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A slow kinetic transient in RNA synthesis catalysed by wheat-germ RNA polymerase II

Claudette JOB, Laure DE MERCOYROL and Dominique JOB*
Centre de Biochimie et de Biologie Moléculaire, Centre National de la Recherche Scientifique, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex 9, France

Progress curves of U-A-primed RNA synthesis catalysed by wheat-germ RNA polymerase II on a poly[d(A-T)] template exhibit a slow burst of activity. In contrast, the progress curves of single-step addition of UMP to U-A primer in the abortive elongation reaction do not exhibit the slow burst of activity. The correlation between the kinetic transient in the productive pathway of RNA synthesis and the rate of abortive elongation is suggestive of the occurrence of a slow conformational change of the transcription complex during the transition from abortive to productive elongation. The exceptional duration of the transient burst (in the region of 4 min) may suggest a transition of a hysteretic type.

INTRODUCTION

Prokaryotic (Oen & Wu, 1978; Sylvester & Cashel, 1980) as well as eukaryotic (Lescure *et al.*, 1981; Vaisius & Wieland, 1982; Dietrich *et al.*, 1985; Luse & Jacob, 1987) RNA polymerases catalyse DNA-dependent single-step addition of a ribonucleotide to a dinucleotide primer, in a process termed abortive elongation. In particular, we showed that cordycepin triphosphate acts as a substrate for wheat-germ RNA polymerase II in the poly[d(A-T)]-dependent synthesis of A-U-A primed by A-U, with an apparent K_m very similar to that measured for the corresponding reaction carried out in the presence of the natural substrate ATP (Dietrich *et al.*, 1985). Surprisingly, the derivative becomes a very potent inhibitor, with a K_i in the order of one-twentieth the apparent K_m for ATP, in the reaction of poly[r(A-U)] synthesis with ATP and UTP as substrates. Apparently, the inhibitory behaviour is not linked to the condensation of cordycepin triphosphate at the 3'-ends of RNA molecules, which could have prevented further incorporation of ribonucleotides, owing to the absence of a 3'-hydroxy group in the ATP derivative (Dietrich *et al.*, 1985; Job *et al.*, 1987*b*). Luse *et al.* (1987) have reported a similar behaviour for dATP, which was found to support different extents of incorporation into RNA transcripts during the course of RNA synthesis catalysed by a HeLa-cell extract. These results would suggest that the eukaryotic RNA polymerases may occur under different states having different reactivities towards the nucleotide substrates during the progress of catalytic polymerization. Consistent with this idea, a recent kinetic study with adenosine 5'-[$\beta\gamma$-imido]triphosphate as substrate in RNA synthesis catalysed by wheat-germ RNA polymerase II also suggested the possibility of 'slow' hysteretic transitions between different states of the transcription complex (Job *et al.*, 1988). Modifications of the ionic conditions in reaction assays containing wheat-germ RNA polymerase II have been correlated with alterations of the catalytic properties of the transcription complex such that a marked inhibition of abortive elongation was always associated with an increased length of RNA chains synthesized in the productive pathway (Dietrich *et al.*, 1986). As outlined above, we postulated that different states of the transcription complex, having different properties and processivities, may be generated depending on the nature of the DNA template and/or the experimental conditions.

In the present paper we show that with wheat-germ RNA polymerase II poly[r(A-U)] synthesis primed by U-A on a poly[d(A-T)] template exhibits a slow burst of activity, extending over about 4 min. In contrast, under the same experimental conditions but in the absence of ATP, the progress curve of U-A-U synthesis is linear. The transient burst of activity in RNA synthesis becomes hardly detectable under experimental conditions known to be associated with a marked inhibition of the process of abortive elongation. These observations are suggestive of the occurrence of a slow transition of the transcription complex in the commitment to productive elongation.

MATERIALS AND METHODS

Reagents

Nucleoside triphosphates and the dinucleoside monophosphates were purchased from Sigma Chemical Co. [^{3}H]UTP (49 Ci/mmol, 1 Ci = 3.7×10^{10} Bq) and [α-^{32}P]UTP (410 Ci/mmol) were from Amersham International. Wheat-germ RNA polymerase IIA was purified by the method of Jendrisak & Burgess (1975), as described previously (Dietrich *et al.*, 1985, 1986).

Reaction assays

Final volumes were 450 μl. Incubations were effected in 1.5 ml stoppered Eppendorf tubes at 35 °C. Unless otherwise noted in the Figure legends, the reaction mixtures leading to poly[r(A-U)] synthesis contained 17.5 μM-poly[d(A-T)], 0.9 mM-U-A, 30 nM-enzyme, 20 μM each of ATP and UTP, 1 μM-[^{3}H]UTP and 1.5 mM-$MnCl_2$ in transcription buffer as described in Job *et al.* (1987*b*). Portions (5 μl) were spotted at timed intervals on Whatman GF/C filters, then quantified for RNA synthesis by the use of trichloroacetic acid precipitation

* To whom correspondence should be addressed.

(Job *et al.*, 1987*b*). For abortive synthesis of U-A-U the conditions were the same, except that the reaction assays contained a single nucleotide substrate, [α-^{32}P]UTP at 5 μM. Portions (2 μl) were spotted at timed intervals on poly(ethyleneimine)-cellulose sheets, which were developed with 1 M-formic acid/0.1 M-LiCl. All spots containing radioactivity were cut out and counted for radioactivity (Job *et al.*, 1987*b*).

Calculations and curve-fitting

Owing to the structure of the deviation of the observed values (v_i), the kinetic data were weighted by $1/v_i^2$ (Wong, 1975) when processed with a program providing an iterative fit to eqn. (1) by using the Gauss–Newton method (Cleland, 1979) or when fitted by linear least-square analysis. For all progress curves, the following equation was used:

$$P = A(1-e^{-\lambda \cdot t})+v_s \cdot t \quad (1)$$

where P stands for the amount of UMP incorporated into product, t is the time in min, A and λ define the transient burst of activity, and v_s is the steady-state rate of product formation. The superiority of eqn. (1) compared with a linear relationship was estimated by the procedure of Bardsley (1986), by computing the variance ratio F from the square of the residuals using eqn. (1) versus the square of the residuals using a linear relationship. Whenever the program failed to fit the data with eqn. (1), then these data were simply fitted by linear least-squares analysis.

RESULTS

Time course of U-A-primed RNA synthesis

Under the standard preincubation conditions the reaction assay in transcription buffer contained enzyme, poly[d(A-T)] template, U-A primer and $MnCl_2$. The duration of the preincubation at 35 °C was fixed at 10 min, allowing all components to equilibrate to that temperature. RNA synthesis was initiated by addition of labelled nucleotide substrates (also preincubated for 10 min at 35 °C). The time course of typical experiments is shown in Fig. 1. Although the experimental points are somewhat scattered, a systematic deviation from a linear relationship seems to exist. Thus the progress curves exhibit a slow burst at the start of the reaction. In these manual mixing and sampling experiments it was difficult to improve the quality of the data. However, the simplest equation fitting these data satisfactorily is eqn. (1). Within the limits of the experimental errors the coefficient λ in eqn. (1) does not seem to depend on the enzyme concentration (Fig. 1 and Table 1), though the burst is hardly detected at low enzyme concentrations. As expected, the steady-state rate v_s varies with enzyme concentration. Furthermore, the magnitude of A considerably exceeds the concentration of enzyme in the reaction assay. For example, at 30 nM-enzyme (0.15 pmol of enzyme/5 μl of reaction mixture) the value of A is 1.6±0.2 pmol (UMP + AMP) incorporated (Fig. 1 and Table 1). From this we conclude that the transient burst involves a catalytic reaction of nucleotide incorporation into RNA. Control experiments showed that the curvature of the plots in Fig. 1 was not due to enzyme inactivation during the assay: there was no loss of enzyme activity on using the standard procedure of

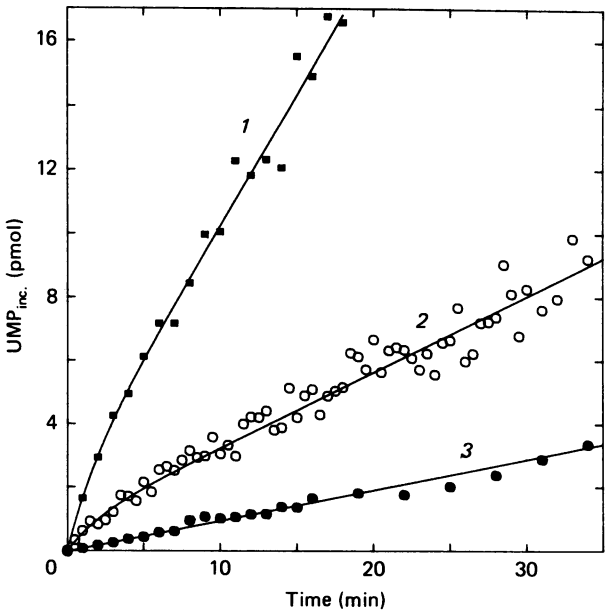

Fig. 1. Progress curves for U-A-primed RNA synthesis

Reactions were performed as described in the Materials and methods section, in the presence of 92 nM- (■, curve *1*), 30 nM- (○, curve *2*) or 9 nM- (●, curve *3*) wheat-germ RNA polymerase II. The amount of UMP incorporated into poly[r(A-U)] is expressed for 5 μl portions. The standard procedure involves a 10 min preincubation at 35 °C of enzyme, 17.5 μM-poly[d(A-T)], 0.9 mM-U-A and 1.5 mM-$MnCl_2$ in transcription buffer. The reaction was started by addition of 20 μM-ATP and 21 μM-[^{3}H]UTP {1 pmol of UMP incorporated into poly[r(A-U)] corresponded to 650 c.p.m.}. The data at 92 nM- and 30 nM-enzyme have been fitted to eqn. (1). The data at 9 nM-enzyme have been fitted by linear least-squares analysis. The values of the best-fit parameters are listed in Table 1.

Fig. 1 but extending the duration of the preincubation at 35 °C up to 60 min (results not shown).

Time course of U-A-primed RNA synthesis under various preincubation procedures

The slow transient burst of activity was further assessed by using different preincubation procedures. In marked contrast with the results in Fig. 1, when the reaction assay medium also receives 40 mM-$(NH_4)_2SO_4$ at the start of the 10 min standard preincubation at 35 °C the U-A-primed RNA synthesis proceeds linearly and no transient is detected (Fig. 2, curve *1*). Similar experiments but carried out in the presence of 10 mM-$MgCl_2$ indicate that the U-A-primed RNA synthesis may proceed through a transient burst (Fig. 2, curve *2*), though to a much lesser extent than for the data in Fig. 1 (Table 1). The results in Fig. 2 are consistent with previous observations that Mg^{2+} enhances and $(NH_4)_2SO_4$ only slightly affects the rate of poly[r(A-U)] synthesis in the presence of Mn^{2+} (Dietrich *et al.*, 1986).

Time course of U-A-primed U-A-U synthesis

Fig. 3 shows typical progress curves of abortive elongation leading to U-A-U synthesis, obtained under the same experimental conditions as in Fig. 1. In contrast

Table 1. Best-fit parameters for progress curves of productive RNA synthesis catalysed by wheat-germ RNA polymerase II

The experimental data are presented in Figs. 1 (*) and 2 (†). They have been fitted to eqn. (1) or by linear least-squares analysis. The variance ratio F (Bardsley, 1986) was calculated as indicated in the Materials and methods section.

[Enzyme] (nM)	Preincubation procedure	A (pmol of $UMP_{inc.}$)	λ (min^{-1})	v_s (pmol of $UMP_{inc.}$/min)	F
92*	Standard	2.1±0.4	0.51±0.15	0.82±0.04	69
30*	Standard	0.8±0.1	0.46±0.11	0.24±0.01	79
9*	Standard			0.096±0.002 ($r^2 = 0.991$)	
30†	Standard + 10 mM-$MgCl_2$	1.2±0.7	0.23±0.17	0.41±0.03	11
30†	Standard + 40 mM-$(NH_4)_2SO_4$			0.31±0.008 ($r^2 = 0.955$)	

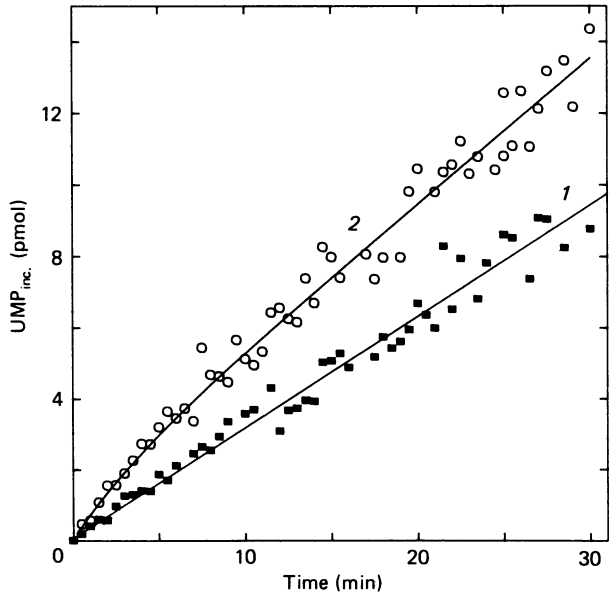

Fig. 2. Effect of Mg^{2+} or of $(NH_4)_2SO_4$ on the time course of U-A-primed RNA synthesis

Reactions were performed as indicated in Fig. 1 legend, in the presence of 30 nM-wheat-germ RNA polymerase II, 17.5 μM-poly[d(A-T)], 0.9 mM-U-A and 1.5 mM-$MnCl_2$, but in the additional presence of 40 mM-$(NH_4)_2SO_4$ (■, curve *1*) or 10 mM-$MgCl_2$ (○, curve *2*) introduced at the start of the preincubation period. After 10 min at 35 °C the reaction of labelled RNA synthesis was started by adding 20 μM-ATP and 21 μM-[^{3}H]UTP with final specific radioactivity as indicated in Fig. 1 legend. The data corresponding to curve *1* have been fitted by linear least-squares analysis, and those corresponding to curve *2* have been fitted to eqn. (1). The values of the best-fit parameters are listed in Table 1. The data corresponding to curve *2* may also be fitted by linear least-squares analysis with v_s = 0.47±0.01 pmol of $UMP_{inc.}$/min, ordinate intercept = 0.27±0.05 pmol of $UMP_{inc.}$, correlation coefficient 0.977 (not shown).

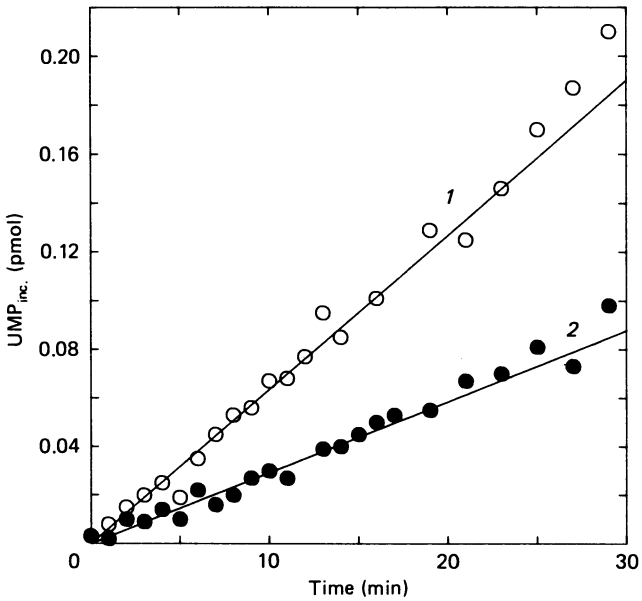

Fig. 3. Progress curve for U-A-primed U-A-U synthesis

Reactions were conducted as in Fig. 1 legend, by using the standard preincubation procedure in the presence of 30 nM- (○, curve *1*) or 12 nM- (●, curve *2*) enzyme. U-A-U synthesis was started by adding 5 μM-[α-^{32}P]UTP (1 pmol of UMP incorporated into U-A-U corresponded to 7000 c.p.m.). The results are expressed for 2 μl portions. From linear least-squares analysis $v_s = 6.3 \times 10^{-3} \pm 0.09 \times 10^{-3}$ pmol of $UMP_{inc.}$/min, correlation coefficient 0.976 (curve *1*), and $v_s = 2.9 \times 10^{-3} \pm 0.06 \times 10^{-3}$ pmol of $UMP_{inc.}$/min, correlation coefficient 0.968 (curve *2*).

with the results obtained for productive RNA chain elongation, U-A-U synthesis proceeded linearly. Linear progress curves were observed by varying the enzyme or the DNA concentration in the range 2–72 nM or 1.7–350 μM respectively (results not shown). This indicates that the curvature of the plots in Fig. 1 was not due to a rate-limiting step in the binding of U-A primer and/or UTP substrate.

DISCUSSION

The results presented in this paper show the existence of a slow burst of activity at the start of the reaction of RNA synthesis catalysed by wheat-germ RNA polymerase II. In this study a systematic deviation from a linear relationship was observed for 29 progress curves obtained by using the standard procedure of Fig. 1. The

numerical values of the variance ratio F for the data in Fig. 1, calculated by the procedure of Bardsley (1986), give strong evidence for the superiority of eqn. (1) compared with linear progress curves. Although the transient becomes hardly detectable at low enzyme concentrations, the half-time of the burst does not seem to depend on enzyme concentration in the reaction assay. In contrast with the results obtained by measuring productive RNA chain elongation, the U-A-primed synthesis of U-A-U proceeds linearly. The results would suggest that the transient burst pertains to unimolecular transition(s) of the transcription complex in the transition from abortive to productive elongation.

The experimental data in Fig. 2 provide evidence to support this contention. Previous studies have shown that Mg^{2+} or $(NH_4)_2SO_4$ facilitates formation of competent elongation complexes in reactions catalysed by wheat-germ RNA polymerase II. Thus, although these effectors inhibited strongly the U-A-primed synthesis of U-A-U, their presence in transcription assays leading to productive RNA chain elongation was associated with marked increase in the length of RNA products (Dietrich *et al.*, 1986; Job *et al.*, 1987*a*). Apparently these experimental conditions are associated with a decrease in the extent of the transient burst of activity at the start of RNA synthesis. The burst was not detectable at 40 mM-$(NH_4)_2SO_4$. Although the data obtained in the presence of 10 mM-$MgCl_2$ could still be fitted by using eqn. (1), it is noteworthy that the A and λ parameters were poorly determined and that the value of the variance ratio F was only 11 as compared with 79 for the control experiment shown in Fig. 1 (Table 1).

From a non-steady-state study of RNA synthesis without enzyme turnover in the presence of *Escherichia coli* RNA polymerase, poly[d(A-T)] as template, U-A as primer and UTP and ATP as substrates, Shimamoto & Wu (1980*a*,*b*) have reported biphasic kinetics in formation of the initiation complex. The data indicated that the rate-limiting step corresponds to unimolecular processes of conformational rearrangement of the initiation complex, essential for subsequent productive incorporation steps. The pre-steady-state results obtained with wheat-germ RNA polymerase II would thus be in good agreement with those obtained by fast kinetic techniques for the prokaryotic RNA polymerase.

With the U-A primer and under the standard incubation procedure, the transient burst occurs with a half-time in the order of minutes. This may suggest an enayme transition of a hysteretic (Frieden, 1970; Neet & Ainslie, 1980) or of a mnemonical (Ricard & Cornish-Bowden, 1987; Cornish-Bowden & Cardenas, 1987) type. Such a possibility has already been invoked to account for the negative co-operative behaviour exhibited by wheat-germ RNA polymerase with adenosine 5′-[$\beta\gamma$-imido]triphosphate as substrate (Job *et al.*, 1988). In this connection it is worth noting that enzyme memory effects have also been invoked in DNA replication. For this latter reaction Papanicolaou *et al.* (1984, 1986) have proposed that the DNA polymerase may switch during catalytic polymerization between two states having different catalytic properties and/or processivities. Interestingly, biphasic kinetics have been observed in single-turnover polymerization and pyrophosphorolysis experiments with DNA polymerase I (Kuchta *et al.*, 1987). The data suggest that the rate is not limited by actual polymerization but by a separate step, assigned to a conformational change of the ternary enzyme–DNA–dNTP complex to a form poised for nucleophilic displacement, possibly important in ensuring fidelity (Kuchta *et al.*, 1987). In this context, it may also be worth pointing out that wheat-germ and HeLa-cell RNA polymerases II utilize differently 'wrong' substrates such as cordycepin triphosphate (Dietrich *et al.*, 1985; Job *et al.*, 1987*b*) or dATP (Luse *et al.*, 1987) in the formation of the first phosphodiester bond rather than in subsequent incorporation steps.

For *E. coli* RNA polymerase there is a strong and inverse correlation between the productive and abortive initiation pathways, suggesting that important control of transcription may occur at the step of commitment to elongation (reviewed in von Hippel *et al.*, 1984). The requirements in formation of transcription complexes having elongation competence *in vitro* have been recently investigated for RNA polymerases II (Job *et al.*, 1987*b*, 1988; Luse *et al.*, 1987; Luse & Jacob, 1987; Rappaport & Weinmann, 1987). In particular, these studies have indicated that RNA polymerases 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. The notion that wheat-germ RNA polymerase II shares striking similarities in enzyme kinetics with its prokaryotic counterpart is supported by our observations that, in the process of commitment to productive elongation, both the rate plots of disappearance of abortive products and appearance of RNA chains have a complex dependence with respect to the concentration of nucleotide substrates (Job *et al.*, 1987*b*, 1988), as reported for *E. coli* RNA polymerase (Shimamoto & Wu, 1980*a*,*b*). In this context, Armaleo (1987) has presented a model that emphasizes the structural similarities between eukaryotic and prokaryotic RNA polymerases.

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