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Abortive intermediates in transcription by wheat-germ RNA polymerase II

Dynamic aspects of enzyme/template interactions in selection of the enzyme synthetic mode

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At constant enzyme concentration and with the full set of nucleotide substrates dictated by template sequence, the chain-length distribution of polymeric product varies with template concentration in reactions catalysed by wheat-germ RNA polymerase II. Under the same conditions, but in the presence of a single ribonucleoside triphosphate, the rate of condensation of the triphosphate substrate to a dinucleotide primer also exhibits a complex dependence with the template concentration. This effect is observed using poly[d(A-T)] as a template. For both reactions there are two extreme types of behaviour in each of which transcription appears to involve a single enzyme synthetic mode, characterized by either a high (at low template concentration) or a low (at high template concentration) probability of releasing the transcripts. A strong correlation is found between these two pathways, such that conditions favouring the abortive release of trinucleotide products in the single-step addition reaction are associated with the synthesis of short-length RNA species in productive elongation, and reciprocally. A model previously developed by Papanicolaou, Lecomte & Ninio [(1986) J. Mol. Biol. **189**, 435–448] to account for the kinetics of polymerization/excision ratios with *Escherichia coli* DNA polymerase I, and by Job, Soulié, Job & Shire [(1988) J. Theor. Biol. **134**, 273–289] for kinetics of RNA-chain elongation by wheat-germ RNA polymerase II provides an explanation for the observed behaviour with the plant transcriptase. The basic requirement of this model is a slow equilibrium between two states of the polymerization complex with distinct probabilities of releasing the product. In the presence of Mn^{2+}, and under conditions allowing the synthesis of poly[r(A-U)], one of these states is involved in the formation of oligonucleotides shorter than 15 bases, whereas the other catalyses the polymerization of chains longer than 40 bases.

INTRODUCTION

A fascinating aspect of DNA-dependent RNA polymerases relies upon the observation that these transcriptases must copy DNA stretches that may extend over 10^5 bases for some eukaryotic genes. As it is quite clear that these enzymes are unable to reinitiate on a preformed primer longer than a di- or a tri-nucleotide, efficient catalysis of RNA-chain elongation requires that the following conditions are met: (i) the probability that the polymerization complex translocates along the template to incorporate yet another nucleotide into the nascent chain should be strictly equal to 1, and (ii) very tight binding should be observed in the interaction between enzyme and template-product polynucleotides (McClure & Chow, 1980). There is evidence, however, for the occurrence of changes in the synthetic mode during the early polymerization steps. Thus the initial phase of RNA synthesis by *Escherichia coli* RNA polymerase involves the reiterative synthesis and abortive release of RNA chains up to 10 bases long while the enzyme is recycled at the promoter. At the end of the abortive phase, the enzyme shifts into the productive elongation mode (Carpousis & Gralla, 1980, 1985). Control of transcription might be exerted at these steps, as the relative probabilities of the abortive and productive pathways appear to correlate with the strength of the promoter site (reviewed by von Hippel *et al.*, 1984).

The mechanisms of transcription at these steps are not defined as well in eukaryotic systems. However, studies with nuclear extracts have suggested that RNA polymerases II behave similarly to their prokaryotic counterpart in that, before being committed to elongation, the enzymes pass through a stage in which transcripts are produced abortively (Coppola & Luse, 1984; Luse & Jacob, 1987; Luse *et al.*, 1987). Our own kinetic studies indicated that wheat-germ RNA polymerase II can display different synthetic modes during the progress of polymerization, and that these modes may be in slow equilibrium (Job *et al.*, 1988*b*,*c*). Conformational transitions of enzyme complexes involved in template-directed polymerization reactions have been invoked for DNA replication (Papanicolaou *et al.*, 1986) and mRNA translation (Ninio, 1986). In the case of *E. coli* DNA polymerase I, Papanicolaou *et al.* (1986) proposed that the enzyme could be in one of two states, a state H of high-excision activity or a state L of low exonuclease. Processive synthesis would favour the L → H transition, but after dissociation, the enzyme would evolve slowly towards the L state, as described for hysteretic enzymes (reviewed by Neet & Ainslie, 1980). This would account for the observation that, at low enzyme/template-primer ratios, replication occurs entirely in the H state, because the enzyme form that dissociates in the state H will immediately bind to another primer-template. Conversely, if the enzyme/template-primer molar ratio is increased, the primers are saturated and a free molecule of enzyme (dissociated in the H state) will take longer to find a free initiator and will return to the L state. These considerations prompted us to investigate whether high or low values of the enzyme/template molar ratio

Abbreviations used: dinucleoside monophosphate primers and trinucleoside diphosphate products are referred to as dinucleotides and trinucleotides respectively.

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permit the trapping of different synthetic modes of wheat-germ RNA polymerase II.

It has been shown that wheat-germ RNA polymerase II catalyses the DNA-dependent reaction of single-step addition of a single nucleotide to a short primer such as a dinucleotide (Dietrich *et al.*, 1985), as described for *E. coli* RNA polymerase (Oen & Wu, 1978). The extent of product formation varies considerably, depending on the ionic conditions, the sequence and conformation of the template (Dietrich *et al.*, 1985, 1986; Job *et al.*, 1988*a*). Therefore kinetic studies of the reaction of single-step addition allow direct and quantitative analysis of the parameters which might affect the stability of the transcription complexes in the early stages of polymerization. For this reason, in the present study we have investigated the effect of varying the enzyme/template molar ratio on the rate of single-step addition reactions catalysed by the plant RNA polymerase II.

Our general finding is that, at constant enzyme concentration, decreasing the DNA template concentration favours the occurrence of a form of the transcription complex having a high probability of releasing the transcripts, whereas the converse applies upon increasing the DNA template concentration. Below, we show that the proposal made by Papanicolaou *et al.* (1986) for *E. coli* DNA polymerase I provides an explanation for the observed behaviour with wheat-germ RNA polymerase II.

MATERIALS AND METHODS

Reagents

Ribonucleoside triphosphates and the dinucleoside monophosphate primers were purchased from Sigma Chemical Co. and Boehringer Mannheim. ^{32}P-labelled ribonucleoside triphosphates and phage-T_4 polynucleotide kinase were from Amersham International. Poly[d(A-T)] was from P-L Biochemicals. Wheat-germ RNA polymerase II was purified by the method of Jendrisak & Burgess (1975).

Reaction assays

Unless noted otherwise, the reaction mixtures contained 18 nM-enzyme, 0.9 mM-UpA primer, 5 μM-UTP, 20 μM-ATP and appropriate amounts of poly[d(A-T)] and metal salts ($MnCl_2$ or mixtures of $MnCl_2$ and $MgCl_2$), in transcription buffer as described previously (Job *et al.*, 1987). The protocol used included a 10 min incubation at 35 °C of all components, except the triphosphate substrates, followed by addition of 5 μM-[α-^{32}P]UTP (synthesis of UpApU) or 5 μM-[α-^{32}P]UTP plus 20 μM-ATP (synthesis of poly[r(A-U)]), and a second incubation at 35 °C as noted. For ApUpA synthesis, the same protocol was used, except that the reaction was conducted in the presence of 0.9 mM-ApU primer and 5 μM-[α-^{32}P]ATP. Final volumes were 25 μl. Reactions were stopped by addition of 2 vol. of stop solution [1 mM-EDTA/80% (v/v) formamide/0.1% (w/v) xylene cyanol].

Activity measurements

Reaction mixtures were processed as follows (Job *et al.*, 1987). (i) Total RNA synthesis was measured by the use of trichloroacetic acid precipitation of 10 μl (reaction mixtures + stop solution) spotted on Whatman GF/C filters. (ii) Trinucleotide synthesis was quantified after t.l.c. on poly(ethyleneimine)–cellulose sheets of 2 μl (reaction mixture + stop solution), with 1 M-formic acid/0.1 M-LiCl as the solvent system, until the front had migrated 10 cm. Under these conditions UpApU and ApUpA migrated with R_F values of 0.4 and 0.75 respectively, whereas unused UTP or ATP remained bound at the origin. After radioautography, all radioactive spots were cut out and counted for radioactivity. (iii) The transcription products were analysed by high-resolution PAGE of reaction assay mixtures, using 20% polyacrylamide [19:1 (w/w) acrylamide/bisacrylamide ratio]/8 M-urea/50 mM-Tris/borate (pH 8.0)/1 mM-EDTA gels (30 cm × 40 cm × 0.03 cm) (Maniatis *et al.*, 1982). Electrophoresis was carried out at 2000 V. Gels were then exposed to Fuji RX film at −80 °C with a Cronex Li-Plus intensifying screen. In these gels UpApU migrated more slowly than did a well-characterized 9-mer (Fig. 1, lanes 9 and 11), as observed by Levin *et al.* (1987) for very short transcripts with free 5′-hydroxy groups. Fig. 1 (lane 12) shows that the expected mobility was mostly recovered upon treating UpApU with phage-T_4 polynucleotide kinase and ATP. In previous work we showed that, under the standard protocol described above, the poly[r(A-U)] product is distributed in the form of a ladder of chains terminating at every other nucleotide, after incorporation of UMP (Dietrich *et al.*, 1985). From pulse–chase experiments we showed that these chains are irreversibly released by the transcription complexes and cannot be re-used for further

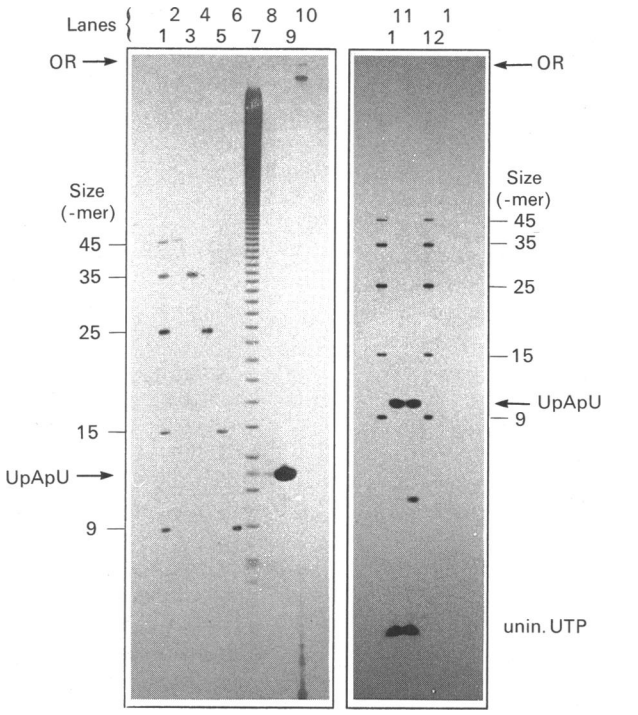


Fig. 1. Poly[d(A-T)]-dependent syntheses of poly[r(A-U)] and UpApU by wheat-germ RNA polymerase II

Reactions were conducted by using the standard protocol described in the Materials and methods section, with 1.5 mM-$MnCl_2$, 18 nM-enzyme, 5 μM-[α-^{32}P]UTP, 100 μM-ATP, 0.9 mM-UpA and 9.47 μM-poly[d(A-T)]. Transcription was allowed to proceed for 30 min at 35 °C. The transcription products were analysed by PAGE as described in the Materials and methods section. Lane 7, complete reaction mixture; lane 8, complete reaction mixture plus 2 μg of α-amanitin/ml; lane 9, complete reaction mixture minus ATP. Lanes 2–6 contain size markers (Baty *et al.*, 1987) of length 9, 15, 25, 35 and 45 nucleotides respectively. Lanes 1 correspond to a mixture of these markers; the numbers alongside the lanes indicate their sizes. In between the migrations of the 45- and 9-mer, 18 bands are seen in lane 7, as termination occurs at every other nucleotide in poly[r(A-U)] synthesis (Dietrich *et al.*, 1985). Lane 10 shows the migration of the poly[d(A-T)] template; the DNA chains were labelled with [γ-^{32}P]ATP and phage-T_4 polynucleotide kinase as described by Maniatis *et al.* (1982). Lane 11 shows the migration of the product UpApU resulting from the single-step addition of UTP to UpA primer (as in lane 9). Lane 12 shows the migration of UpApU upon treatment with phage-T_4 polynucleotide kinase and unlabelled ATP (Maniatis *et al.*, 1982). OR, origin; unin., unincorporated.

elongation by the enzyme (Durand *et al.*, 1982). Furthermore, as the product distribution pattern is not affected by varying the concentration of triphosphate substrates up to 100 μM, that is, well above the K_m values for the substrates (Job *et al.*, 1987), this behaviour was not complicated by a coupling between triphosphate binding and enzyme processivity. Fig. 1 (lane 10) shows that the mean size of the poly[d(A-T)] chains was greater than the longest synthesized poly[r(A-U)] chains, testifying that the observed product distribution did not involve the possibility that the plant enzyme could synthesize products longer than the template, as reported for *E. coli* RNA polymerase (Nishimura *et al.*, 1964). Formation of UpApU and of ApUpA was strictly dependent on the presence of enzyme, poly[d(A-T)], bivalent cation and appropriate primer-substrate combinations. Neither poly(A) nor poly(U) synthesis could be detected from these assays with a single nucleotide. For all reactions, 50% inhibition was obtained at about 0.05 μg of α-amanitin/ml. Therefore the enzyme preparation is not contaminated by the other types of nuclear RNA polymerases (Jendrisak & Guilfoyle, 1978). However, and as previously noted (de Mercoyrol *et al.*, 1989*a*), a sizeable proportion of the formation of UpApU escaped inhibition by the fungal toxin (Fig. 1, lane 8).

Data analysis

The kinetic data were analysed with a program providing an iterative fit to the appropriate rate equation by using the Gauss–Newton algorithm (Cleland, 1979) or were fitted by linear least-square analysis. Distinction between rival models was effected as described by Bardsley (1986) and Mannervik (1981). Size distributions of poly[r(A-U)] products were analysed by densitometry of the autoradiographs, using a GS300 scanning densitometer (Hoefer Scientific Instruments) or a data-processing microdensitometer (1010A; Perkin–Elmer Corp.) (Rasigni *et al.*, 1985).

RESULTS

Formation of poly[r(A-U)] at various template/enzyme molar ratios

Fig. 2 shows that, with reaction assays leading to poly[r(A-U)] synthesis, functional saturation of a fixed amount of enzyme was achieved by varying the concentration of the template. Saturation was observed at a template/enzyme molar ratio in the range of 100 bp/enzyme, indicative of high template occupancy, as the size of the site occupied by wheat-germ RNA polymerase II on simian-virus-40 DNA is 35–41 bp (Chandler & Gralla, 1980). The simplest equation fitting these data is:

$$v = \frac{p_1[T] + p_2[T]^2}{1 + p_3[T] + p_4[T]^2} \quad (1)$$

where T is the template, p_1–p_4 are collections of rate constants (see Scheme 1 below) and v is the steady-state rate of poly[2(A-U)] synthesis. By employing the discrimination criteria of Bardsley (1986) and Mannervik (1981), we found that neither the polynomial functions differing from eqn. (1) by omitting either one of the p_1–p_4 parameters, nor a Michaelis–Menten-type cofactor saturation equation (i.e. p_2 and $p_4 = 0$ in eqn. 1) would allow us to fit satisfactorily the data in Fig. 2.

Poly[r(A-U)] synthesized under these conditions was analysed for size distribution by PAGE (Fig. 3). Two prominent features were observed. (i) At low (0.42 μM) or high (54.9 μM) template concentration, the poly[r(A-U)] chains were of short length (shorter than 17 nucleotides) or long length (longer than 40 nucleotides) respectively. (ii) At intermediate template concentrations, the distribution of RNA product exhibited a bimodal

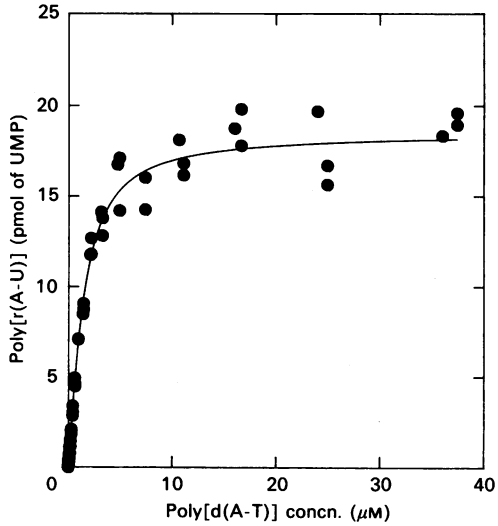


Fig. 2. Saturation of enzyme by template in poly[r(A-U)] synthesis

Reactions were conducted using the standard protocol described in the Materials and methods section, with 1.5 mM-$MnCl_2$, 18 nM-enzyme, 5 μM-[α-^{32}P]UTP (1 pmol corresponded to 18973 c.p.m.), 20 μM-ATP, 0.9 mM-UpA and various amounts of poly[d(A-T)]. Poly[r(A-U)] synthesis was quantified after trichloroacetic acid precipitation. The molar amount of UMP incorporated into product relates to the initial 25 μl reaction mixtures. Transcription was allowed to proceed for 30 min at 35 °C; poly[d(A-T)] concentration was varied up to 37 μM. The smooth line is the best fit to eqn. (1), with $p_1 = 6.2 \pm 0.3$, $p_2 = 9.4 \pm 5.2$, $p_3 = 0.7 \pm 0.4$, and $p_4 = 0.5 \pm 0.2$.

character. It is noteworthy that, upon increasing the template, there was a continuous change in the size-distribution pattern (Figs. 3*c*–3*f*). Thus the population associated with the longest products showed a migration that was progressively shifted to longer chains, whereas the amount of the short products in the other population was respectively diminished, features that are characteristic of an increased stability of the transcriptional complex in poly[r(A-U)] synthesis.

The pattern of behaviour was not changed whether the autoradiography was conducted for 18 or 72 h (Figs. 3*a* and 3*b*), even though in the latter case the intensity of the various bands was increased. With the longer exposure, faint bands became detectable between two successive major bands, confirming the finding that the main ladders originate from termination at every other nucleotide in transcription of poly[d(A-T)] (Fig. 1, lane 7; Dietrich *et al.*, 1985). Control experiments using the ^{32}P-labelled poly[r(A-U)] chains ensured that neither the enzyme nor poly[d(A-T)] contained ribonuclease activity.

Poly[r(A-U)] was formed at a template/enzyme molar ratio of 530 bp/enzyme. Aliquots of reaction mixtures were taken at various times during the reaction. After 2 min incubation, the enzyme had synthesized mostly products of length shorter than 15 nucleotides. Then the distribution patterns appeared stationary and not only were the synthesized polymers longer, but the amount of long chains greatly increased compared with that of short chains (Fig. 4). It must be stressed that the different distributions seen for the longest polymers in Figs. 4(*a*) and 4(*b*) rule out the possibility that the apparent distribution patterns were complicated by non-linearity in the response of the X-ray film. Furthermore, these patterns were not changed by conducting the autoradiography for longer exposures (results not shown).

Formation of UpApU at various template/enzyme molar ratios

In the case of UpApU synthesis, the titration behaviour with respect to template was more complicated than that observed for

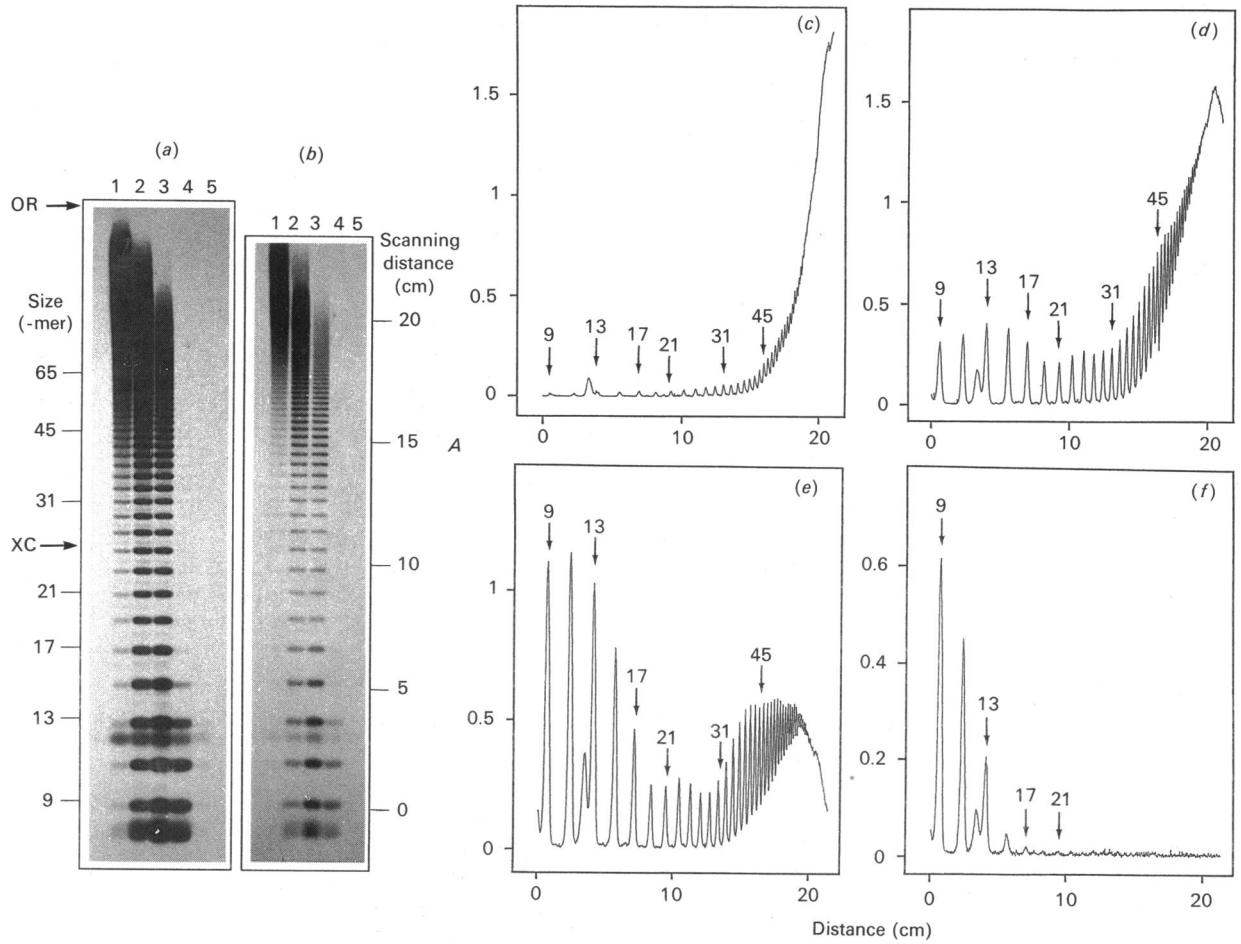


Fig. 3. Radioautographic and microdensitometric analyses of the product-length distributions in poly[r(A-U)] synthesis at various poly[d(A-T)]/enzyme molar ratios

Poly[r(A-U)] was formed by using the standard protocol described in the Materials and methods section, with 18 nM-enzyme, 1.5 mM-$MnCl_2$, 5 μM-[α-^{32}P]UTP (1 pmol corresponded to 8960 c.p.m.), 20 μM-ATP, 0.9 mM-UpA, and variable amounts of poly[d(A-T)]. Transcription was allowed to proceed for 30 min at 35 °C. A 5 μl portion (reaction mixture + stop solution) was loaded on to the gel and analysed by PAGE. Template concentration was 54.9, 9.47, 2.2, 0.42 or 0.076 μM for lanes 1–5 respectively. For the sake of scaling, the numbers alongside the lanes in (*a*) and in (*c*)–(*f*) indicate the sizes of some of the transcripts (termination occurs at every other nucleotide). In (*a*) the exposure was for 72 h; in (*b*) for 18 h. Portions of 21 cm of the lanes of the autoradiography in (*b*) were analysed by microdensitometry. XC, xylene cyanol; OR, origin. (*c*, *d*, *e* and *f*) Tracings of lanes 1, 2, 3 and 4 in (*b*) respectively, over the distance (cm) as represented in (*b*). The maximal scanning length with the microdensitometer is 21 cm; therefore the first fast-migrating species at the bottom of the lanes (i.e. the 5- and 7-mer), and the longest products in lanes 1 and 2, are not shown.

poly[r(A-U)] synthesis (Fig. 5*a*). The enzyme activity first increased with the DNA concentration and was maximal for a template occupancy of 78 bp/enzyme. The value of $V_{max.}$ indicated that UpApU synthesis proceeded catalytically, as the maximum amount of trinucleotide synthesized (12.5 pmol) largely exceeded the amount of enzyme (0.45 pmol) in the assay. When the concentration of poly[d(A-T)] was increased 20 times there was a decrease in the rate of single-step addition reaction to a plateau value that was nearly 5 times smaller than the amount of UpApU synthesized under the optimal conditions. The simplest rate equation fitting these data is eqn. (1). Similar results were obtained for the ApU-primed reaction of ApUpA synthesis with ATP substrate (Fig. 5*b*).

In further experiments, UpApU was formed at fixed enzyme concentration (6, 10, 15, 30, 50 or 60 nM) and the template was varied. Results identical with those presented in Fig. 5(*a*) were obtained. Furthermore the $V_{max.}$ values showed a linear dependence on the enzyme concentration, ruling out the possibility that aggregation of the enzyme played a role in the saturation behaviour (results not shown).

In the case of the reaction of UpApU synthesis, functional saturation was observed by varying the enzyme concentration and keeping constant that of the template (Fig. 6*a*). At 0.19 μM-poly[d(A-T)], the rate data can be fitted to a simple hyperbolic equation (Fig. 6*a*). The calculated occupancy of the template by the enzyme at half saturation was of the order of 50 bp, indicating that the saturation of the poly[d(A-T)] template was complete and that the observed UpApU synthesis was directed most presumably at double-stranded sequences rather than at any putative single-stranded nicks.

In additional experiments, UpApU was synthesized at fixed template concentration (0.38 or 0.57 μM) and the enzyme was varied. Similar results to those presented in Fig. 6(*a*) were obtained. Furthermore, at the plateau level, the $V_{max.}$ values were proportional to the template concentration in the assays. At 1.9 μM-poly[d(A-T)], functional saturation was not observed by varying the enzyme in the 0–72 nM concentration range (Fig. 6*b*). It is noticeable, however, that, as for the data in Fig. 6(*a*), there was no indication of sigmoidicity in this rate plot, as linear least-squares analysis yielded a zero intercept. As stated above, these results rule out the possibility that interactions between enzymes on the template played an important role in the reactions.

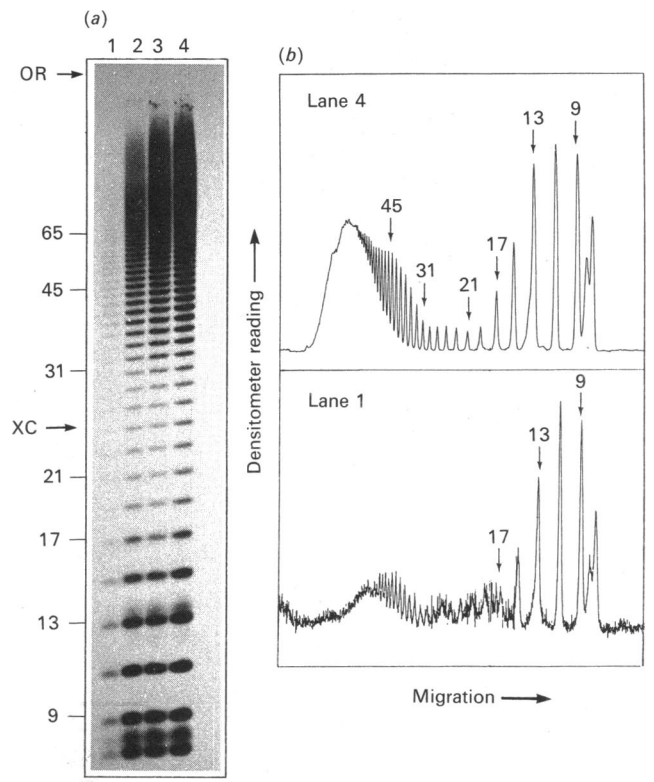


Fig. 4. Radioautographic analysis by high-resolution gel electrophoresis of the product-length distribution during the time course of poly[r(A-U)] synthesis

Poly[r(A-U)] was formed, as in Fig. 3, at 18 nM-enzyme and 9.47 μM-poly[d(A-T)]. Aliquots were taken at 2 min (lane 1), 10 min (lane 2), 19 min (lane 3), or 28 min (lane 4) and analysed by PAGE. XC, xylene cyanol; OR, origin. For the sake of scaling, the numbers alongside the lanes indicate the sizes of some of the transcripts (termination occurs at every other nucleotide). The densitometric scans of lanes 1 and 4 are also shown. The gain of the densitometer was adjusted so as the short-length species within the two lanes displayed equal amplitudes on the recordings. The product distributions in lanes 2 and 3 are similar to that corresponding to lane 4.

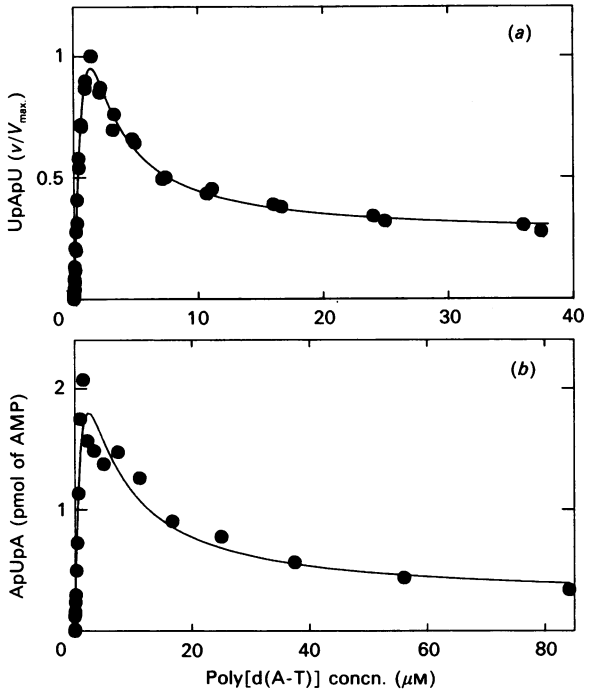


Fig. 5. Saturation of enzyme by template in synthesis of UpApU and ApUpA

(*a*) Synthesis of UpApU. UpApU was formed and analysed using the standard protocol described in the Materials and methods section, with 18 nM-enzyme (0.45 pmol), 1.5 mM-$MnCl_2$, 5 μM-[α-^{32}P]UTP and 0.9 mM-UpA. The concentration of poly[d(A-T)] was varied up to 38 μM. Transcription was allowed to proceed for 30 min at 35 °C. The molar amount of UMP incorporated into UpApU (v) was determined for the initial 25 μl reaction mixtures. Rate measurements were normalized to the $V_{max.}$ value (12.5 pmol of UMP incorporated/30 min incubation). The smooth line is the best fit to eqn. (1), with $p_1 = 1.34 \pm 0.03$, $p_2 = 0.17 \pm 0.018$, $p_3 = 0$, and $p_4 = 0.67 \pm 0.04$. From these values, the concentration of template for which the rate of UpApU synthesis is maximum can be calculated to be 1.41 ± 0.03 μM (bp) (Cleland, 1979). (*b*) Synthesis of ApUpA. ApUpA was formed and analysed using the standard protocol described in the Materials and methods section, with 18 nM-enzyme (0.45 pmol), 1.5 mM-$MnCl_2$, 5 μM-[α-^{32}P]ATP and 0.9 mM-ApU. The concentration of poly[d(A-T)] was varied up to 189.4 μM. The Figure only shows the lower-concentration part of the data. Transcription was allowed to proceed for 30 min at 35 °C. The molar amount of AMP incorporated into ApUpA relates to the initial 25 μl reaction mixtures. The smooth line is the best fit to eqn. (1), with $p_1 = 2.6 \pm 0.5$, $p_2 = 0.05 \pm 0.03$, $p_3 = 0.62 \pm 0.3$, and $p_4 = 0.2 \pm 0.05$.

Further substantiation of this finding is the fact that the data in Fig. 6(*a*) can also be fitted to the theoretical saturation isotherms described by McGhee & von Hippel (1974) for non-co-operative binding of large ligands to a homogeneous lattice (see the legend to Fig. 6*a*).

Effect of Mg^{2+} on UpApU and poly[r(A-U)] syntheses

Figs. 7(*a*) and 7(*b*) show that Mg^{2+} favoured the elongation of poly[r(A-U)] transcripts and considerably reduced the formation of UpApU. For the reaction of poly[r(A-U)] synthesis, functional saturation of a fixed amount of enzyme with respect to template was achieved under these ionic conditions (Fig. 7*c*). However, Fig. 7(*d*) shows that the RNA-chain-length distribution was unaffected by varying the concentration of template from 55 to 0.076 μM in the $MgCl_2$-containing assays. Thus, apart for a low level of trinucleotide UpApU, the short-length species observed with incubations in $MnCl_2$ were not present, and only the long chains could be detected (compare Figs. 3*a* and 3*b* with Fig. 7*d*).

DISCUSSION

The present results indicate that, under certain conditions, transcription by wheat-germ RNA polymerase II proceeded through a single synthetic mode having a low or a high probability of releasing the reaction products. The former pertains to the cases of high values of the template/enzyme molar ratio, or if the assays contained Mg^{2+}, whereas the latter is observed for low values of the template/enzyme molar ratio. This property is detected either by measuring the extent of trinucleotide formation in the single-step addition reaction or by analysing the size distributions of polymeric product in the presence of the full set of substrates. Under all experimental conditions presently examined, there exists a strong correlation for the enzyme synthetic mode in the two pathways, extending our previous results (Dietrich *et al.*, 1986; Job *et al.*, 1988*a*). The results suggest a model in which the transcription complex has two states, having different probabilities of releasing the transcript. In Scheme 1 we consider the possibility that, during the progress of the reaction, the enzyme could evolve from an initial C state to a kinetically different final C* state, and that the transitions between these states are slow compared with the rate of catalysis.

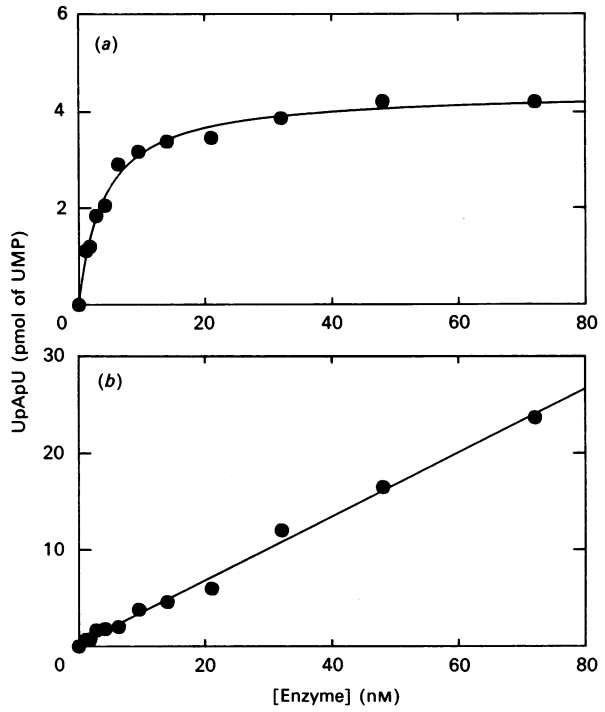


Fig. 6. Saturation of poly[d(A-T)] template by enzyme in synthesis of UpApU

Reactions were conducted as described in Fig. 5(*a*), with fixed amounts of poly[d(A-T)] and the concentration of enzyme was varied. Transcription was allowed to proceed for 15 min at 35 °C. The molar amount of UMP incorporated into UpApU product relates to the initial 25 μl reaction mixtures. (*a*) The template was at 0.19 μM. The smooth curve was obtained by fitting the data to the hyperbolic saturation equation: $v = p_1[E]/(p_2+[E])$, where [E] is the enzyme concentration. Non-linear regression analysis yielded the following parameter values: $p_1 = 4.4 \pm 0.1$ pmol of UMP; $p_2 = (4.1 \pm 0.4) \times 10^{-9}$ M. The sum of the residual squares was 0.212. A slightly better fit (sum of the residual squares 0.197) was obtained by fitting the data to the theoretical saturation isotherms described by McGhee & von Hippel (1974) for non-co-operative binding of large ligands to a homogeneous lattice, although statistical analysis as described by Bardsley (1986) does not give evidence for the superiority of this model compared with the hyperbolic equation. In this latter approach, non-linear curve-fitting through a simplex strategy (Nelder & Mead, 1965) yielded values of n (the size of the site that wheat-germ RNA polymerase II occupies on poly[d(A-T)]), v_m (the maximal velocity per nM-enzyme) and K (the intrinsic association constant of the enzyme to the template) of 48.5 bp, 1.5 pmol of UpApU per enzyme and 8.7 10^6 M^{-1} respectively. The plot resulting from the simulated data with the equation of McGhee & von Hippel (1974) is not distinguishable from that obtained with the hyperbolic equation. (*b*) The template was at 1.9 μM. The straight line was obtained by linear least-squares analysis. The numerical values of the intercepts of this line with the vertical and horizontal axes are: 0.274 ± 0.232 pmol of UMP and -0.829 ± 0.717 nM-enzyme respectively. Slope, $(330.2 \pm 8.3) \times 10^{-3}$ pmol of UMP/15 min per 1 nM-enzyme; correlation coefficient, 0.9937.

We also assume that both C and C* bind the template, but with different affinities. Upon departure from the template, the C* form slowly evolves towards the C state, as proposed previously by Papanicolaou *et al.* (1986) to describe the kinetics of *E. coli* DNA polymerase I.

Single-step addition reactions

We assume that both C and C* are active in the synthesis of UpApU (or ApUpA), but that C* releases the product at a slower rate compared with C. In support of Scheme 1 it is worth pointing out, as suggested by Shimamoto & Wu (1980), in the first incorporation step of UpA-primed RNA synthesis catalysed by *E. coli* RNA polymerase on a poly[d(A-T)] template, that the rate-limiting step corresponds to unimolecular processes of conformational rearrangements of the transcription complex, essential for subsequent incorporation steps. Furthermore, for wheat-germ RNA polymerase II, UpA primer, UTP substrate and poly[d(A-T)] template, the complex dependence of the rates of disappearance of UpApU and appearance of poly[r(A-U)] with the concentration of elongating substrate ATP can be accounted for by a model, analogous to Scheme 1, in which the C and C* states {i.e. ternary transcription complexes composed of enzyme, poly[d(A-T)] and UpApU product} are assumed to occur in slow equilibrium and exhibit different rates of UpApU release and different reactivities towards ATP (Job *et al.*, 1988*b*).

Under steady-state conditions, eqn. (1), used to fit the data in Fig. 5, can be deduced from Scheme 1. We examined the possibility that a more complex equation than eqn. (1), in which the denominator contains an additional term, p_5T^3, might fit the data in Fig. 5(*a*). This was of special interest because such a rate expression can be derived under steady-state hypothesis from a model in which the template is assumed to contain a non-competitive inhibitor of the reaction of UpApU synthesis, as invoked by Pays (1978) to account for inhibition of transcription by excess DNA template with rat liver RNA polymerase II. However the value of p_5 was only poorly determined [$(6.9 \pm 6.5) \times 10^{-3}$], and statistical analysis as described by Bardsley (1986) does not give any evidence for the superiority of this model compared with eqn. (1). Finally, we examined the possibility that the complex titration behaviour in Figs. 5 and 6 arose from the existence of fixed heterogeneity in the template or enzyme molecules, assuming all enzyme–template complexes under a 'tight binding' hypothesis. However, attempts to use the rate equations deduced from these models in order to fit the data in Figs. 5 and 6 proved that such rate equations were inferior to eqn. (1), and therefore it must be stressed that the slow-transition model as depicted in Scheme 1 is the simplest and most straightforward model to account for these data.

Productive RNA synthesis

Scheme 1 proposes that the transcription complex starts RNA synthesis with a mode C exhibiting a high-probability of abortive dissociation and gradually shifts to a state C* associated with increased stability with respect to product. The fact that poly[r(A-U)] synthesis by wheat-germ RNA polymerase II is more sensitive to inhibition by heparin if the polyanion is introduced to the assay shortly after the start of RNA synthesis (e.g. at 2 min) than in later stages (e.g. at 10 min) (de Mercoyrol *et al.*, 1989*b*) is consistent with this hypothesis. Furthermore, Coppola & Luse (1984) and Cai & Luse (1987) showed that the AD2 major late transcription complex with HeLa-cell RNA polymerase II undergoes a transition from an unstable to a stable transcription complex near the formation of a 10-mer transcript. Scheme 1 permits the following predictions.

(i) At low-template concentration, transcription is impeded (presumably in this case there is hindrance of the progress of the polymerase by other polymerase molecules) and will involve the C form, which will be continuously recycled.

(ii) In the opposite situation, at high-template concentration, after a transient period involving the C mode, the enzyme shifts to C* to perform the subsequent polymerization steps (Fig. 4). As free DNA is in large excess, upon dissociation from the template the C* form immediately finds a new site to reinitiate RNA synthesis, and so transcription appears to be performed primarily with the C* synthetic mode.

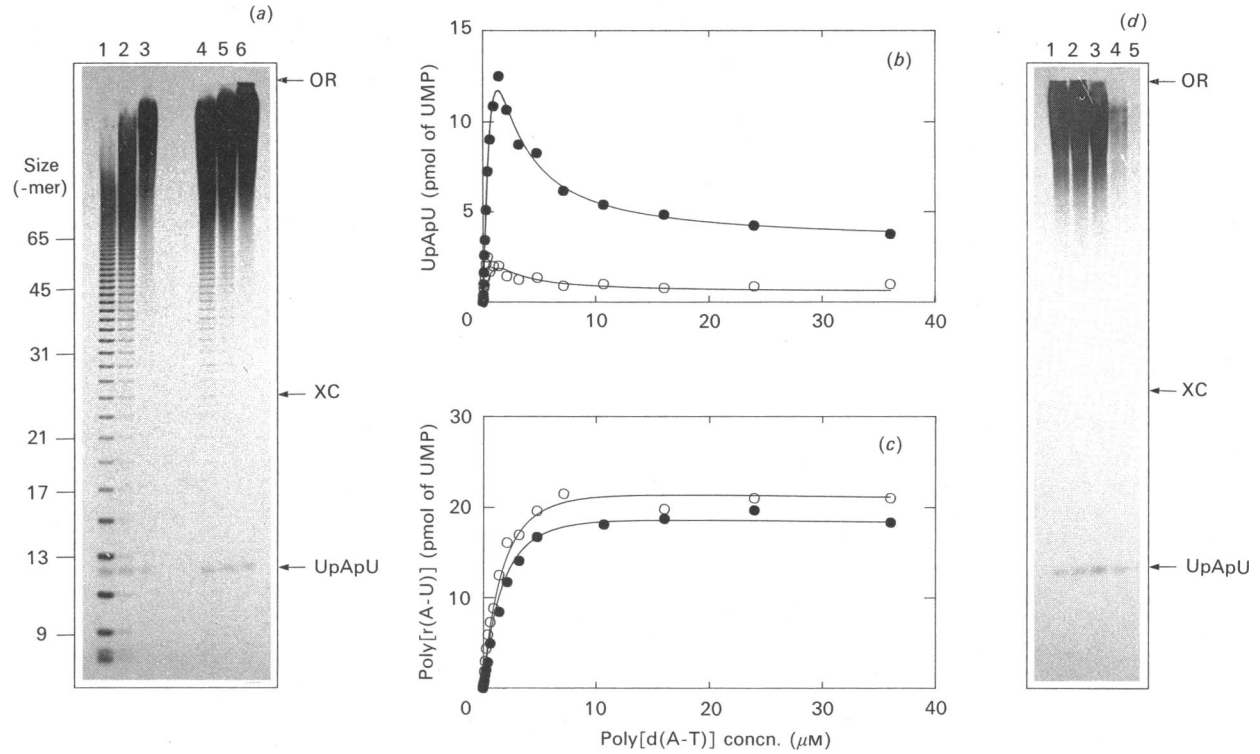
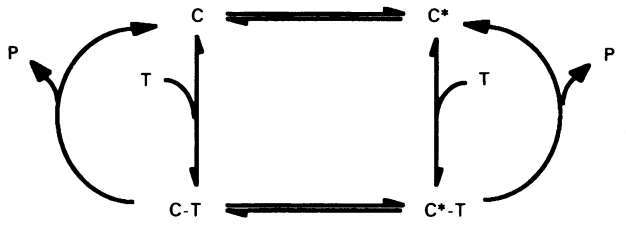


Fig. 7. Effect of Mg^{2+} on UpApU and poly[r(A-U)] syntheses

Poly[r(A-U)] and UpApU were formed and analysed as described in the Materials and methods section, at 1.5 mM-$MnCl_2$, 18 nM-enzyme, and various amounts of poly[d(A-T)] and of $MgCl_2$. Transcription was allowed to proceed for 30 min at 35 °C. (*a*) Effect of Mg^{2+} on poly[r(A-U)] synthesis at constant DNA concentration: poly[d(A-T)] was at 1.42 μM (lanes 1–3) or at 57 μM (lanes 4–6). Conditions for bivalent metal: 1.5 mM-$MnCl_2$ (lanes 1 and 4); 1.5 mM-$MnCl_2$ + 1.46 mM-$MgCl_2$ (lanes 2 and 5); 1.5 mM-$MnCl_2$ + 7.4 mM-$MgCl_2$ (lanes 3 and 6). XC, xylene cyanol; OR, origin. For the sake of scaling, the numbers alongside the lanes indicate the sizes of some of the transcripts (termination occurs at every other nucleotide). (*b*) Effect of poly[d(A-T)] concentration on UpApU synthesis and (*c*) effect of poly[d(A-T)] concentration on poly[r(A-U)] synthesis. The molar amount of UMP incorporated into UpApU (*b*) or poly[2(A-U)] (*c*) relates to the initial 25 μl reaction mixtures. Conditions for bivalent metal: ●, 1.5 mM-$MnCl_2$; ○, 1.5 mM-$MnCl_2$ + 15 mM-$MgCl_2$. Smooth lines are the best fits to eqn. (1). (*d*) Radioautographic analysis after gel electrophoresis of the data in (*c*), at 1.5 mM-$MnCl_2$ + 15 mM-$MgCl_2$. Template concentrations were 54.9, 9.47, 2.2, 0.42 or 0.076 μM for lanes 1–5 respectively. XC, xylene cyanol; OR, origin. The chain-length distributions corresponding to ● are given in Fig. 3.



Scheme 1. Slow-transition model

C and C* are defined in the text; T is the template, P are the reaction products and the p_1–p_4 parameters in eqn. (1) are collections of rate constants in this Scheme.

(iii) For the case of intermediate template concentrations, consider that state C_j^* is attained after incorporation of nucleotide N_j in the nascent RNA chain. Then the enzyme has two possible fates.

In the first, it dissociates from the template under the mode C_j^*. The nature of the enzyme which will be recycled for polymerization will now depend on the length of time spent by the enzyme in finding a new site on the template compared with the duration of the relaxation process from the C_j^* state towards the initial C state. The lower the template concentration, the higher its occupancy and the longer the enzyme will take to reinitiate, i.e. the C mode will be favoured. The converse will apply upon increase of the template concentration, and therefore the C_j^* mode will be progressively favoured. As Scheme 1 assumes that the transitions between these different states are neither too slow nor too fast compared with the rate of catalysis, at steady state there will be a *flux* between catalytically different modes and this allows to account for the bimodal nature of RNA synthesis, because otherwise the enzyme would function entirely under a single synthetic mode, as in cases (i) and (ii) discussed above.

In the second, those molecules of C_j^* that escaped dissociation will progress in the polymerization and reach the C_{j+1}^* state. However, by reason of the recycling phenomenon, the concentration of enzyme productively engaged in steps beyond the incorporation of N_j will rapidly decrease. Therefore, the higher the template occupancy the lower the amount of chains synthesized by the final C* state in Scheme 1, such that this amount may become so low that these chains cannot be detected in the gel. The converse will apply by increasing the template and this would account for the observation that the length of the longest products varies with the template concentration in the reactions (Fig. 3).

Altogether, these considerations show that the experimental behaviour observed in Figs. 3 and 4 validates the predictions of Scheme 1 with respect to the RNA-chain-length distribution patterns, as it does for the kinetic behaviour of the single-step addition reactions.

An important prediction of Scheme 1 is that, owing to the nature of the transitions postulated in this model, kinetic transients are to be expected (Neet & Ainslie, 1980). The data in Fig. 4 are in agreement with this prediction. Furthermore, progress curves of RNA synthesis catalysed by wheat-germ RNA polymerase II on the poly[d(A-T)] template exhibit a slow burst of activity extending over about 3 min (Job *et al.*, 1988*c*). We also reported that the kinetic transient becomes hardly detectable if reactions are conducted with added effectors such as Mg^{2+} or $(NH_4)_2SO_4$ (Job *et al.*, 1988*c*), which favour elongation by RNA polymerase II (Dietrich *et al.*, 1986; Sluder *et al.*, 1988). It is noteworthy that these conditions are associated with increased lengths of poly[r(A-U)] products (Fig. 7*a*), while the present study provides direct evidence that these conditions are precisely those that involve transcription of poly[d(A-T)] by only a *single* enzyme synthetic mode (Fig. 7*d*). In the frame of the slow-transition model, the differential effect of Mg^{2+} on the long and short classes of products would thus be explained by assuming that this metal ion stabilizes the C* state of the enzyme.

In conclusion, several studies have established that abortive cycling is a fundamental aspect of early transcription by prokaryotic RNA polymerase. The present study shows that kinetics of wheat-germ RNA polymerase II can best be explained by this concept. An attractive feature of the slow-transition model is that functionally distinct forms of the transcription complex are generated during catalysis. These transitions could possibly be modulated by the nature of the transcribed sequence [for example wheat-germ RNA polymerase II seems to be committed to an abortive synthetic mode on left-handed *Z*-DNA sequences (Job *et al.*, 1988*a*) or to an elongating mode on dT-rich sequences (Dietrich *et al.*, 1985)] and by external factors, as exemplified by the effect of Mg^{2+} described in the present study, giving support to regulatory models of gene expression exploiting the possibility of modulating the balance between abortive dissociation and productive elongation. In the context of Scheme 1 it is worth pointing out the proposal by Telesnitsky & Chamberlin (1989), that *E. coli* RNA polymerase could exist in either of two conformations which might be generated in the early stages of the polymerization reaction, a termination-proficient T state or a termination-resistant R state, to account for the observation that the efficiency of transcription termination at certain well-defined prokaryotic rho-independent terminators, depends on the promoter unit from which transcription is initiated.

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