleoside triphosphate exhibiting the highest inhibitory behavior. However, ATP and to a lesser extent UTP, are frequently found to be very potent inhibitors of enzyme activity for the template-primer combinations studied.

Inhibition kinetics by substrate derivatives

From the above results, and in order to further characterize the inhibition behavior, several derivatives such as AMP, UMP and 3'-dATP (cordycepin triphosphate) were assayed for their potential inhibitory capacities. The results in Figure 5 show that neither AMP nor UMP can inhibit RNA synthesis with poly(dA), poly(dC) or poly(dAT) as templates, in the presence of appropriate dinucleotide primers and substrates. Same results were obtained with poly(dT) as template (not shown). On the other hand, 3'-dATP inhibits strongly the transcription of poly(dAT) and to a somewhat smaller extent than ATP, the transcription of poly(dA) and poly(dC) under similar experimental conditions. 3'-dATP also inhibits poly(G) synthesis in the presence of poly(dC)-poly(dG) and excess GpG. However, as observed with ATP (Figure 4 f), prior to inhibition, slight activation of poly(G) synthesis was noted in the presence of 3'-dATP in the micromolar concentration range (not shown).

DISCUSSION

For E. coli RNA polymerase, it has been shown that the requirement for a high level of the initiating purine nucleotide could be bypassed by employing a dinucleotide primer (24, 27). Transcription at low concentrations of nucleotide substrates is dramatically stimulated in the presence of dinucleotides complementary to the DNA transcription start site. These primers are themselves incorporated into RNA where they replace the initiating purine. Various results (3, 18-21, 23) demonstrated that eucaryotic RNA polymerases of class II can use these primers to initiate RNA synthesis or to catalyze DNA dependent trinucleotide formation. Moreover, Shaw and Saunders (28) showed that RNA synthesis catalyzed by wheat germ RNA polymerase II was markedly increased in the presence of dinucleotides. Results reported by Yarbrough (20) indicate that in these reactions, the dinucleotide primer is incorporated into RNA. Considerable improvement of poly(dA) transcription by wheat germ RNA polymerase II in the presence of oligo-RNA primers has also been recently reported (29). Dinucleotide

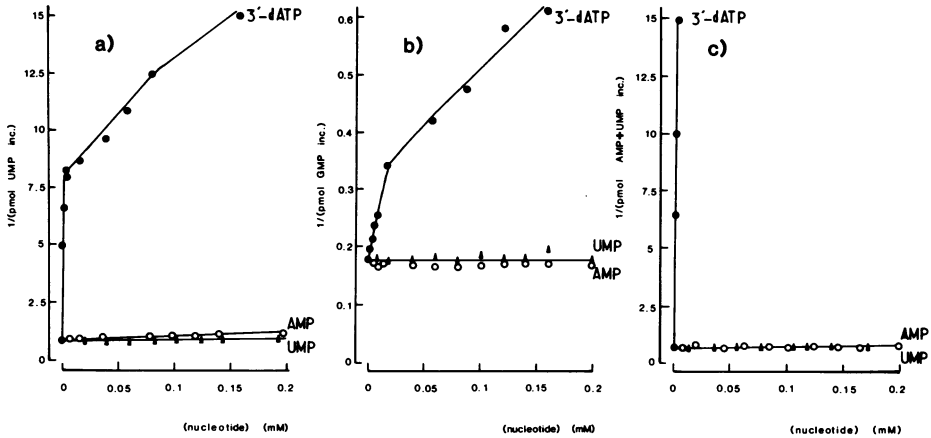

FIGURE 5: Initial velocity patterns for transcription of synthetic templates in the presence of complementary dinucleotide primers and nucleotide derivatives.
All assay conditions are given under Materials and Methods. Concentration of complementary 3H - labelled nucleoside triphosphate substrates was fixed at 1 μM and initial velocities were measured as a function of AMP, UMP or 3'-dATP concentration. Data are plotted as reciprocal velocities as a function of nucleotide derivative concentration. Template-dinucleotide primer pairs are: a) poly(dA) + UpU, b) poly(dC) + GpG, c) poly(dAT) + UpA.

primers have also been used in transcription reactions catalyzed by mouse RNA polymerases I and III (22). From these studies it was deduced that the requirement for a high concentration of initiating purine could be circumvent by the use of sequence-specific dinucleotides (22). The results in Table 1 show that the dinucleotide monophosphates complementary to the DNA template markedly enhanced RNA synthesis with wheat germ RNA polymerase II, in agreement with previous results (28). Furthermore, in the case of transcription of poly(dT), where AMP incorporation is exclusively dependent on the presence of ApA, the mechanism of ATP and ApA utilization is sequential, indicating that both dinucleotide primer and triphosphate substrate must bind to the enzyme before reaction occurs. Therefore, it might be reasonably assumed that the elongation-translocation step is rate-limiting in the experimental conditions used. The results obtained in this steady-state kinetic study can be compared and discussed in the light of those reported for E.coli RNA polymerase, where they are best documented. Thus, for the latter enzyme: (a) the double reciprocal plots 1/v versus 1/(nucleoside triphosphate substrate) are usually linear when the initiation step is bypassed (6, 24, 30, 31), in contrast to some of the data in Figure 2.

(b) for DNA templates of alternating nucleotide sequence a so-called "ping-pong" kinetic model is obtained in which the enzyme oscillates alternately between two states, each of them is specific for a different substrate (6). This model predicts that the plots as shown in Figure 3 should be parallel lines (equations 1 to 5 in Ref. 6), which is not observed.
(c) under conditions where inhibition of RNA chain elongation by nucleoside triphosphates and derivatives occurs, linear plots of 1/v versus (inhibitor) are obtained, for which the slopes are proportional to inhibition constants (equations 6 to 11 in Ref. 6), in contrast to the curvature of some of the plots in Figure 4. Surprisingly, cordycepin triphosphate not only strongly inhibits AMP incorporation, as usually reported (18, 32), but also that of UMP and GMP on appropriate templates (Figure 5).
(d) for most of the noncomplementary nucleoside triphosphates and derivatives, inhibition constants have very close numerical values (6), i.e. the slopes of the plots 1/v versus inhibitor concentration are similar, in contrast to the results in Figures 4 and 5.

Therefore, it appears that the reaction of RNA chain elongation by wheat germ RNA polymerase II is rather complex. Apparent positive or negative cooperativity is observed for the transcription of homopolymer templates with respect to nucleotide substrates. One hypothesis to explain this kinetic behavior would be that several substrate molecules can bind to the enzyme before the reaction of incorporation of a nucleoside monophosphate occurs. Since the rate measurements relied solely on substrate incorporation into RNA, it should be stressed that we do not know whether these additional molecules, simply bind to the enzyme or are further utilized in as yet undefined reactions which could be of importance for the elongation step to occur. In this context, it has been shown that RNA polymerases from different sources may contain various nucleoside triphosphate-dependent enzymatic activities such as phosphohydrolases (23, 33, 34) or kinases (35). On the basis of numerous experimental results, the possibility that both procaryotic and eucaryotic RNA polymerases may contain several ligand binding sites has already been invoked (7-9, 36-40). The interaction between these ligand binding sites could provide a means of modulation of the rate of the transcription process. Thus, occupancy of the additional ligand binding sites could result in activation or inhibition in the rate of incorporation of a given nucleoside monophosphate, which would account for the results presented in Figures 2 and 4. A different explanation would be that the kinetic parameters of the enzyme for incorporation of a given nucleoside monophosphate into RNA are dependent on the length of the RNA chain (22, 41) and/or the nucleotide sequence

surrounding the complementary base of the DNA template (5, 42). The numerical values of the kinetic parameters obtained for the various template-primer combinations listed in Table 2 would agree with this notion. This would explain both the pausing (3, 5) and the non processivity (4) of the RNA polymerase. Further experiments on the kinetics of RNA chain elongation, using a reconstituted system capable of selective initiation such as recently reported (3) are necessary to clarify these points.

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