

Accelerated Publications

T7 RNA Polymerase Does Not Interact with the 5'-Phosphate of the Initiating Nucleotide[†]Craig T. Martin[†] and Joseph E. Coleman*

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Received December 29, 1988; Revised Manuscript Received February 8, 1989

ABSTRACT: The study of transcription kinetics by T7 RNA polymerase is facilitated by the small size of its promoter, allowing the use of synthetic oligonucleotide templates with carefully defined sequences. We have previously used this approach to measure Michaelis–Menten steady-state kinetics for production of the five-base runoff transcript GGACU. In particular, K_m for the interaction between enzyme and template under saturating levels of all four nucleotide triphosphates was shown to be approximately 0.02 μM . We now show that the corresponding K_m and V_{max} for initiation on a similar template coding for the runoff transcript GACU are the same as for the earlier study ($K_m = 0.02 \mu\text{M}$; $k_{\text{cat}} = 40\text{--}50 \text{ min}^{-1}$). This new template allows the measurement K_m for association of the initial nucleotide GTP with enzyme or with the enzyme–DNA complex. The results show that K_m^{GTP} (0.60 mM) is somewhat higher than earlier approximations of K_m for addition of elongating GTP during the later phase of processive elongation. As expected, the (initiating) K_m for the GTP analogue ITP (K_m^{ITP}) is increased (by about 2-fold), presumably as a result of weakened Watson–Crick base pairing. However, comparison of K_m values for the GTP analogues GMP and guanosine shows little effect on substitution of the 5'-triphosphate by monophosphate or by a hydroxyl, respectively. This result suggests that a single active site has been evolutionarily adapted to accept from the 5' end of a waiting nucleotide both a 5'-triphosphate at initiation and a 5'-monophosphate ester (RNA) during elongation. This dual functionality is achieved at the loss of any binding interaction, positive or negative, with the 5' end of the waiting nucleotide.

Transcription by T7 RNA polymerase begins at a uniquely defined base relative to the promoter DNA sequence (Chamberlin & Ryan, 1982). We have recently characterized the initiation of transcription (Martin & Coleman, 1987) and the early postinitiation phase in which the enzyme shows a high degree of abortive cycling (Martin et al., 1988; Muller et al., 1988). In order to better understand the catalytic steps that comprise initiation in this model system, we now examine the effects of substitution at the 5'-phosphate position of the initiating nucleotide. The first nucleotide incorporated into an RNA transcript is unique in that the 5'-triphosphate is not utilized in a bond-formation step. Formation of the first bond is also unique in that the elongating RNA consists of a single nucleoside triphosphate (the first base in the message). An immediate question which arises is whether the triphosphate group of this first nucleotide is involved (positively or negatively) in binding to the enzyme or to the enzyme–DNA complex.

We have previously characterized the steady-state kinetics of the initiation of transcription by T7 RNA polymerase on a template coding for the five-base runoff transcript GGACU. By construction of a synthetic template in which each base occurs only once, we can probe the effects of nucleotide analogues on specific steps in this initial phase of transcription. As a specific example of this approach, we now compare substitution of GTP by GMP and guanosine, in transcription

from the synthetic template coding for the four-base message GACU. In particular, we can measure not only K_m and k_{cat} for association of enzyme and template DNA (Martin & Coleman, 1987) but also the K_m for association of the complex with the individual GTP analogues. We also compare these data with those for the more weakly hydrogen-bonding analogue ITP. The results show quite clearly that, for the initial nucleotide, the 5' region (normally containing a triphosphate substituent) is not involved in binding to the protein and/or to the DNA template. As expected, however, Watson–Crick base pairing is clearly involved. These results suggest that a single active site has been optimized to satisfy the sometimes conflicting requirements for initiation and elongation during transcription.

MATERIALS AND METHODS

T7 RNA polymerase was prepared from *Escherichia coli* strain BL 21 containing plasmid pAR1219 (kindly supplied by William Studier and John Dunn), with T7 gene 1 (RNA polymerase) cloned under inducible control of the *lac* UV5 promoter (Davanloo et al., 1984). Enzyme was purified as previously described (King et al., 1986) by fractionation with Polymin P (less than 1.25%) and ammonium sulfate, followed by chromatography on SP-Trisacryl (LKB), TSK CM-Fractogel (EM Science), and TSK DEAE-Fractogel (EM Science). A molar extinction coefficient of $\epsilon_{280} = 1.4 \times 10^5 \text{ M}^{-1}$ was used to determine enzyme concentrations (King et al., 1986). Enzyme routinely showed a standard activity on T7 DNA of 300 000–400 000 units/mg (Chamberlin et al., 1970; Chamberlin & Ring, 1973).

[†] This work was supported by NIH Grant GM21919-13.

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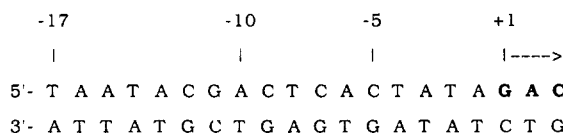
Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems Model 380B synthesizer and purified and prepared as described previously (Martin & Coleman, 1987).

Kinetic assays of transcription on oligonucleotide templates were carried out as previously described (Martin & Coleman, 1987) in 20 μL of 40 mM Tris-HCl, pH 7.8; 20 mM MgCl_2 ; 10 mM NaCl; 0.25 mM EDTA; 1 mM DTT; 0.05 mg/mL BSA (Boehringer Mannheim); 0.4 mM each of UTP, CTP, and ATP; and GTP or analogue as indicated. Unless otherwise indicated, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ was added to approximately 100 $\mu\text{Ci/mL}$. A 10.0- μL aliquot of a 2 \times mix (55 mM Tris, 40 mM MgCl_2 , 2 mM DTT, 0.1 mg/mL BSA, 1.6 mM GTP, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, and 0.8 mM each of UTP, CTP, and ATP) on ice was premixed with 5.0 μL of 4 \times oligonucleotide in TE buffer and incubated in 0.5-mL plastic tubes for 3 min at 37 $^\circ\text{C}$. To start the reaction, 5.0 μL of 4 \times enzyme (freshly diluted into 40 mM Tris-HCl, pH 7.8, 40 mM NaCl) on ice was added and the sample returned to 37 $^\circ\text{C}$. Aliquots of 5.0 μL were withdrawn at 3, 6, and 9 min and spotted onto individual lanes of Whatman 3MM filter paper, prespotted with 10 μL of 200 mM EDTA. The samples were then chromatographed in 60% saturated ammonium sulfate, pH 8, to separate products from unincorporated nucleotide (Mulligan et al., 1985). For each sample, a distinct band of radioactivity migrating away from the origin, but well separated from free nucleotide, was visualized by exposure to X-ray film and then cut out and quantified with OptiFluor (United Technologies Packard) liquid scintillant.

Analysis of transcription products was performed by denaturing gel electrophoresis as previously described (Martin & Coleman, 1987). For very short RNA products, the use of various labeled nucleoside triphosphates allows direct sequence determination (Martin et al., 1988). In all of the measurements with GTP and its analogues, the expected four-base runoff transcript was the predominant product observed on denaturing gels.

RESULTS AND DISCUSSION

The first nucleotide base incorporated into an RNA transcript is unique in that the 5'-triphosphate is not utilized in any bond-formation step. In order to determine the requirements for this initiating nucleotide, we constructed a synthetic oligonucleotide template similar to one that we had previously prepared for kinetic studies of initiation of transcription (Martin & Coleman, 1987). Like the previously characterized template, the new oligonucleotide sequence contains the 17-base promoter consensus sequence (from position -17 to -1). However, the promoter in the current case is followed by DNA coding for a four-base runoff transcript in which each base is present only once:



The resulting RNA transcript, GACU, allows determination of the effect of the incorporation of GTP analogues on the formation of the first phosphodiester bond.

Characterization of the Template. Measurement of steady-state kinetics with respect to enzyme and DNA on this template (under saturating levels of all four nucleotides) yields results very similar to those of the previous study. A fit of the data presented in Figure 1 produces the following kinetic parameters for the new template: $K_m = 0.027$ (0.012–0.061)

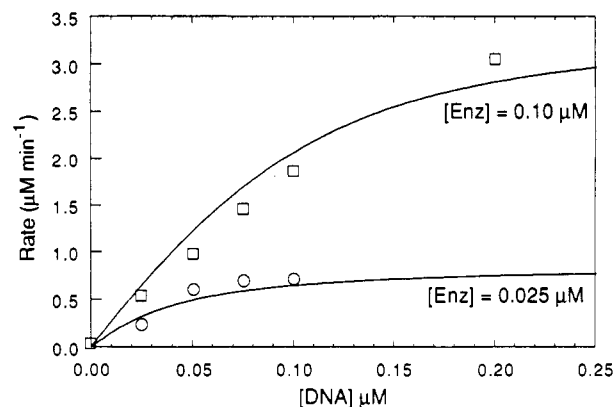


FIGURE 1: Determination of K_m and k_{cat} for the interaction of enzyme and template under saturating concentrations of all four nucleoside triphosphates. The single best fit to all data points ($K_m = 0.027 \mu\text{M}$; $k_{cat} = 35 \text{ min}^{-1}$) is shown for the various enzyme concentrations.

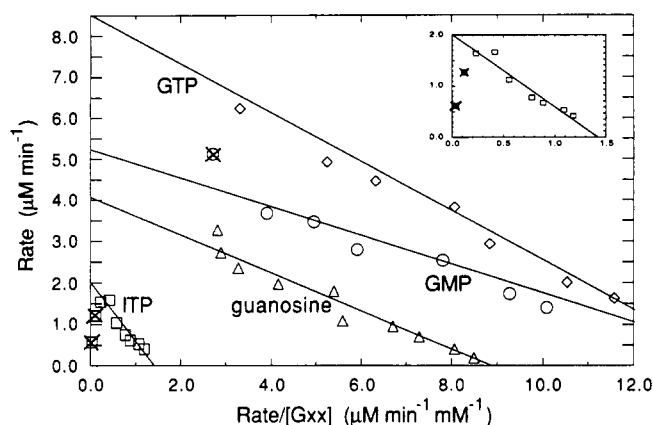


FIGURE 2: Eadie-Hofstee plot of kinetic data for various analogues of GTP. The inset shows expansion of scales for ITP. Note that the y intercept for the best-fit straight line provides an estimate of V_{max} , while the slope equals the negative of K_m .

μM and $k_{cat} = 35$ (28–42) min^{-1} . Comparison of these results with those for the previously characterized template (Martin & Coleman, 1987), $K_m = 0.015$ (0.008–0.029) μM and $k_{cat} = 52$ (46–58) min^{-1} , shows that the synthesis of the four-base message GACU follows steady-state kinetics similar to those for synthesis of the five-base message GGACU. These results suggest that the formation of the initial dinucleotide GA follows kinetics similar to the initial formation of GG and that promoter binding and initiation may not be significantly affected by this change in reactants. The values for K_m measured in these assays are the Michaelis constants for the interaction of enzyme and template DNA under conditions of saturating nucleoside triphosphates. Our previous results suggest that K_m is a good approximation to the dissociation constant, K_d , for the interaction between enzyme and promoter (Martin & Coleman, 1987; Martin et al., 1988; Muller et al., 1988). The interpretation of k_{cat} may be more complicated, but this value must reflect the rate-limiting step in the initiation of transcription.

Dependence of Kinetics on the Concentration of the First Nucleotide. We can now also measure the Michaelis constant for association of the initial nucleotide with the enzyme-DNA complex under conditions of saturating promoter. From the Eadie-Hofstee plot (the initial rate of the reaction vs the ratio of the rate to the concentration of GTP) shown in Figure 2 we determine that K_m^{GTP} for the initiating nucleoside triphosphate GTP is approximately 0.60 mM, with $k_{cat}^{\text{GTP}} = 43 \text{ min}^{-1}$. This value for K_m is higher than the average K_m for

Table I: Dependence of Steady-State Kinetic Data on Various GTP Analogues

Xxx	$k_{\text{cat}}^{\text{Xxx}}$ (min^{-1})	K_m^{Xxx} (mM)
GTP	43	0.60
GMP	25	0.33
guanosine	20	0.46
ITP	10	1.4

GTP incorporation measured during elongation ($K_m^{\text{GTP}} \approx 0.16$ mM; Chamberlin & Ring, 1973), again emphasizing the different roles of the initiating nucleotide and the subsequent elongating nucleotides.

Initiation with Nucleotide Analogues of the First Base.

Using this assay, we can now determine the effect of the structure of the initiating nucleotide on the steady-state kinetics of initiation. The results shown in Figure 2 and summarized in Table I show that, as expected, binding of ITP (as measured by K_m) is decreased by more than a factor of 2. This is to be expected, since the rI-dC base pair has one less Watson-Crick hydrogen bond compared to rG-dC. The catalytic rate of initiation (reflected in k_{cat}) is decreased 4-fold for ITP and may reflect a loose positioning of the 3'-hydroxyl on ITP at the active site. A similar imprecise positioning of UTP (and corresponding decrease in forward catalytic rate) has been previously proposed to explain the high rate of abortive falloff following incorporation of UTP during abortive cycling (Martin et al., 1988).

Noting that the 5'-triphosphate of the initiating nucleotide is not involved in bond formation, we can determine whether it is required for binding to protein or to the DNA template. Comparison of data for the series GTP, GMP, and guanosine in Table I reveals that the 5'-triphosphate is not involved in any favorable binding interactions and, in fact, may provide slightly negative binding energy (again, to the extent that K_m reflects K_d). Guanosine monophosphate is preferred as the initial nucleotide over GTP, and competition experiments (not shown) confirm this conclusion. Presumably the monophosphate form most resembles the (mono)phosphodiester linkage of a growing RNA chain.

Conclusions. If the same active site on the enzyme is used for formation of both the initial and subsequent phosphodiester bonds, the binding site for the nucleotide donating the reactive 3'-hydroxyl must in vivo be able to accommodate not only a phosphodiester linkage at the 5' end of the nucleotide during chain elongation but also a triphosphate moiety during formation of the initial linkage between the first two nucleotides. The simplest way to satisfy these requirements is for the enzyme-DNA complex to have little direct interaction with the immediate 5' end of this nucleotide during any stage of transcription. Our results suggest that this is the case since the kinetics of the latter reaction are only slightly affected by the complete absence of the normal triphosphate on the nucleotide at position 1. Finally, we note that interactions between the enzyme and the growing RNA chain *more distant* from the active site have been demonstrated (Muller et al., 1988) and would seem necessary to achieve the high degree of processivity characteristic of transcription by T7 RNA polymerase.

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