Effects of Solution Conditions on the Steady-State Kinetics of Initiation of Transcription by T7 RNA Polymerase†

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ABSTRACT: The T7 family of DNA-dependent RNA polymerases presents an ideal model system for the study of fundamental aspects of transcription. The small size of the promoter allows a variety of studies based on simple steady-state kinetics in the synthesis of a five-base runoff transcript. This assay can be used to characterize the effects on the initiation of transcription of simple modifications to potential protein or DNA specificity contacts. In the current work, *in vitro* conditions for this assay have been identified which optimize the apparent K_m for the interaction between the enzyme and the promoter DNA. The addition to the reaction mixture of 0.05% Tween-20 and the substitution of 10 mM NaCl by 100 mM potassium glutamate not only improves the quality of the kinetic assays but also decreases K_m by about an order of magnitude (strengthening the interaction between polymerase and its promoter). As observed for DNA binding in other systems, the parameter K_m increases substantially with increasing [NaCl], but the salt dependence is shifted to higher concentrations as a function of [KGlu]. Thermal denaturation of the protein, monitored by circular dichroism spectroscopy, confirms the effects of salt and supports a model in which Cl^- and other anions compete for phosphate binding sites on the protein. Finally, while K_m is highly dependent on [NaCl], the measured k_{cat} is relatively insensitive to salt. These data indicate that the parameters K_m and k_{cat} reflect changes respectively in promoter binding and in a rate-limiting step or steps leading to the initiation of transcription.

The recognition of a promoter DNA sequence by RNA polymerase represents the simplest and most fundamental mechanism for the regulation of transcription. The family of DNA-dependent RNA polymerases from the bacteriophages T7, T3, and SP6 presents an ideal model system in which to understand this recognition process (Chamberlin & Ryan, 1982). These single-subunit RNA polymerases are highly specific for an approximately 20 base pair, nonsymmetric promoter sequence (Oakley & Coleman, 1977; Oakley *et al.*, 1979). As such, this presents a recognition problem potentially an order of magnitude more complex than recognition of smaller, palindromic sequences by more simple DNA binding proteins. In addition, since the initiation of transcription is a complex process (McClure, 1985), the possibility exists for specificity at stages beyond simple binding. To begin to understand recognition in the initiation of transcription, we have recently exploited a steady-state kinetic assay (Martin & Coleman, 1987), coupled with the chemical synthesis of promoter DNA containing simple functional group modifications, to probe recognition contacts within the promoter DNA (Maslak & Martin, 1993; Maslak *et al.*, 1993; Schick & Martin, 1993).

Previous *in vitro* studies of T7 RNA polymerase (Chamberlin & Ring, 1973b; Smeekens & Romano, 1986; Gunderson *et al.*, 1987) and of the RNA polymerase from *Escherichia coli* (Mangel & Chamberlin, 1974; Miller & Burgess, 1978; Leirmo *et al.*, 1987) have shown a strong dependence of overall activity on various solution conditions. In particular, both enzymes show a substantial negative correlation of activity with increasing NaCl or KCl concentrations. In the case of *E. coli* RNA polymerase (Leirmo *et al.*, 1987), and preliminarily with the enzyme from T7 (Chapman & Burgess, 1987), this has been shown to be a function additionally of the type of anion, chloride inhibiting at lower concentrations than glutamate. These results have been interpreted in terms of a competition by chloride for anion binding sites on the protein which are normally involved in binding of DNA backbone phosphates. It is argued that glutamate binds less tightly to these sites on the protein (Ha *et al.*, 1992) and that it is the more physiologically relevant anion for use in *in vitro* studies (Leirmo *et al.*, 1987).

Since the above considerations indicate that T7 RNA polymerase binds its promoter more tightly at low chloride concentrations, the use of low concentrations of salt would seem desirable in studies of *in vitro* transcription. However, the enzyme has also been shown to aggregate at low ionic strengths (Zawadski & Gross, 1991). Such nonspecific adsorption would make estimates of effective protein concentrations in solution less reliable, and quantitative assays of the concentration dependence of reaction kinetics may suffer as a consequence. In the current study, we characterize the dependence of the steady-state kinetic parameters k_{cat} and K_m on a variety of solution conditions, in order both to improve the quality of fits of experimental data to simple models and to provide insight into the mechanisms underlying these parameters.

EXPERIMENTAL PROCEDURES

T7 RNA Polymerase. T7 RNA polymerase was prepared from *E. coli* strain BL21 carrying the overproducing plasmid pAR1219 (kindly supplied by William Studier and John Dunn, Brookhaven National Laboratories), which has the polymerase gene under inducible control of the *lac* UV5 promoter (Davanloo *et al.*, 1984). T7 RNA polymerase was purified as previously described (King *et al.*, 1986) by fractionation with Polymin P (less than 1.25%) and ammonium sulfate,

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followed by chromatography on Trisacryl SP-M (Sepracor), TSK-GEL Toyopearl CM-650 M (Supelco), and TSK-GEL Toyopearl DEAE-650 M (Supelco). A molar extinction of $\epsilon_{280} = 1.4 \times 10^5$ M^{-1} cm^{-1} was used to determine the enzyme concentration (King *et al.*, 1986). Purity of the enzyme was verified by SDS–polyacrylamide gel electrophoresis. Oligonucleotides were synthesized by the phosphoramidite method on a Milligen/Biosearch Cyclone Plus DNA synthesizer and were purified as previously described (Schick & Martin, 1993).

Kinetic Assay of Transcription Initiation. Kinetic assays of transcription (Martin & Coleman, 1987) were carried out at 37 °C in a total volume of 20 μL containing 30 mM HEPES,[1] pH 7.8; 0.25 mM EDTA; 1 mM DTT; 0.8 mM GTP; ~100 μCi/mL [α-^{32}P]GTP; 0.4 mM ATP; and magnesium salts, other salts, carrier protein, and detergent as indicated in the tables and figures, as described in Maslak *et al.* (1993). Transcription products were separated from unincorporated nucleotides by ascending paper chromatography (Mulligan *et al.*, 1985; Maslak *et al.*, 1993). For extremes of conditions, the correct synthesis of primarily the five-base runoff product was verified by denaturing (7 M urea) polyacrylamide (18–20%) gel electrophoresis (data not shown). In some cases, four-base products were also produced in low amounts and were included as total product formed. In no case were poly(G) products corresponding to stalled slippage at the promoter observed (Martin *et al.*, 1988).

For each template, reaction velocities were measured at various enzyme and DNA concentrations. The error in each velocity was approximated as the higher of 0.1 μM/min or the *t* distribution 80% confidence interval of the fitted slope for the three time points. Velocity data were then fit as previously described (Martin & Coleman, 1987) to the exact solution of the steady-state equation, using a weighted nonlinear least-squares minimization algorithm based on the Gauss–Newton method (Johnson *et al.*, 1981). Ranges in the values represent a 67% joint confidence interval of the fitted parameters. As a direct result of the nonlinear nature of the velocity equation, the confidence intervals are not symmetric. In particular, given the enzyme and promoter concentration ranges of the current study, increases in K_m (weakening of binding) have more confidence than do decreases in K_m. For a given promoter construct, the predicted velocity curves presented for the various enzyme concentrations all correspond to the single pair of best fit values to K_m and k_{cat}.

Circular Dichroism Measurements. Circular dichroism experiments were carried out with an Aviv 60 DS spectrometer in a 1-mm path length cell. Denaturation of T7 RNA polymerase was monitored at 222 nm in 1 °C temperature increments, with an acquisition time of 10 s per point. Reaction mixtures contained 9.8 μM each of T7 RNA polymerase and duplex 22 base pair promoter DNA, in 20 or 80 mM potassium phosphate, pH 7.8, and 0.05% Tween-20. In all cases, the denaturation was completely irreversible (at 50 °C, visible precipitation was substantial).

RESULTS

The assay for the initiation of transcription used in these studies employs a 22 base pair synthetic DNA template containing the consensus promoter sequence from positions

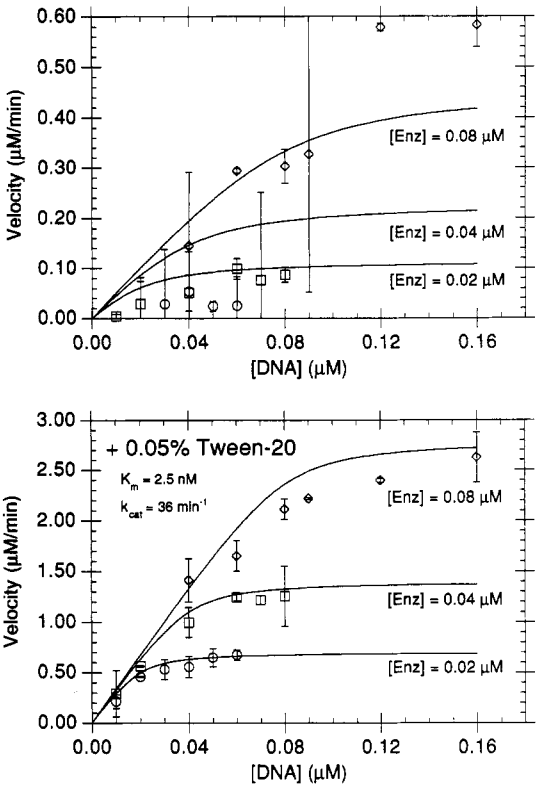


FIGURE 1: Effects of 0.05% Tween-20 on the concentration dependence of the velocity of the initiation of transcription in the absence of carrier protein. Final reaction conditions in each case were 100 mM KGlu, 30 mM HEPES, pH 7.8, 15 mM $Mg(OAc)_2$, 1 mM DTT, 0.25 mM EDTA, 0.8 mM GTP, and 0.4 mM ATP, with enzyme (○, 0.02 μM; □, 0.04 μM; ◇, 0.08 μM) and DNA concentrations as shown. The sample representing the lower panel additionally contained 0.05% Tween-20 (w/v). The curves in the lower panel correspond to the best fit kinetic parameters shown. The data in the upper panel did not fit well to simple theory; the curves shown correspond to K_m = 8 nM and k_{cat} = 5.7 min^{-1}.

−17 to −1 and coding for the five-base runoff transcript GGGAA.

As such, the assay is relatively insensitive to effects on stages of transcription beyond the immediate initiation of transcription. Analysis of RNA products generated from this DNA template (not shown) verifies that the expected five-base RNA is the predominant product.

Detergent Stabilizes the Enzyme. Initial studies of T7 RNA polymerase reported a requirement for BSA or spermidine for optimal activity in transcription of full-length templates (Chamberlin *et al.*, 1970; Chamberlin & Ring, 1973a; Butler & Chamberlin, 1982). We have verified that in the absence of spermidine the removal of carrier protein (BSA or *N,N*-dimethylated casein) from the protein buffer and assay mixture impairs *in vitro* transcription by T7 RNA polymerase. As shown in Figure 1 and summarized in Table 1, initiation is low and the appearance of product RNA is not reproducibly linear over a 9-min time course (as evidenced by large errors in the velocities). Consequently, the data are not fit well by simple steady-state kinetics (estimates of K_m and k_{cat} are relatively meaningless in this case). Comparison of velocities at two different enzyme concentrations (*e.g.*, 0.04 and 0.08

[1] Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KGlu, potassium glutamate; $Mg(OAc)_2$, magnesium acetate.

Table 1: Comparison of the Effects of Carrier Protein and Detergent on Steady-State Kinetic Parameters[a]

[Tween-20] (%)	[casein] (mg/mL)	[BSA] (mg/mL)	K_m (nM)	k_{cat} (min^{-1})
			unable to fit	
		0.1	6.5 (3.4–12)	35 (32–38)
	0.1		1.1 (0.4–3.0)	30 (29–31)
0.05			3.6 (2.2–5.7)	33 (31–35)
0.05		0.1	2.4 (1.2–4.8)	35 (33–37)
0.05	0.1		2.0 (0.8–4.2)	28 (27–30)

[a] All reaction mixtures contained, in addition to the components indicated, 100 mM KGlu, 30 mM HEPES, pH 7.8, 15 mM $Mg(OAc)_2$, 1 mM DTT, 0.25 mM EDTA, 0.8 mM GTP, and 0.4 mM ATP.

μM), illustrated in Figure 1, demonstrates that transcription at the lower protein concentration yields substantially lower velocities than predicted by simple extrapolations from the higher concentration data.

The addition of either BSA or *N,N*-dimethylated casein improves substantially the initiation of transcription, confirming previously identified requirements for so-called carrier protein. The inclusion of 0.05% (w/v) Tween-20, however, completely removes the requirement for carrier protein. As for the addition of carrier protein, overall synthesis of the five-base message is substantially increased (compare the velocity scales in the two panels of Figure 1). Of equal importance, the velocities in the presence of detergent fit very well to velocities predicted from the simple steady-state equation, providing the kinetic parameters summarized in Table 1. Note that, in addition to the two parameters K_m and k_{cat}, one can also include as an adjustable-fit parameter the ratio of active enzyme to DNA. Under these conditions, allowing all three parameters to be optimized simultaneously, the ratio of enzyme to DNA is very close to 1.0 (fits not shown).

The concentration dependence of the activation by Tween-20 has been examined over the range from 0.025% to 0.15% (w/v), yielding a relatively broad maximum in overall kinetics near 0.05% (data not shown). We have also observed that other detergents can substitute in this activation; less complete studies with 0.05–0.20% octyl glucoside suggest that it substitutes equally well in the assay. These results suggest that the stabilization afforded by detergent is a general stabilization of the system. The nonionic detergent Tween-20 is now a routine component of both assay and enzyme storage and dilution buffers.

Sulfhydryl Reducing Agents Are Not Required. During the course of our studies we have found that, contrary to previous reports (Chamberlin & Ring, 1973a), the presence of either of the reducing agents dithiothreitol and β-mercaptoethanol is not required for full activity. Although it remains possible that such reagents stabilize very long term storage of the enzyme, we have maintained enzyme solutions without reducing agents for as long as 6 months at 4 °C with no loss in activity (unpublished results). It is possible that previous observations of such requirements reflected impurities present in the enzyme solution.

K_m, but Not k_{cat}, Is Strongly Dependent on [NaCl]. Many DNA binding proteins, including T7 RNA polymerase, show a strong negative correlation of DNA binding with increasing concentration of NaCl (Chamberlin & Ring, 1973b; Oakley *et al.*, 1975; Shaner *et al.*, 1982; Smeekens & Romano, 1986; Wheeler *et al.*, 1987; Gunderson *et al.*, 1987). The kinetic data presented in Figure 2 demonstrate that binding (as represented by K_m) is substantially weaker in 100 mM NaCl compared with that in 100 mM KGlu. A more complete comparison of steady-state kinetic parameters for the initiation of transcription by T7 RNA polymerase as a function of

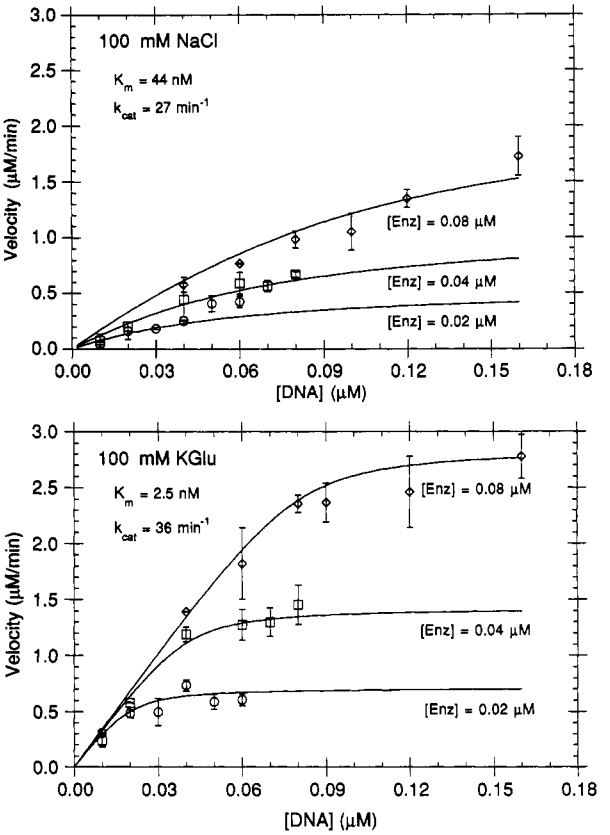


FIGURE 2: Effects of anion type on the concentration dependence of the velocity of the initiation of transcription. Final reaction conditions in each case were 100 mM NaCl or KGlu, 0.05% Tween-20 (w/v), 30 mM HEPES, pH 7.8, 15 mM $Mg(OAc)_2$, 0.1 mg/mL *N,N*-dimethylated casein, 1 mM DTT, 0.25 mM EDTA, 0.8 mM GTP, and 0.4 mM ATP, with enzyme (○, 0.02 μM; □, 0.04 μM; ◇, 0.08 μM) and DNA concentrations as shown.

increasing [NaCl], summarized in Table 2, shows that the apparent Michaelis constant, K_m, depends strongly on the concentration of NaCl. As discussed below, the large increase in K_m almost certainly parallels a large increase in the dissociation constant for complex formation (reflecting a decrease in stability of the protein–DNA complex). In contrast to the behavior of K_m, the kinetic parameter k_{cat} shows little dependence on the concentration of NaCl over the range from 10 to 200 mM. This strongly suggests that DNA binding does not contribute to the rate-limiting step in initiation, measured by k_{cat}, and further supports the independence of these two parameters. Finally, note that the joint confidence intervals for the fit parameters generally increase at higher concentrations of NaCl, indicating less ideal behavior of the system at high salt.

Potassium Glutamate Is Tolerated at Higher Concentrations Than NaCl. The results in Table 2 demonstrate that, as for other DNA binding proteins (Leirmo *et al.*, 1987), the effect of increasing concentrations of NaCl is much larger

Table 2: Comparison of the Effects of Salt Concentration and Type on Steady-State Kinetic Parameters[a]

[NaCl] (mM)	[KGlu] (mM)	K_m (nM)	k_{cat} (min^{-1})
10		1.3 (0.4–3.7)	28 (26–30)
25		7.1 (4.3–12)	32 (30–34)
100		44 (27–73)	27 (22–32)
150		100 (50–180)	38 (32–44)
200		330 (230–470)	38 (32–45)
	10	0.4 (<0.1–2.0)	27 (26–29)
	25	2.2 (1.1–4.3)	30 (29–31)
	50	5.2 (2.6–10)	37 (35–39)
	100	2.0 (0.8–4.2)	28 (27–30)
	175	11 (4.5–25)	38 (33–43)
	250	26 (6.4–96)	41 (27–54)

[a] All reaction mixtures contained, in addition to the components indicated, 0.05% Tween-20 (w/v), 30 mM HEPES, pH 7.8, 15 mM $Mg(OAc)_2$, 1 mM DTT, 0.25 mM EDTA, 0.1 mg/mL *N,N*-dimethylated casein, 0.8 mM GTP, and 0.4 mM ATP.

than that of the same concentrations of potassium glutamate, KGlu. Although the replacement of NaCl by KGlu improves the overall quality of the steady-state kinetic fits using oligonucleotide templates, preliminary results (data not shown) show no change in the pattern or amount of abortive transcripts on longer DNA templates (Martin *et al.*, 1988). This further supports the conclusion that the anion effect operates only at the level of initial promoter binding and/or general protein stability.

The Requirement for Magnesium. With improved reaction conditions in hand it is instructive to revisit the requirement for magnesium in the reaction. Results summarized in Table 3 show that the kinetics are insensitive to the replacement of 15 mM $Mg(OAc)_2$ by the same concentration of $MgCl_2$. This result is consistent with the chloride dependence shown in Table 2. Also note that the nonlinear nature of the joint confidence intervals for the fit parameters indicates that the determined K_m values for the first six entries in Table 3, which compare the effects of decreasing concentrations of $Mg(OAc)_2$ and varying anion and cation type at low salt concentrations, are statistically indistinguishable (Maslak *et al.*, 1993). Finally, under the reaction conditions described here (including 100 mM potassium glutamate), the dependence of overall transcription on magnesium concentration is similar to that in previous reports (Chamberlin & Ring, 1973a). Preliminarily, limiting levels of $Mg(OAc)_2$ (2.0 mM) affect both K_m and k_{cat}. Thus, as expected, the steps monitored by k_{cat} require magnesium, but additionally optimal binding (as reflected in K_m) also requires a minimal level of magnesium, as reported previously (Gunderson *et al.*, 1987).

Binding of Ions Increases Protein Stability. The above results suggest that anions compete with DNA for specific binding sites on the protein. The energetically favorable binding of a ligand to a specific binding site on a folded protein necessarily stabilizes the protein to unfolding. Since T7 RNA polymerase is very largely α-helical (Sousa *et al.*, 1993), the stability of the protein to thermal denaturation can be studied by monitoring the circular dichroism (CD) at 222 nm as a function of temperature. Although the unfolding transition is completely irreversible, the midpoint of the unfolding transition (T_m) provides a qualitative measure of protein stability.

The midpoints of unfolding of T7 RNA polymerase are compared in Figure 3 in the presence of promoter DNA and/or increased levels of phosphate (buffer choice is restricted in this case by the need for solution components which do not contribute to the CD spectrum at 222 nm). At low ionic concentrations (20 mM potassium phosphate), the T_m is 42.8 °C. An increase in phosphate concentration to 80 mM raises the T_m to 45.8 °C ($\Delta T_m^{phosphate}$ = 3.0 °C), confirming the ion-induced stabilization of the protein. Similarly, under the lower salt conditions, the addition of equimolar amounts of 22 base pair promoter DNA increases the T_m to 49.3 °C ($\Delta T_m^{promoter}$ = 6.5 °C). At the higher salt concentration, however, the addition of promoter DNA results in a smaller increase in stability ($\Delta T_m^{promoter}$ = 2.6 °C), consistent with the competition of DNA and anions for the ion binding sites on the protein. Preliminary studies using differential scanning microcalorimetry to measure protein unfolding provided similar results (data not shown). The phosphate anion competes in binding, as confirmed by steady-state kinetic measurements. At low (20 mM) potassium phosphate, K_m = 3.5 nM and k_{cat} = 27 min^{-1}, similar to kinetic parameters observed at low concentrations of chloride. At 80 mM potassium phosphate, K_m increases to 26 nM and k_{cat} decreases to 11 min^{-1}. The latter result may indicate a competition of phosphate for binding at the active site nucleotide triphosphate binding site(s), but the increase in K_m strongly supports a competition for promoter binding.

DISCUSSION

In the earliest characterizations of T7 RNA polymerase, a variety of requirements were described for optimal transcription from long templates (Chamberlin *et al.*, 1970; Chamberlin & Ring, 1973a). A sulfhydryl reducing agent, Mg^{2+}, low salt, and BSA were found to be necessary for optimal activity. In the current work, we examine some of the requirements for the initiation phase of transcription (independent of later stages), using highly purified, overproduced enzyme and chemically synthesized DNA templates. In addition to providing comparisons to reported requirements for overall activity, we have obtained conditions which substantially improve the fits of experimental data to a simple steady-state kinetic scheme.

Original measurements of an apparent Michaelis constant, K_m, in this steady-state kinetic system found values of 15–20 nM under conditions of 10 mM NaCl and 20 mM $MgCl_2$ (Martin & Coleman, 1987). By comparison, the best measurements of static promoter binding (under conditions of 10 mM NaCl and 2 mM $MgCl_2$) have found a dissociation constant (K_d) of 10–25 nM (Gunderson *et al.*, 1987). The set of reaction conditions presented in Table 4 provides measured steady-state kinetics which not only fit very well to the simple kinetic model discussed below, and are more reproducible than in early studies, but also result in a K_m value (1–3 nM) which is 10-fold lower than these values. Although the latter is of practical importance only at low concentrations of enzyme and DNA, these conditions may, in some cases, lead to improved synthesis of RNA for biochemical and biophysical studies (Milligan & Uhlenbeck, 1989). More importantly, in studies of promoter recognition, *in vitro* conditions should be employed which both optimize binding strength and more faithfully reflect intracellular conditions (Cayley *et al.*, 1991).

Detergent Stabilizes the Enzyme. Previous studies indicated that BSA or spermidine was required for optimal activity, presumably to bind inhibitory polyanions (Chamberlin & Ring, 1973a). The high purity of our conditions (apparent lack of inhibitory polyanions), combined with the fact that Tween-20, a nonionic detergent, can replace the need for carrier protein, argues against this explanation. We have observed that T7 RNA polymerase binds nonspecifically to glass surfaces (J. Gelles and C. T. Martin, unpublished results). It has also been observed that the enzyme has some tendency

Table 3: Comparison of the Effects of Magnesium Salts on Steady-State Kinetic Parameters[a]

[NaCl] (mM)	[KCl] (mM)	[KGlu] (mM)	[$MgCl_2$] (mM)	[$Mg(OAc)_2$] (mM)	K_m (nM)	k_{cat} (min^{-1})
	10		15		2.7 (0.9–6.7)	26 (23–28)
	10			15	0.1 (<0.1–1.5)	27 (25–28)
		10	15		0.3 (0.4–2.5)	29 (27–31)
10			15		2.0 (0.9–4.2)	34 (31–36)
10				15	1.3 (0.4–3.7)	28 (26–30)
		100		15	2.0 (0.8–4.2)	28 (27–30)
		100		8.0	1.9 (0.7–3.6)	28 (26–29)
		100		2.0	50 (26–105)	15 (11–19)

[a] All reaction mixtures contained, in addition to the components indicated, 0.05% Tween-20 (w/v), 30 mM HEPES, pH 7.8, 1 mM DTT, 0.25 mM EDTA, 0.1 mg/mL *N,N*-dimethylated casein, 0.8 mM GTP, and 0.4 mM ATP.

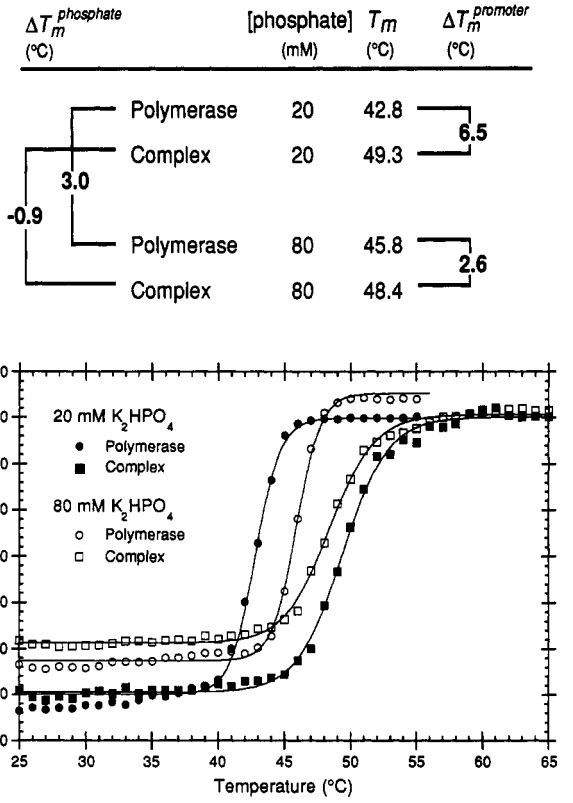


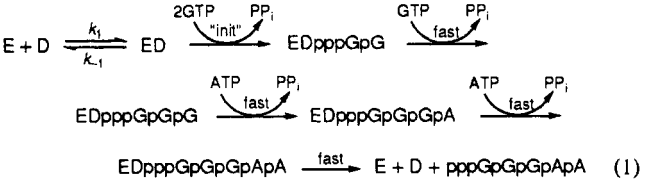
FIGURE 3: Effects of phosphate concentration on thermal unfolding of the enzyme as measured by circular dichroism at 222 nm. The midpoint melting temperature (T_m) for the unfolding of T7 RNA polymerase measured by CD was measured at low ionic strength (20 mM potassium phosphate) and at high ionic strength (80 mM potassium phosphate) for enzyme alone and for enzyme complexed with the 22 base pair promoter DNA. In order to facilitate the CD studies, potassium phosphate served as both the buffer and the ionic species. The difference in the midpoint melting temperature (ΔT_m) reflects stabilization of the protein by complexation with the promoter ($\Delta T_m^{promoter}$) or by increased phosphate concentration ($\Delta T_m^{phosphate}$). Final reaction conditions in each case were 0.05% Tween-20 (w/v) and either 20 or 80 mM potassium phosphate, pH 7.8.

Table 4: Current Reaction Conditions for Initiation Kinetics

0.05%	Tween-20 (w/v)
100 mM	KGlu
30 mM	HEPES, pH 7.8
15 mM	$Mg(OAc)_2$
0.25 mM	EDTA
0.1 mg/mL	*N,N*-dimethylated casein (optional)
1 mM	DTT (optional)
0.8 mM	GTP
0.4 mM	each of ATP, CTP, and UTP (as required)

to aggregate at low ionic strength (Zawadzki & Gross, 1991), as observed for the enzyme from *E. coli* (Shaner *et al.*, 1982). Indeed, the first characterizations of the T7 enzyme indicated that enzyme activity was lost when diluted in the absence of BSA (Chamberlin *et al.*, 1970). The detergent-mediated improvement in the fit of the initiation kinetics to simple theory is likely due at least in part to a stabilization of the enzyme to nonspecific adsorptions (aggregation and/or adsorption to container walls), providing more reliable estimates of concentration and uniformity of the enzyme in solution. In this regard, detergent must be included in enzyme stock dilutions prepared prior to the kinetic assay. This stabilization is only due to such stabilizations against adsorptions and not to overall stabilization of the folding of the enzyme, as evidenced by the fact that detergent does not significantly alter the thermal stability of the enzyme to unfolding (circular dichroism and differential scanning microcalorimetry data not shown).

Implications for Steady-State Kinetics. Although the steady-state kinetic assay has been used primarily to identify functional group interactions between the polymerase and the promoter DNA (Maslak *et al.*, 1993; Schick & Martin, 1993), it is instructive to consider potential mechanistic contributions to the determined kinetic parameters. A simple mechanistic scheme for the synthesis of the five-base transcript GGGAA is shown in eq 1.



The apparent equilibrium described by k_1 and k_{-1} represents the specific association of enzyme and promoter. It may also include isomerizations to open and/or activated complexes as seen for the enzyme from *E. coli* (McClure, 1980; Buc & McClure, 1985), although previous studies with the T7 enzyme suggest that DNA melting contributes only slightly to the observed kinetics (Maslak & Martin, 1993). The process denoted "init" in eq 1 is certainly a multistep reaction, including at least the binding of two nucleoside triphosphates and the first catalytic event. Under the conditions used in these studies, GTP is at saturating concentrations, such that this process can be represented by an apparent first-order rate constant (k_{cat}, first order in [ED]). Despite the apparent complexity of eq 1, the steady-state synthesis of a five-base transcript fits well to the simpler form given by eq 2.

$$E + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED \xrightarrow{k_{cat}} E + D + pppGpGpGpApA \qquad (2)$$

Evidence Supporting the Assignment of k_{cat} to Initiation. In order to understand the simple behavior of the steady-state kinetics, note that during processive elongation the average turnover rate per nucleotide addition is approximately 230 s^{-1} (Golomb & Chamberlin, 1974), while the turnover number

for synthesis of the five-base transcript (k_{cat}) is 0.5–1.0 s^{-1} (Martin & Coleman, 1987; Maslak *et al.*, 1993). If the elongation rate during this early stage of polymerization is the same order of magnitude as during processive elongation, the three subsequent elongation events, labeled "*fast*" in eq 1, will be too fast to enter into the measured kinetic parameters and can be ignored. The conclusion that the final step in eq 1, dissociation of the ternary complex, is not rate limiting stems from a variety of indirect evidence. Specifically, changes in the *nontranscribed* sequence of the promoter can lead to substantial increases in K_m, with no increase in k_{cat} (Martin & Coleman, 1987; Maslak & Martin, 1993; Maslak *et al.*, 1993). Modifications in this region of the DNA might be expected to disrupt promoter binding and initiation, but are less likely to influence runoff termination five-bases downstream. More importantly, k_{cat} is the same for the synthesis of the RNA transcripts GGGAA and GACU (Martin & Coleman, 1989), sequences with very different expected heteroduplex stabilities. Finally, the fact that, in the synthesis of the four-base transcript GACU, substitution of ITP for GTP reduces k_{cat} at saturating levels of ITP (Martin & Coleman, 1989) argues strongly that catalysis contributes to this kinetic parameter (we have additionally found that initiation is immeasurably weak for the incorporation of inosine into the message GGGAA). While incorporation of inosine might weaken a heteroduplex and lead to *higher* rates of product release, weaker hydrogen bonding between the substrate inosine and cytosine in the template could lead to less rigid positioning of the substrate and a reduced catalytic rate, as predicted for subsequent nucleoside additions (Martin *et al.*, 1988). Although the kinetic constant k_{cat} may contain contributions from several substeps within the initiation process (*e.g.*, DNA melting and/or catalysis), the above analysis argues strongly that changes in k_{cat} reflect changes in the rate-limiting step or steps in the initial synthesis of the RNA.

Evidence Supporting the Assignment of K_m to Complex Dissociation. As in any kinetic analysis of a complex reaction process, the Michaelis constant, K_m, for the steady-state solution to eq 2 represents an underlying series of events. Nevertheless, previous evidence, combined with our current results, suggests that K_m is dominated by binding interactions between polymerase and promoter. Indeed, prior to the development of the improved reaction conditions reported here, native values for K_m, 15–20 nM (Martin & Coleman, 1987), were comparable to the best reported measurements of dissociation constants, 10–25 nM, determined by quantitative footprinting (Gunderson *et al.*, 1987).

The dependence of the steady-state kinetic parameters on solution conditions presented in the current study, combined with kinetic measurements from modified promoters (Martin & Coleman, 1987; Maslak & Martin, 1993; Maslak *et al.*, 1993; Schick & Martin, 1993), demonstrates that K_m and k_{cat} can vary independently, as expected in the simplest of kinetic schemes (eq 2). The results summarized in Table 2 show that while K_m is very sensitive to increasing concentrations of NaCl, k_{cat} is relatively unaffected. Early studies of transcription of T7 DNA in a 2-min assay showed that overall transcription is highly sensitive to 200 mM NaCl if the salt is added prior to transcription, but that the addition of 200 mM NaCl 15 s after initial mixing allowed substantially increased activity (Chamberlin & Ring, 1973b). Subsequent studies have confirmed this result and indicate that the elongation rate is not affected by 200 mM KCl (McAllister & Carter, 1980). Similarly, it has been shown that the pattern of abortive products produced immediately following initiation is not sensitive to 200 mM NaCl (Martin *et al.*, 1988). The inhibitory effect of salt must be acting at the level of initial promoter binding. The circular dichroism measurements of ligand-induced (promoter-induced) stabilization of the protein lend further support to this conclusion and to the proposal that certain anions compete for sites on the protein which normally serve to bind the DNA phosphate backbone (Ha *et al.*, 1992).

Finally, various groups have reported the salt sensitivity of simple promoter binding (Oakley *et al.*, 1975; Smeekens & Romano, 1986; Gunderson *et al.*, 1987; Chapman *et al.*, 1988). Those results combined with the data presented here argue strongly that the steady-state parameter K_m measures either specific promoter binding or a step dominated by binding. Furthermore, the parameter k_{cat} is relatively independent of perturbations to binding and likely measures a rate-limiting step or steps leading to the promoter-dependent initiation of catalysis. The independence of these parameters allows a preliminary assignment of the effects of DNA, protein, or solution modifications to specificity effects at the level of binding or catalysis.

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