Kinetic co-operativity of wheat-germ RNA polymerase II with adenosine 5'-[$\beta\gamma$ -imido]triphosphate as substrate

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A kinetic study of productive RNA chain elongation indicates that adenosine 5'-[$\beta\gamma$ -imido]triphosphate can serve as substrate in reactions catalysed by purified wheat-germ RNA polymerase II on a poly[d(A-T)] template. However, in contrast with the results obtained with the natural substrate ATP, the double-reciprocal plots, $1/\text{velocity}$ versus $1/[\text{nucleotide}]$, are not linear but characteristic of apparent negative co-operativity. The extent of the kinetic co-operativity is modified when the reactions are conducted in the additional presence of fixed amounts of an alternative substrate such as ATP or inhibitors such as dATP or cordycepin triphosphate. Analogous results are obtained whether the reactions are carried out in the presence of Mg^{2+} or Mn^{2+} as the metal ion cofactor. However, the data show that with Mn^{2+} the RNA polymerase is less specific in substrate recognition than with Mg^{2+} . Tentative kinetic models are proposed to account for the rate measurements.

INTRODUCTION

Previous results have shown that purified wheat-germ RNA polymerase II is able to catalyse efficiently the condensation of ribonucleotides to dinucleotide primers, leading to trinucleotide product formation (Dietrich *et al.*, 1985, 1986). A kinetic study of the transition between the initiation complexes (those having formed a single phosphodiester bond) and the elongation complexes suggested the participation of additional nucleotides for the stabilization of transcription ternary complexes (Job *et al.*, 1987b), as proposed in related studies with *Escherichia coli* RNA polymerase (Shimamoto & Wu, 1980a,b). Interestingly, a phenomenological study of the first steps of transcription *in vitro* with a HeLa-cell extract, adenovirus DNA as template and dinucleotide primers (Luse *et al.*, 1987) also indicates many similarities between the purified plant enzyme and the cellular extract, which in addition to the RNA polymerase II does contain a number of transcription factors: (i) complexes involved in the formation of a single phosphodiester bond are unstable, (ii) complexes having formed at least two phosphodiester bonds have an increased stability, and (iii) substrate derivatives such as dATP (Luse *et al.*, 1987) or cordycepin triphosphate (Job *et al.*, 1987b) are utilized differently in the early steps than in the course of RNA chain elongation. An additional observation is the failure of adenosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppA), an ATP derivative showing altered sensitivity to hydrolysis of the γ - β bond (Yount *et al.*, 1971), to stabilize the mammalian transcription complexes (Luse *et al.*, 1987). However, adenosine 5'-[γ -thio]triphosphate was found to replace ATP with the same efficiency in reactions catalysed by wheat-germ RNA polymerase II (Job *et al.*, 1987b), yet this compound also shows an altered sensitivity to hydrolysis of the γ - β bond (see, for instance, Chabbert *et al.*, 1987).

In order to examine further the comparison between

the various transcription systems, we have investigated the possibility that the ATP analogue p[NH]ppA could interfere with some of the steps of RNA synthesis catalysed by purified wheat-germ RNA polymerase II, on a poly[d(A-T)] template. Since transcription *in vitro* with the purified plant enzyme has often been conducted in the presence of Mn^{2+} , whereas the reactions with the more complex transcription systems derived from nuclear extracts are usually carried out with Mg^{2+} as the metal ion cofactor, we also performed comparative kinetic measurements with these two bivalent metal ions. In the present paper we show that, in contrast with the results with the natural substrate ATP, the rate measurements as a function of [p[NH]ppA] lead to an apparent negative co-operative behaviour. The results could support the view that productive RNA chain elongation with the purified plant enzyme necessitates some specific requirement for ATP that cannot be fulfilled by p[NH]ppA. Minimal kinetic schemes are presented to account for these kinetic data.

MATERIALS AND METHODS

Reagents

Nucleotides and the dinucleoside monophosphates were from Sigma Chemical Co. and Boehringer. [α - ^{32}P]UTP (410 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) was from Amersham International. Poly[d(A-T)] was from P-L Biochemicals. All buffer components were reagent grade. Solutions were prepared with ultrapure water from a Millipore SQ system.

Wheat-germ RNA polymerase II was purified by the method of Jendrisak & Burgess (1975), as described previously (Job *et al.*, 1984; Dietrich *et al.*, 1985, 1986). Inhibition experiments with α -amanitin, a toxic compound that is highly specific for the class II RNA polymerases (Cochet-Meilhac & Chambon, 1974), yielded linear Dixon plots and an inhibition constant of

0.06±0.01 µg/ml, in good agreement with previously obtained values for plant RNA polymerases II (Jendrisak & Burgess, 1975; Guilfoyle & Jendrisak, 1978). Therefore the enzyme preparation is not significantly contaminated by the other classes of nuclear RNA polymerases.

Reaction assays

Unless otherwise noted in the Figure legends, the reaction mixtures contained 55 nM enzyme, 5 µg of poly[d(A-T)]/ml, 1.7 mM-U-A dinucleotide primer, 20 µM-[α-³²P]UTP (0.3–5 Ci/mmol) and appropriate amounts of unlabelled nucleotide substrates, ATP derivatives and metal salts (1.5 mM-MnCl₂ or 10 mM-MgCl₂). 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, 1.1 mM-dithiothreitol, 0.05 mM-EDTA, 0.05% (v/v) Triton X-100 and 1.5 mM-NaF. Final volumes were 10 µl, and assays were usually incubated for 60 min at 35 °C. Reactions were stopped by mixing the 10 µl reaction mixtures with 20 µl of stop buffer containing 1 mM-EDTA and 80% (v/v) formamide.

Activity measurements

Total RNA synthesis was measured by trichloroacetic acid precipitation of 10 µl reaction mixtures spotted on Whatman GF/C filters (Job *et al.*, 1984; Job *et al.*, 1987b). After eight washing steps of the filters, the background was less than 1/2000 of the input radioactivity. Consumption of substrates was less than 10%, ensuring steady-state conditions. In some instances, the poly[r(A-U)] chains synthesized were analysed for length distribution by high-resolution gel electrophoresis on 8 M-urea/50 mM-Tris/borate (pH 8.0)/15% polyacrylamide gels (0.03 cm × 30 cm × 40 cm) as described previously (Dietrich *et al.*, 1985). Electrophoresis was conducted at a constant 80 W until the marker dye, Xylene Cyanol, had migrated for 25 cm. The gels were autoradiographed at -70 °C with Fuji RX films with Cronex Li-Plus intensifying screens from Dupont. As previously observed (Durand *et al.*, 1982; Dietrich *et al.*, 1985; Job *et al.*, 1987b), ladders of RNA products of increasing lengths were obtained, which terminated after the steps of UMP incorporation. The analysis of *P*, the probability of releasing the poly[r(A-U)] products, was conducted from densitometric recordings of the ladders (for 12 bands centred around the position of Xylene Cyanol) by the procedure of McClure & Chow (1980). With 1.5 mM-MnCl₂ and 0.5 µM-, 0.75 µM-, 1 µM-, 1.5 µM-, 2.5 µM-, 5 µM-, 7.5 µM-, 10 µM- and 15 µM-ATP or -adenosine 5'-[γ-thio]triphosphate or 5 µM-, 7.5 µM-, 10 µM-, 15 µM-, 25 µM-, 50 µM-, 75 µM-, 100 µM- and 150 µM-p[NH]ppA, we calculated that *P* = 0.17±0.03.

Abortive synthesis of U-A-U was quantified after t.l.c. on poly(ethyleneimine)-cellulose sheets of 2 µl portions (Randerath & Randerath, 1967) with 1 M-formic acid/0.1 M-LiCl. Under these conditions U-A-U migrated with an *R_f* of 0.4, whereas unused UTP remained bound at the origin. The synthesis of U-A-U was absolutely dependent on the presence of enzyme, poly[d(A-T)] template, bivalent cation and U-A primer in the reaction assays. No trinucleotide product was detected in complete reaction mixtures lacking the U-A primer. After autoradiography, all spots containing radioactive material were cut out and counted for radioactivity (Job *et al.*, 1984).

Table 1. Steady-state kinetic parameters for nucleotide substrates associated with poly[r(A-U)] formation

Reaction assays were prepared as described in the Materials and methods section. The labelled substrate was UTP at 20 µM with a final specific radioactivity of 1.12 × 10⁶ c.p.m./nmol. Incubation was for 60 min at 35 °C. The concentration of ATP (a) or p[NH]ppA (b) was varied in the range 0–30 µM or 0–300 µM respectively. The bivalent cation was Mn²⁺ at 1.5 mM or Mg²⁺ at 10 mM. Poly[r(A-U)] synthesis was quantified by the use of trichloroacetic acid precipitation of 10 µl reaction mixtures spotted on GF/C filters.

(a) ATP as substrate*

Conditions	<i>K_m^{app}</i> (µM)	<i>V_{max}</i> (c.p.m./60 min)
MnCl ₂	2.5	20944
MgCl ₂	3	23592

(b) p[NH]ppA as substrate†

Conditions	<i>P</i> ₁ (µM)	<i>A</i> ₁	<i>P</i> ₂ (µM)	<i>A</i> ₂
MnCl ₂	2.6	0.18	139	1.7
MgCl ₂	8.9	0.17	213	0.6

* The experimental data are presented in Figs. 1(a) and 1(c).

† The experimental data are presented in Figs. 1(b) and 1(d). The parameters correspond to eqn. (1). For both MnCl₂ and MgCl₂ the rate measurements obtained by varying the p[NH]ppA concentration were normalized to the corresponding *V_{max}* values determined with ATP as substrate and listed in (a).

Calculations and curve-fitting

From a compilation of 300 triplicate measurements made by using the GF/C-filter method (the rate values, *v*_i, ranged from 79 to 593052 c.p.m.), we calculated the following relationship:

$$\log(\text{Deviation of the observed rate values}) = (0.87 \pm 0.03) \times \log(\text{observed rate}) - (0.76 \pm 0.11)$$

Owing to the structure of the deviation, the kinetic data were weighted by 1/*v*_i² (Wong, 1975) when processed with a program providing an iterative fit to the appropriate equation, by using the Gauss-Newton method (Cleland, 1979). Calculations and plots were effected with a Plessey 6623 minicomputer system provided with a HP 7475 A plotter.

RESULTS

p[NH]ppA as substrate

RNA synthesis was carried out in the presence of ATP or p[NH]ppA as the substrate whose concentration was varied. UTP concentration was held constant at 20 µM. The metal salt was MnCl₂ at 1.5 mM in Figs. 1(a) and 1(b) or MgCl₂ at 10 mM in Figs. 1(c) and 1(d). With the natural substrate ATP, the double-reciprocal plots,

Fig. 1. Effect of ATP or p[NH]ppA concentration on poly[d(A-T)]-dependent poly[r(A-U)] synthesis

Transcription assays were prepared as described in the Materials and methods section, in the presence of wheat-germ RNA polymerase II, poly[d(A-T)] as template, U-A as primer and $20 \mu\text{M}$ - $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (1.12×10^6 c.p.m./nmol) as substrate. The bivalent cation was Mn^{2+} at 1.5 mM (a and b) or Mg^{2+} at 10 mM (c and d). In addition, before incubation for 60 min at 35°C , they received various amounts of ATP (a and c) or p[NH]ppA (b and d). Poly[r(A-U)] synthesized under these experimental conditions was quantified by the use of trichloroacetic acid precipitation of reaction mixtures spotted on GF/C filters. The rate measurements are expressed as double-reciprocal plots. The lines in (a) and (c) were obtained by linear least-squares analysis. The kinetic parameters are listed in Table 1(a). The data in (b) and (d) were normalized to the corresponding V_{max} values determined with ATP as the varied substrate, fitted by using eqn. (1) and a non-linear least-squares program, then plotted in a double-reciprocal form. Continuous lines are the non-linear correlations derived from eqn. (1), for the best-fit parameters listed in Table 1(b). The data in (c) may also be fitted by using eqn. (1), for the following values of the parameters: $A_1 = 0.33$; $P_1 = 1 \mu\text{M}$; $A_2 = 1.1$; $P_2 = 13.3 \mu\text{M}$ (not shown).

$1/\text{velocity}$ versus $1/[\text{ATP}]$, are reasonably linear, although there is a slight but significant deviation from linearity with Mg^{2+} (Fig. 1c). Linear least-squares analysis of these data yielded the apparent K_m values for ATP as substrate listed in Table 1(a). They are in the region of $3 \mu\text{M}$. In contrast, the double-reciprocal plots obtained with p[NH]ppA as the variable substrate are no longer linear and exhibit an apparent negative cooperativity, with either Mg^{2+} or Mn^{2+} (Figs. 1b and 1d). These data have been normalized to the corresponding V_{max} values determined with ATP as substrate (Table 1a), and fitted by using empirical eqn. (1):

$$v/V_{\text{max}} = \frac{A_1 \cdot [\text{p[NH]ppA}]}{P_1 + [\text{p[NH]ppA}]} + \frac{A_2 \cdot [\text{p[NH]ppA}]}{P_2 + [\text{p[NH]ppA}]} \quad (1)$$

Use of a non-linear least-squares program led to the non-linear correlations shown in Figs. 1(b) and 1(d) and the best-fit parameters given in Table 1(b).

The results in Fig. 1 and Table 1 also show the following. (i) Productive poly[r(A-U)] synthesis requires much larger p[NH]ppA concentrations than when the natural substrate ATP is used. (ii) With MnCl_2 the maximal rate value ($A_1 + A_2$; Table 1b) in the presence of p[NH]ppA is about twice that with ATP, which argues against the possibility that contamination of p[NH]ppA by ATP contributes significantly to the observed behaviour. Furthermore, it can be easily shown that, for the case of an enzyme having a single substrate-binding site, this kind of contamination can by no way result in the curvature observed in Figs. 1(b) and 1(d), because

Fig. 2. Effect of p[NH]ppA concentration on poly[d(A-T)]-dependent U-A-U and poly[r(A-U)] synthesis

Transcription assays leading to labelled U-A-U formation were prepared as described in Fig. 1 legend and in the Materials and methods section. The bivalent cation was Mn^{2+} at 1.5 mM. In addition, before incubation for 60 min at 35 °C, they received various amounts of unlabelled p[NH]ppA. The labelled substrate was UTP at 20 μM with a final specific radioactivity of 1.12×10^6 c.p.m./nmol. The poly[r(A-U)] synthesis (○) corresponds to the data in Fig. 1(b). The continuous line is derived from the computer simulation of eqn. (1), for the values of the parameters in Table 1(b). The U-A-U synthesis (●) was quantified after t.l.c. on poly(ethyleneimine)-cellulose sheets, as described in the Materials and methods section. The rate measurements were normalized to the V_{max} value determined in the absence of p[NH]ppA (1.6 pmol of UMP incorporated into U-A-U). The continuous line is the computer simulation of eqn. (2), for the following values of the parameters: $P'_1 = 0.084 \mu M^{-1}$; $P'_2 = 0.17 \mu M^{-1}$.

Fig. 3. Effect of ATP on poly[d(A-T)]-dependent poly[r(A-U)] synthesis in the presence of p[NH]ppA

Reaction mixtures leading to poly[r(A-U)] synthesis were prepared as described in Fig. 1 legend and in the Materials and methods section. p[NH]ppA was the varied substrate. The labelled substrate was UTP at 20 μM with a final specific radioactivity of 0.79×10^6 c.p.m./nmol in (a) or 0.7×10^6 c.p.m./nmol in (b) and (c). The bivalent cation was Mg^{2+} at 10 mM in (a) or Mn^{2+} at 1.5 mM in (b) and (c). Reaction assays were incubated for 60 min at 35 °C in the absence or in the presence of ATP. The ATP concentration was: (a) 0 μM (○), 0.5 μM (●) or 2.5 μM (◇); (b) 0 μM (○), 1 μM (●) or 5 μM (◇). In (a) the curves were calculated from non-linear least-squares fits of the data to eqn. (1). The whole data in (b) have been fitted to eqn. (4). The best-fit parameters yielding the non-linear correlations are $a_1 = 950$, $a_2 = 51$, $a_3 = 3610$, $a_4 = 1160$, $a_5 = 0.4$, $a_6 = 4.24 \times 10^{-3}$, $a_7 = 0.36$ and $a_8 = 72 \times 10^{-3}$. (c) The data presented in (b) are analysed in the form of Hill plots. The symbols have the same significance as in (b). The straight lines were determined by linear least-squares analysis. The Hill coefficient h is 0.7 ± 0.016 (○), 0.45 ± 0.046 (●) and 0.3 ± 0.03 (◇).

Fig. 4. Effect of dATP on poly[d(A-T)]-dependent poly[r(A-U)] synthesis in the presence of p[NH]ppA

Reaction mixtures leading to poly[r(A-U)] synthesis were prepared as described in Fig. 1 legend and in the Materials and methods section, in the presence of p[NH]ppA as the varied substrate. The labelled substrate was UTP at 20 μM with a final specific radioactivity of 4.97×10^6 c.p.m./nmol in (a) or 2.42×10^6 c.p.m./nmol in (b). The bivalent cation was Mg^{2+} at 10 mM in (a) or Mn^{2+} at 1.5 mM in (b). Reaction assays were incubated for 60 min at 35 $^{\circ}\text{C}$ in the absence or in the presence of dATP. The dATP concentration was: (a) 0 μM (●) or 25 μM (○); (b) 0 μM (●) or 12.5 μM (○). The curves were calculated from non-linear least-squares fits of the data to eqn. (1).

Fig. 5. Effect of cordycepin triphosphate on poly[d(A-T)]-dependent poly[r(A-U)] synthesis in the presence of p[NH]ppA

Reaction mixtures leading to poly[r(A-U)] synthesis were prepared as described in Fig. 1 legend and in the Materials and methods section, in the presence of p[NH]ppA as the varied substrate. The labelled substrate was UTP at 20 μM with a final specific radioactivity of 0.88×10^6 c.p.m./nmol in (a) or 0.66×10^6 c.p.m./nmol in (b). The bivalent cation was Mg^{2+} at 10 mM in (a) or Mn^{2+} at 1.5 mM in (b). Reaction assays were incubated for 60 min at 35 $^{\circ}\text{C}$ in the absence or in the presence of cordycepin triphosphate. The 3'-dATP concentration was: (a) 0 μM (●) or 0.1 μM (○); (b) 0 μM (●), 0.1 μM (○) or 0.3 μM (▲). In (a) the curves were calculated from non-linear least-squares fits of the data to eqn. (1). The whole data in (b) have been fitted to eqn. (5) for the best-fit parameters $a'_1 = 1050$, $a'_2 = 250$, $a'_3 = 0.92$, $a'_4 = 0.03$, $a'_5 = 393$ and $a'_6 = 31$. Attempts to fit the data with a simplest equation containing only five parameters were unsatisfactory since mathematical convergence could only be obtained for negative values of some of the parameters and with worst sum of the residual squares. The inset in (b) shows the data in the form of a Hill plot. Linear least-squares analysis yielded the following values of the Hill coefficient h : 0.8 ± 0.02 (●), 1.09 ± 0.03 (○) and 1.13 ± 0.03 (▲).

both the K_m^{app} and the V_{max} values for p[NH]ppA would be affected, but the enzyme would still obey Michaelis-Menten kinetics. (iii) The P_1 and P_2 values (i.e. the apparent K_m values) in Table 1(b) for the ATP analogue are smaller with MnCl_2 than with MgCl_2 , although the nature of the metal ion cofactor has no significant effect on the apparent K_m values for ATP utilization.

Effect of p[NH]ppA concentration on U-A-U synthesis

Reaction mixtures were prepared in the presence of U-A as primer and [α - ^{32}P]UTP as substrate, thereby leading to labelled U-A-U synthesis. Various amounts of unlabelled p[NH]ppA were added before incubation at 35 $^{\circ}\text{C}$. In these experiments the metal ion cofactor was

Fig. 6. Inhibitory effect of cordycepin triphosphate on poly[d(A-T)]-dependent poly[r(A-U)] synthesis in the presence of p[NH]ppA

Reactions mixtures leading to poly[r(A-U)] synthesis were prepared as described in Fig. 1 legend and in the Materials and methods section, in the presence of fixed amounts of p[NH]ppA, and 10 mM-MgCl₂. The labelled substrate was UTP at 20 μM with a final specific radioactivity of 2.26×10^6 c.p.m./nmol. The concentration of cordycepin triphosphate was varied in the range 0–1.5 μM. The results are presented in the form of Dixon plots. The straight lines were obtained by linear least-squares analysis. The p[NH]ppA concentration was: 9.37 μM (▲); 18.75 μM (◇); 37.5 μM (■); 75 μM (○); 300 μM (●).

Mn²⁺ since, as we have noted previously (Dietrich *et al.*, 1986; Job *et al.*, 1987a), reactions of abortive elongation in the presence of Mg²⁺ are too low to allow quantification. Increasing the p[NH]ppA concentration is associated with the following effects (Fig. 2). (i) There is a decrease in the rate of U-A-U synthesis and an increase in the rate of poly[r(A-U)] formation. (ii) At saturating p[NH]ppA concentrations (above 100 μM) about 40 % of U-A-U still remains synthesized. Therefore a significant proportion of U-A-U escapes elongation by p[NH]ppA, and this to a higher extent than with the natural substrate ATP. In the latter case, at saturating [ATP], there is only 20 % U-A-U synthesis (Job *et al.*, 1987b). This precludes the possibility that the results in Figs. 1 and 2 could be simply explained by a direct competition between p[NH]ppA and U-A primer for initiation of RNA synthesis. (iii) The two rate plots in Fig. 2 do not vary concomitantly: thus the apparent K_m for p[NH]ppA utilization is of the order of 6 μM for the U-A-U reaction, compared with an apparent value of over 80 μM for the poly[r(A-U)] rate plot.

The data in Fig. 2 have been fitted by using eqn. (1) for poly[r(A-U)], with the best-fit parameters listed in Table 1(b), and for U-A-U:

$$v/V = \frac{1 + P'_1 \cdot [p[NH]ppA]}{1 + P'_2 \cdot [p[NH]ppA]} \quad (2)$$

where V stands for the maximal velocity of U-A-U synthesis measured in the absence of p[NH]ppA. The best fit is obtained for the values of the parameters in the legend to Fig. 2.

Effect of ATP or dATP on poly[r(A-U)] synthesis in the presence of p[NH]ppA

Experiments such as those presented in Figs. 1(b) and 1(d) were performed but in the additional presence of fixed amounts of ATP (Fig. 3) or dATP (Fig. 4).

With either Mn²⁺ or Mg²⁺ the rate of poly[r(A-U)] synthesis is enhanced in the presence of fixed amounts of the alternative substrate ATP (Fig. 3). One can also observe that increasing the ATP concentration is associated with a dramatic increase in the extent of apparent negative co-operativity with increasing [p[NH]ppA], as indicated by Hill replot of the data in Fig. 3(c). Activation of RNA synthesis is obtained at lower ATP concentrations with Mg²⁺ than with Mn²⁺ as the bivalent metal ion, which may be related to the fact that the plot in Fig. 1(c) shows a slight deviation from linearity.

dATP at 25 μM has no effect on the reaction of RNA synthesis in the presence of p[NH]ppA and Mg²⁺. In contrast, the same compound at 12.5 μM is slightly inhibitory in reactions conducted with Mn²⁺. Furthermore, inhibition of poly[r(A-U)] synthesis is accompanied by a diminution in the extent of apparent negative co-operativity (Fig. 4).

Effect of cordycepin triphosphate on poly[r(A-U)] synthesis in the presence of p[NH]ppA

Cordycepin triphosphate (3'-dATP) is a competitive inhibitor of ATP in the reaction of poly[r(A-U)] synthesis catalysed by wheat-germ RNA polymerase II on a poly[d(A-T)] template (Dietrich *et al.*, 1985; Job *et al.*, 1987b). 3'-dATP is also a strong inhibitor of poly[r(A-U)] synthesis in the presence of p[NH]ppA (Fig. 5). Similarly to what is observed with dATP (Fig. 4), the inhibition is accompanied by a decrease in the extent of apparent negative co-operativity, as evidenced by the Hill replot of the data in the inset of Fig. 5(b). However, the inhibitory effect is greater with MnCl₂ than with MgCl₂.

Poly[r(A-U)] was synthesized in the presence of poly[d(A-T)], 10 mM-MgCl₂, 1.7 mM-U-A, 20 μM-[α-³²P]UTP, various fixed amounts of p[NH]ppA and 3'-dATP, which was varied in the concentration range 0–1.5 μM. The results are shown in Fig. 6, in the form of Dixon plots. These plots are linear.

DISCUSSION

In view of the strict requirement for hydrolysable ATP in the early steps of reactions of transcription catalysed by transcription systems derived from nuclear extracts (Bunick *et al.*, 1982; Sawadogo & Roeder, 1984; Luse *et al.*, 1987), we have examined the possibility that p[NH]ppA could serve as substrate for purified wheat-germ RNA polymerase II on a poly[d(A-T)] template. The remarkable features encountered during this steady-state kinetic study are: (i) poly[r(A-U)] synthesis necessitates at least 10-fold greater concentrations of p[NH]ppA than of ATP, indicating that some of the steps in transcription are somehow impeded in the presence of the substrate analogue; (ii) a greater proportion of U-A-U synthesized in the abortive elongation reaction escapes elongation by p[NH]ppA (Fig. 2), as compared with the results obtained in the presence of ATP (Job *et al.*, 1987b), suggesting that

p[NH]ppA is less efficient than ATP in the formation of stable transcription complexes; (iii) the rate plots, $1/\text{velocity}$ versus $1/[\text{p[NH]ppA}]$, are curvilinear and characteristic of apparent negative kinetic co-operativity, as opposed to the linear plots obtained with ATP as the varied substrate; (iv) the extent of apparent negative co-operativity varies when reactions are conducted in the additional presence of an alternative substrate such as ATP or inhibitors such as dATP or 3'-dATP.

However, other general properties of the reaction of transcription are common to both ATP (the present study; Dietrich *et al.*, 1985; Job *et al.*, 1987b) and p[NH]ppA. Thus the rate of U-A-U trinucleotide formation is diminished in the presence of the elongating substrate, and the rate plots corresponding to disappearance of abortive products and formation of poly[r(A-U)] are not characterized by same apparent K_m value for the elongating substrate.

As reviewed by Neet (1980), Neet & Ainslie (1980) and Ricard & Cornish-Bowden (1987), there are at least two different possibilities to explain the kinetic results in Fig. 1. On one side, the negative kinetic co-operativity may arise from the existence of at least two nucleotide-binding sites on the enzyme molecule. For the sake of simplicity, we only consider the situation of two non-identical binding sites. On the other hand, a slow conformational change of the enzyme might produce 'co-operative' kinetics deviating from Michaelis-Menten behaviour. These two situations are depicted below.

For two nucleotide-binding sites:

where E refers to the transcription complex, S to p[NH]ppA and P to poly[r(A-U)].

For a hysteretic enzyme:

where E and E' refer to two conformational states of the transcription complex having different reactivities.

Under quasi-equilibrium conditions scheme (I) results in rate eqn. (3) (Laidler & Bunting, 1973):

$$v = \frac{V_{\max}([S]^2 + a[S])}{[S]^2 + b[S] + c} \quad (3)$$

The latter is fundamentally equivalent to empirical eqn. (1) used to fit the experimental data in Fig. 1. Eqn. (3) is also obtained under steady-state hypothesis for the hysteretic scheme (II), provided that the isomerization steps and the off constants of the substrate are 'slow' compared with the maximal velocity of the reaction (Neet & Ainslie, 1980).

The rate plots, $1/\text{velocity}$ versus $1/[\text{p[NH]ppA}]$, are affected by the additional presence in the transcription

assay of ATP, dATP or 3'-dATP (Figs. 3-5). Furthermore, the Dixon plots in Fig. 6 are linear, indicating the participation of a single molecule of cordycepin triphosphate in the inhibition of poly[r(A-U)] formation. Assuming that the enzyme forms E and/or SE in the two-sites model, or that the forms E and/or E' in the hysteretic model can bind the effector, allows one to deduce rate equations predicting modifications of the amplitude of the kinetic co-operativity, such as that observed experimentally, by using Hill-slope (Neet & Ainslie, 1980) or Γ -coefficient (Ricard *et al.*, 1974) determinations. These equations are of the following form.

With an alternative substrate such as ATP:

$$v = \frac{a_1[S] + a_2[S]^2 + a_3[S'] + a_4[S][S']}{1 + a_5[S] + a_6[S]^2 + a_7[S'] + a_8[S][S']} \quad (4)$$

where [S] stands for [p[NH]ppA], [S'] stands for [ATP] and a_i ($i = 1-8$) are collections of rate constants.

With an inhibitor such as dATP or 3'-dATP:

$$v = \frac{a'_1[S] + a'_2[S]^2}{1 + a'_3[S] + a'_4[S]^2 + a'_5[I] + a'_6[I][S]} \quad (5)$$

where S is p[NH]ppA, I is the inhibitor and a'_i ($i = 1-6$) are collections of rate constants.

Eqn. (5), which gives an account of the linear Dixon plots in Fig. 6, has been used to fit the whole data in Fig. 5(b). Similarly, the whole data in Fig. 3(b) can be satisfactorily fitted by using eqn. (4). It is noteworthy that this complex fitting allows one to deduce the apparent K_m of the reaction with ATP in the absence of p[NH]ppA as $1/a_7 = 2.8 \mu\text{M}$ (Fig. 3b), which compares well with the value of $2.5 \mu\text{M}$ given in Table 1(a). Therefore the present results do not allow a clear distinction between the above models. As Neet & Ainslie (1980) have pointed out, since hysteretic co-operativity can only occur in kinetic studies, not in equilibrium techniques, direct-nucleotide-binding studies are needed to clarify this point. There are indications that may support either of the two above models.

Thus several reports have implicated the large polypeptides of eukaryotic RNA polymerases in the binding of nucleotide substrates (Valenzuela *et al.*, 1978; Bateman & Nicholson, 1984; Grachev *et al.*, 1986), in agreement with sequence data on the 140 kDa subunit of yeast RNA polymerase II (Sweetser *et al.*, 1987). However, a nucleotide-binding site was also identified on the 37 kDa subunit of calf-thymus RNA polymerase II by using photoaffinity analogues of ATP and GTP (Freund & McGuire, 1986). From kinetic studies of dinucleotide-primed RNA synthesis catalysed by *E. coli* RNA polymerase, Shimamoto & Wu (1980a,b) have proposed that two nucleotide molecules may bind to the enzyme-DNA complex during the initiation process and that the additional nucleotide may be crucial in driving the initiation reaction or in discriminating between abortive or productive initiation.

On the other hand, in order to explain the results in Fig. 2, as well as the corresponding results obtained in the presence of ATP (Job *et al.*, 1987b), at least one additional rate-limiting step must be considered before the step of condensation of the purine nucleotide. Otherwise, the extent of U-A-U formation should decrease to a zero value at saturating elongating substrate concentrations, which is not observed. It is possible that

such an isomerization step could form the basis for the availability of an additional state of the enzyme that has different kinetic properties, as implied in the hysteretic model described by Neet & Ainslie (1980). Isomerization steps of the ternary transcription complexes have also been invoked to explain the non-processive synthesis of poly[r(A-U)] catalysed by wheat-germ RNA polymerase II (Durand *et al.*, 1982), or to account for the kinetics of formation of the first phosphodiester bonds in poly[r(A-U)] synthesis catalysed by *E. coli* RNA polymerase (Shimamoto & Wu, 1980*a,b*). A memory effect has been postulated to account for the variation of the polymerization/excision ratio or for the change in processivity in reactions of DNA replication catalysed by *E. coli* DNA polymerase I (Papanicolaou *et al.*, 1984, 1986). In this context, it may be noted that a change of the regime of processivity is also well established for transcription *in vitro* with *E. coli* RNA polymerase: during the process of abortive initiation, the enzyme can form and release short oligonucleotides, even in the presence of all four ribonucleoside triphosphates, rather than to continue to elongate RNA chains (for a review see von Hippel *et al.*, 1984). Our results, as well as those of Luse *et al.* (1987) and of Flynn *et al.* (1987), indicate that RNA polymerases of class II behave similarly to prokaryotic RNA polymerase in that, before being committed to the elongation mode, the enzymes pass through a stage in which transcripts are produced abortively. For wheat-germ RNA polymerase II it must be stressed, however, that the maximum rate of catalysis is quite low (Jendrisak & Burgess, 1975), the highest value being 2 s^{-1} with poly[d(C)] as template (Job *et al.*, 1982). It is then hardly probable that the substrate off constants might be in this range and allow the hysteretic model to give a complete account of the enzyme behaviour. However, only fast kinetic studies will answer this question.

In conclusion, the results presented allow a further comparison of the catalytic properties of purified wheat-germ RNA polymerase II with more complex class II transcription systems derived from nuclear extracts. We found that RNA synthesis, especially with Mg^{2+} as the metal ion cofactor, is not a favoured process in the presence of p[NH]ppA as substrate. This would agree with the general finding that formation of stable initiation transcription complexes cannot be achieved in the presence of the ATP analogue (Bunick *et al.*, 1982; Sawadogo & Roeder, 1984; Luse *et al.*, 1987). Similar conclusions have been reached for transcription of human mitochondrial DNA in a HeLa-cell mitochondrial lysate (Narasimhan & Attardi, 1987). In this latter study it was also shown that p[NH]ppA greatly stimulated the transcription of mitochondrial DNA in the presence of a low amount of ATP (Narasimhan & Attardi, 1987). The catalytic properties of purified wheat-germ RNA polymerase II in the process of transition from initiation to elongation complexes, as documented previously (Dietrich *et al.*, 1985, 1986; Job *et al.*, 1987*b*) and in the present study, seem thus very similar to those of the RNA polymerase in the cellular extracts (Luse *et al.*, 1987).

Another interesting point relies upon the comparison of the results obtained with Mn^{2+} or Mg^{2+} as the metal ion cofactor. The present results indicate that in the presence of Mn^{2+} the wheat-germ enzyme has the propensity to become less selective in substrate recogni-

tion than in the presence of Mg^{2+} . With *E. coli* RNA polymerase, bivalent metal ions other than Mg^{2+} increase misincorporation (Niyogi & Feldman, 1981) or modify the stereospecificity in nucleotide binding (Armstrong *et al.*, 1979).

Note added in proof (received 23 February 1988)

The following papers dealing with mechanistic studies of transcription catalysed by HeLa-cell extracts (abortive initiation and purine β - γ bond hydrolysis requirements) have appeared since this paper was submitted: Luse & Jacob (1987) and Rappaport & Weinmann (1987).

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