Determinants of Ribose Specificity in RNA Polymerization: Effects of Mn^{2+} and Deoxynucleoside Monophosphate Incorporation into Transcripts†

Ying Huang,‡ Amber Beaudry,§ Jim McSwiggen,§ and Rui Sousa*,‡

Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, Texas 78284-7760, and Ribozyme Pharmaceuticals Incorporated, 2950 Wilderness Place, Boulder, Colorado 80301

Received July 3, 1997; Revised Manuscript Received August 26, 1997⊗

ABSTRACT: The catalytic specificity of T7 RNA polymerase (RNAP) for ribonucleoside triphosphates vs deoxynucleoside triphosphates $\{(k_{cat}/K_m)_{rNTP}/(k_{cat}/K_m)_{dNTP}\}$ during transcript elongation is ∼80. Mutation of tyrosine 639 to phenylalanine reduces specificity by a factor of ∼20 and largely eliminates the K_m difference between rNTPs and dNTPs. The remaining specificity factor of ∼4 is k_{cat}-mediated and is nearly eliminated if Mn^{2+} is substituted for Mg^{2+} in the reaction. Mn^{2+} substitution does not significantly affect the K_m difference between rNTPs and dNTPs. Mn^{2+} substitution also enhances the activity of poorly active mutant enzymes carrying nonconservative substitutions in the active site, and its effects are generally consistent with the Mn^{2+}-catalyzed reaction being less restrictive in its requirements for alignment of the reactive groups. In addition to discrimination occurring at the level of nucleoside monophosphate (NMP) incorporation, it is also found that transcripts containing deoxynucleoside monophosphates (dNMPs) are more poorly extended than transcripts of canonical structure, though a severe barrier to transcript extension is seen only when the 3′ region of the transcript is heavily substituted with dNMPs. The barrier to extension of transcripts heavily substituted with dNMPs is reduced for sequences known to be amenable to forming A-like helices and is larger for sequences that resist transformation from B-form DNA·DNA structures. The barrier to extension of dNMP-substituted transcripts is also reduced by solution conditions known to destabilize B-form DNA and to stabilize A-form structures. These observations imply a requirement for a non-B-form, possibly A-like, conformation in the transcript·template hybrid that is disrupted when the transcript is of predominantly deoxyribose structure.

The molecular mechanisms used by nucleic acid polymerases to discriminate substrate structure so as to specify the synthesis of particular products (DNA, RNA, or nucleic acids incorporating noncanonical nucleotides) have, until recently, been obscure. However, over the past few years a number of studies have emerged identifying single amino acid substitutions in the active sites of RNA and DNA polymerases that have dramatic effects on discrimination between ribose 3′ H- or OH-substituted triphosphates (*2*), and between ribose 2′ H- or OH-substitutions (*3*−*5*; see ref *6* for a review).

During transcript elongation T7 RNAP[1] incorporates rNMPs with, on average, ∼80-fold higher efficiency than it incorporates dNMPs. The value of ∼80 for the $(k_{cat}/K_m)_{rNTP}/(k_{cat}/K_m)_{dNTP}$ term during elongation is accounted for by an average ∼20-fold difference in K_m values and an average ∼4-fold difference in k_{cat} values. The difference in K_m values is essentially eliminated by mutation of active-site tyrosine 639 to phenylalanine (*5*). The effect of this mutation is remarkably similar to the effect of a Y to F substitution at position 34 in tyrosyl-tRNA synthetase (*7*): in both cases the Y to F substitution has little effect on kinetic parameters for utilization of the canonical substrate (rNTP or tyrosine) but enhances utilization of a noncanonical substrate (dNTP or phenylalanine) that differs from the canonical substrate only in having a hydroxyl group replaced by a hydrogen atom. In both cases the molecular mechanism of the reduced specificity of the mutant enzyme is attributed to loss of a hydrogen bond between the tyrosine hydroxyl and the relevant -OH group on the canonical substrate. That the loss of this H-bonding interaction gives rise to reduced specificity through enhanced utilization of the noncanonical substrate (rather than reduced utilization of the canonical substrate) is attributed to the fact that this interaction makes little *net* contribution to polymerase·substrate association because a water molecule H-bonded to the tyrosine hydroxyl must be displaced to allow catalytically optimal substrate binding.

Previous results have also suggested that, in addition to mechanisms that discriminate at the level of substrate incorporation, extension of transcripts containing dNMPs is less efficient than extension of transcripts of canonical structure (*3*). The determinants of specificity in transcript extension—as opposed to substrate incorporation—have not yet been elucidated. Furthermore, while substitution of Mg^{2+} with Mn^{2+} in the synthesis reaction was found to reduce the substrate specificity of both the wt and Y639F enzymes, the mechanism of the Mn^{2+} effect on substrate specificity and enzyme activity was not addressed in detail.

† Supported by NIH Grant GM52522-01 (to R.S.) and funds from the Texas ARP/ATP.

* To whom correspondence should be addressed: Phone 210-567-3760; Fax 210-567-6595; e-mail sousa@bioc02.uthscsa.edu.

‡ University of Texas Health Science Center.

§ Ribozyme Pharmaceuticals Inc.

⊗ Abstract published in *Advance ACS Abstracts,* October 15, 1997.

[1] Abbreviations: RNAP, RNA polymerase; DNAP, DNA polymerase; dNTP, deoxynucleoside triphosphate; rNTP, ribonucleoside triphosphate; rNMP, ribonucleoside monophosphate; dNMP, deoxynucleoside monophosphate.

S0006-2960(97)01609-7 CCC: $14.00

Here we address these questions. We find that whether a transcript can be efficiently extended is not primarily determined by whether the 3′-NMP is a dNMP or an rNMP but by the level of dNMP substitution in the 3′ region of the transcript. This appears to reflect a requirement for a specific helix conformation in the transcript·template hybrid that is disrupted when the transcript is composed primarily of dNMPs. We also find that the Mn^{2+}-catalyzed reaction is less sensitive to nonconservative active-site mutations and that the reduced substrate specificity of the polymerase in the presence of Mn^{2+} is due largely to an increase in k_{cat} for the noncanonical substrate relative to the canonical substrate. We interpret these observations as indicating that the Mn^{2+}-catalyzed reaction is compatible with a wider range of reactive group geometries than is the Mg^{2+}-catalyzed reaction.

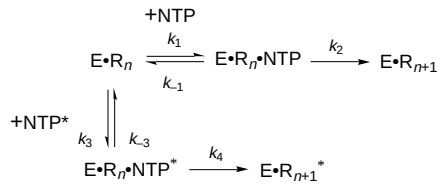
MATERIALS AND METHODS

Mutant and wt enzymes were purified as described (*8*). Activity determinations and NTP K_m determinations were carried out as described (*5*) using supercoiled pT75, which carries a single copy of the T7 ϕ10 promoter (*9*), or homopolymers as templates.

Kinetic parameters for utilization of rGTP and dGTP as elongating substrates for dinucleotide synthesis were also determined as described in ref *5* with reaction conditions modified as follows for particular experiments: (1) Substitution of Mg^{2+} with Mn^{2+} in the reaction was done by replacing the 15 mM $MgCl_2$ in the standard reaction buffer with 15 mM $MnCl_2$ and (2) methanol or ethanol was added to the some reactions to a final percentage (v/v) as specified in the tables and figure captions while maintaining all other reactant and buffer components at constant (moles/volume) concentrations.

Meaning of the rNTP/dNTP Selectivity Term Obtained in the Incorporation Assays. Determination of the catalytic specificity of T7 RNAP for rNTPs vs dNTPs during elongation (as presented in Tables 1 and 8) was carried out as described in ref *5*. In these assays the four unlabeled rNTPs are present at 0.5 mM and a single α-^{32}P-labeled rNTP or dNTP is added to a final concentration of 33 nM. The ratio of the rate of percent incorporation of the radioactive rNTP relative to the radioactive dNTP is then equal to $(k_{cat,rNTP}/K_{m,rNTP})/(k_{cat,dNTP}/K_{m,dNTP})$, as is shown below.

Consider the reaction scheme for an elongation complex that can incorporate either a cold NTP or a radioactively labeled NTP* ($E{\cdot}R_n$ = elongation complex containing transcript of length n):



The rate of incorporation of the labeled NTP is then equal to

$$V_{NTP} = \frac{k_4[E_{tot}]K_{m1}[NTP^*]}{K_{m2}K_{m1} + [NTP^*]K_{m1} + [NTP]K_{m2}}$$

where $K_{m1} = (k_2 + k_{-1})/k_1$ and $K_{m2} = (k_4 + k_{-3})/k_3$. If the cold NTP is present in great excess of the labeled NTP (i.e., in our case [NTP] = ~10 000[NTP*]) and K_{m1} is not much larger than K_{m2} (in our case $K_{m1} \leq K_{m2}$), then the above equation, for *fractional* incorporation, reduces to

$$\frac{V_{NTP^*}}{[NTP^*]} = \frac{k_4[E_{tot}]K_{m1}}{K_{m2}K_{m1} + [NTP]K_{m2}}$$

or

$$\frac{V_{NTP^*}}{[NTP^*]} = \frac{k_4[E_{tot}]K_{m1}/K_{m2}}{K_{m1} + [NTP]}$$

If the labeled NTP is chemically identical to the unlabeled NTP (i.e., both are rNTPs) then obviously $K_{m1} = K_{m2} = K_{m,rNTP}$ and $k_2 = k_4 = k_{cat,rNTP}$. However, if the labeled NTP is a dNTP, then $K_{m1} = K_{m,rNTP} \neq K_{m2} = K_{m,dNTP}$ and $k_2 = k_{cat,rNTP} \neq k_4 = k_{cat,dNTP}$. The ratio of the rate of fractional incorporation of the labeled rNTP relative to the dNTP is then equal to

$$(V_{rNTP^*}/[rNTP^*])/(V_{dNTP^*}/[dNTP^*]) = \{k_{cat,rNTP}[E_{tot}]/(K_{m,rNTP} + [NTP])\}/\{(k_{cat,dNTP}[E_{tot}]K_{m,rNTP}/K_{m,dNTP})/(K_{m,rNTP} + [NTP])\} = (k_{cat,rNTP}/K_{m,rNTP})/(k_{cat,dNTP}/K_{m,dNTP})$$

Kinetic parameters for utilization of rGTP and dGTP as initiating substrates for dinucleotide synthesis were determined using the synthetic hairpin T7 promoter template described in (ref *5*) (HP-GA) in 20 μL reactions containing 15 mM $MgCl_2$, 40 mM Tris, pH 8.0, 5 mM DTT, 1 mM EDTA, with [template] = 1×10^{-7} M, [polymerase] = 1×10^{-6} M, [ATP] = 0.5 mM, and dGTP or rGTP varying from 0.018 to 2.25 mM in serial 2-fold dilutions. [α-^{32}P]ATP was added in trace amounts for labeling. Reactions were initiated by addition of polymerase and were run at 37 °C. Aliquots (5 μL) were taken at 10 and 20 min, mixed with an equal volume of 90% formamide, 0.01% xylene cyanol, and 50 mM EDTA, and electrophoresed on 20% acrylamide/4% bisacrylamide/6 M urea gels. Dinucleotide and unincorporated radioactivity (mononucleotide) bands were quantitated using a Molecular Dynamics phosphorimager. Percent incorporation was translated into the rate of dinucleotide synthesis as a function of [GTP] and rate data were fit by nonlinear least squares (NONLIN) using the Michaelis−Menten equation to derive K_m and k_{cat} values.

Determination of the extension efficiency of transcripts of different ribose/deoxyribose composition was carried out at 37 °C for 10 min in 25 μL reaction volumes using pT75 as a template at 10^{-7} M and wt or Y639F polymerase at 10^{-8} M in 15 mM $MgCl_2$, 40 mM Tris, pH 8.0, 5 mM DTT, and 1 mM EDTA. Varying combinations of rNTPs, dNTPs, and rGMP were used in different reactions as indicated in the tables and figures. rNTPs were always present at 0.5 mM, and dNTPs and rGMP were added to final concentrations of 1 mM. Reactions contained either [α-^{32}P]GTP or dGTP for labeling, depending on whether cold rGTP or dGTP was being used in the reaction. Reactions were terminated by addition of an equal volume of 90% formamide, 0.01% xylene cyanol, and 50 mM EDTA and electrophoresed on 20% acrylamide/4% bisacrylamide/6 M urea gels, which were quantitated on a Molecular Dynamics phosphorimager.

Table 1: Relative Activity and rNTP/dNTP Specificity of wt and Y639 Mutant Polymerases in Mg^{2+} or Mn^{2+} Catalyzed Reactions[a]

RNAP	activity	A	C	G	U	average
			Mg^{2+}			
wt	200	121 ± 33	89 ± 13	60 ± 11	34 ± 8.5	76
Y639F	200 ± 32	5.0 ± 1.8	7.5 ± 2.0	2.0 ± 0.41	1.8 ± 0.39	4.1
Y639M	104 ± 15	8.8 ± 1.5	8.5 ± 2.0	2.3 ± 0.41	2.5 ± 0.84	5.5
Y639L	86 ± 12	8.2 ± 1.1	21 ± 4.7	12 ± 3.2	4.0 ± 0.97	11
Y639C	15 ± 5.6	14 ± 2.3	14 ± 5.3	9.2 ± 2.3	6.6 ± 2.4	11
Y639V	8.6 ± 4.6	22 ± 6.6	21 ± 3.9	26 ± 5.0	11 ± 1.7	19
Y639T	2.6 ± 0.86	8.8 ± 1.7	7.1 ± 2.4	4.9 ± 1.0	5.0 ± 9.8	6.5
Y639Q	2.0 ± 0.34	5.3 ± 1.5	6.8 ± 1.8	3.5 ± 1.6	2.4 ± 1.4	4.5
Y639H	0.74 ± 0.08	nd	nd	nd	nd	nd
S641A	186 ± 26	125 ± 28	77 ± 10	59 ± 7.4	30 ± 9.0	73
Y639F/S641A	178 ± 22	12 ± 0.85	10 ± 4.2	4.1 ± 1.6	2.7 ± 0.57	7.3
			Mn^{2+}			
wt	21 ± 1.9	40 ± 14	18 ± 2.5	14 ± 5.6	9.6 ± 3.7	20
Y639F	12 ± 1.2	1.9 ± 0.50	1.7 ± 0.50	1.2 ± 0.34	2.0 ± 0.6	1.7
Y639M	11 ± 0.96	3.3 ± 0.25	1.7 ± 0.15	1.2 ± 0.50	2.2 ± 0.65	2.1
Y639L	13 ± 1.4	3.1 ± 0.15	4.2 ± 0.50	2.6 ± 0.25	2.5 ± 0.55	3.1
Y639C	16 ± 4.0	1.0 ± 0.05	1.0 ± 0.50	0.97 ± 0.14	1.8 ± 0.30	1.2
Y639V	14 ± 2.4	32 ± 8.1	14 ± 4.6	7.1 ± 1.2	4.8 ± 0.60	14
Y639T	5.2 ± 1.0	25 ± 8.9	13 ± 3.5	4.3 ± 1.4	6.1 ± 0.25	12
Y639Q	3.4 ± 0.34	5.1 ± 1.5	1.9 ± 0.30	1.5 ± 0.35	1.4 ± 0.34	2.3
Y639H	1.0 ± 0.19	nd	nd	nd	nd	nd

[a] A, C, G, and U indicate the $(k_{cat}/K_m)_{rNTP}/(k_{cat}/K_m)_{dNTP}$ values for the NTP (see Materials and Methods) with the corresponding base. Average indicates the average rNTP/dNTP catalytic specificity factor for all four NTPs. Data for reactions with Mg^{2+} have been presented previously (*5*) and are presented for comparison. Values given are ±SE.

Table 2: Kinetic Parameters for wt T7 RNAP Utilization of rUTP and dUTP during Elongation in Mg^{2+} and Mn^{2+} Reactions[a]

cofactor	$K_{m,rU}$ (mM)	$K_{m,dU}$ (mM)	$\frac{(k_{cat}/K_m)_{rU}}{(k_{cat}/K_m)_{dU}}$	$\frac{\{(k_{cat}/K_m)_{rU}/(k_{cat}/K_m)_{dU}\}_{Mg}}{\{(k_{cat}/K_m)_{rU}/(k_{cat}/K_m)_{dU}\}_{Mn}}$	$\frac{(K_{m,dU}/K_{m,rU})_{Mg}}{(K_{m,dU}/K_{m,rU})_{Mn}}$	$\frac{(k_{catrU}/k_{catdU})_{Mg}}{(k_{catrU}/k_{catdU})_{Mn}}$
Mg^{2+}	0.041 ± 0.018	1.7 ± 0.33	35 ± 9.5	3.6	1.2	3.0
Mn^{2+}	0.036 ± 0.028	1.2 ± 0.077	9.6 ± 3.7			

[a] Values given are ±SE for $n = 3$.

Poly(dG) and poly(rG) ladders generated in reactions in which dGTP or rGTP was the only NTP present were used as markers for assigning the length and sequence of different transcripts. Percent extension for a transcript of length n was determined by dividing the molar amount of all transcripts of length $>n$ by the molar amount of all transcripts $\geq n$.

RESULTS

Effect of Substituting Mn^{2+} for Mg^{2+} on Specificity and Activity. Table 1 presents values for the effect of Mn^{2+} substitution on the relative activity and rNTP/dNTP specificity of polymerases carrying different substitutions at position 639. Substituting Mn^{2+} for Mg^{2+} in the reaction reduces wt activity by ~10-fold on a plasmid template. Nonconservative substitutions at position 639 were previously shown to markedly reduce activity (*5*), but with Mn^{2+} the effects of nonconservative substitutions are ameliorated. Thus Y639T, for example, is ~75-fold less active than the wt enzyme in Mg^{2+} buffer but is only ~4-fold less active than wt in Mn^{2+} buffer. Mn^{2+} substitution also affects rNTP/dNTP specificity $\{(k_{cat}/K_m)_{dNTP}/(k_{cat}/K_m)_{rNTP}\}$, reducing it by an average factor of ~4 for the wt enzyme and by ~3-fold for Y639F.

Effect of Mn^{2+} Substitution on Kinetic Parameters for dNTPs and rNTPs. The reduction in rNTP/dNTP selectivity obtained with Mn^{2+} could be due to effects on the relative K_m and/or k_{cat} values for dNTPs vs rNTPs. To determine which parameter was being affected by Mn^{2+} we measured NTP K_m values on a supercoiled plasmid template in Mg^{2+} and Mn^{2+} buffer (Table 2). Measurement of kinetic parameters on such templates is complicated by the occurrence of phases of transcription (initiation, abortive cycling, elongation) with distinct processivities and different apparent NTP K_m values. Previously we found that, with supercoiled templates in Mg^{2+} buffer, the elongation phase of the reaction dominated the kinetics so that NTP K_m values for elongation could be determined (*5*). However, when we examined transcript patterns in reactions with the wt enzyme and with one rNTP substituted with a dNTP in Mn^{2+} buffer, it was apparent that the reduced activity of the wt enzyme under these conditions greatly increased the level of abortive cycling (not shown) and the assumption that the reaction kinetics would be dominated by the elongation phase of the transcription reaction no longer appeared valid. We therefore limited our measurement of elongation phase K_m values to rUTP and dUTP because, with the canonical T7 promoters used in this study, these nucleotides are incorporated only during the elongation phase of transcription. While K_m values can be determined in this way, k_{cat} values for dUMP incorporation cannot be determined directly in these assays because it is not possible to separate the contributions to V_{max} of a reduced incorporation rate for dUMP and a reduced rate of extension of dUMP substituted transcripts. However, the ratio of $k_{cat,rU}/k_{cat,dU}$ can be determined from the catalytic specificity $(k_{cat}/K_m)_{rU}/(k_{cat}/K_m)_{dU}$ determined previously (Table 1) and from the ratio of $K_{m,dU}/K_{m,rU}$. We found that Mn^{2+} substitution had little or no effect on the ratio of the K_m values

Table 3: Kinetic Parameters for Utilization of rGTP and dGTP as the Elongating Substrate during Dinucleotide Synthesis in Mg^{2+} and Mn^{2+} Reactions[a]

pol (cofactor)	$K_{m,rG}$ (μM)	$K_{m,dG}$ (μM)	$k_{cat,rG}$	$k_{cat,dG}$	$\frac{(k_{cat}/K_m)_{rG}}{(k_{cat}/K_m)_{dG}}$	$\frac{\{(k_{cat}/K_m)_{rG}/(k_{cat}/K_m)_{dG}\}_{Mg}}{\{(k_{cat}/K_m)_{rG}/(k_{cat}/K_m)_{dG}\}_{Mg}}$	$\frac{(k_{cat,rG}/k_{cat,dG})_{Mg}}{(k_{cat,rG}/k_{cat,dG})_{Mn}}$	$\frac{(K_{m,dG}/K_{m,rG})_{Mg}}{(K_{m,dG}/K_{m,rG})_{Mn}}$
wt (Mg)	17.5 ± 2.5	850 ± 76	2.55 ± 0.15	0.018 ± 0.001	6.9×10^3	26	20	1.3
wt (Mn)	10.3 ± 3.3	387 ± 23	1.2 ± 0.15	0.17 ± 0.034	2.7×10^2			
Y639F (Mg)	10.4 ± 0.65	15 ± 0.70	1.8 ± 0.1	0.054 ± 0.005	50	21	23	0.9
Y639F(Mn)	15.3 ± 2.7	25 ± 7.0	1.1 ± 0.25	0.75 ± 0.16	2.4			

[a] Data for reactions in Mg^{2+} buffer have been presented previously (*5*) and are presented here for comparison. Values given are $\pm$SE for $n = 3$.

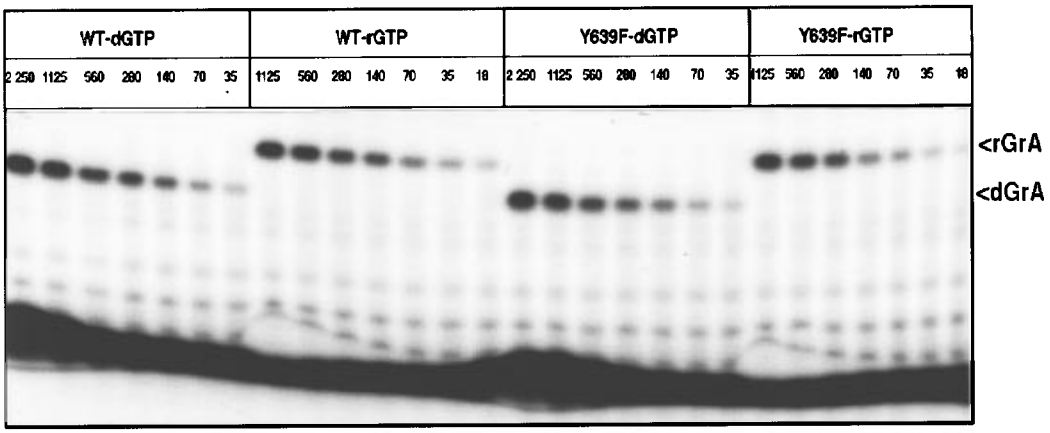


FIGURE 1: Dinucleotide synthesis reactions were run for 10 min at 37 °C with the indicated polymerases and micromolar concentrations of rGTP or dGTP to serve as the initiating substrate. ATP was present at 0.5 mM in all reactions and [α-^{32}P]ATP was used in trace amounts for labeling. Neither polymerase shows strong discrimination against utilization of dGTP as an initiating nucleotide.

for rUTP and dUTP, which allows us to attribute the reduced substrate specificity in Mn^{2+} buffer largely to an effect on the relative k_{cat} values for these substrates (Table 2). Since both ATP and GTP are incorporated during abortive cycling on all canonical T7 promoters, K_m values for purines could not be evaluated with this assay. We therefore used dinucleotide synthesis to evaluate the effects of Mn^{2+} substitution on the kinetic parameters for utilization of rGTP and dGTP as elongating substrates (Table 3). This assay also allows apparent k_{cat} for incorporation of dGMP to be determined directly without having to consider the effect of dGMP incorporation on subsequent extension. Dinucleotide synthesis is characterized by a much higher level of rGTP/dGTP specificity than the elongation reaction. This is primarily due to a decrease in k_{cat} for incorporation of dGTP relative to rGTP (Table 3) in dinucleotide synthesis vs elongation. The effects of the Y639F mutation and Mn^{2+} substitution are unambiguously distinct. The Y639F mutation eliminates the 40−50-fold K_m difference between rGTP and dGTP but affects the ~140-fold difference in k_{cat} between these two substrates by only a factor of ~4. Mn^{2+} substitution, however, reduces the ratio of the k_{cat} values of rGTP and dGTP by more than 20-fold but has no significant effect on their relative K_m values.

Kinetic Parameters for Initiation with a dNTP vs an rNTP. In order to characterize kinetic parameters for utilization of a dNTP at the initiating (or 3′-NMP) substrate site we measured the rates of GA dinucleotide synthesis on a self-complementary hairpin−promoter template in the presence of saturating concentrations of the elongating substrate (ATP) and varying concentrations of GTP or dGTP. The data in Table 3 reveal that, in Mg^{2+} buffer, wt enzyme utilization of dGTP as an *elongating* substrate in a dinucleotide synthesis reaction is characterized by a ~48-fold higher K_m and a ~140-fold lower k_{cat} than utilization of rGTP. However, while the use of a dNTP in the elongating substrate site is strongly discriminated against by the wt enzyme, the utilization of a dNTP as an initiating substrate is only modestly disfavored (Figure 1): the wt enzyme shows only a 3-fold higher K_m and a virtually identical k_{cat} when using dGTP vs rGTP for initiation (Table 4). The Y639F and S641A mutations and the Y639F/S641A double mutation do not markedly affect these kinetic parameters (Table 4). The S641A mutation was tested because of a previous report that this substitution has a dramatic effect on the substrate/product specificity of T7 RNAP (*10*). Previously, we showed that, contrary to this report, the S641A substitution does not affect the specificity of incorporation of the elongating substrate (*5*). As shown in Table 4 it also appears that the S641A substitution does not affect specificity for the initiating substrate.

Table 4: Kinetic Parameters for the Initiating Nucleotide in rGrA or dGrA Synthesis[a]

	wt	Y639F	S641A	Y639F/S641A
$K_{m,rGTP}$ (mM)	0.25 ± 0.04	0.32 ± 0.02	0.21 ± 0.04	0.22 ± 0.090
$K_{m,dGTP}$ (mM)	0.88 ± 0.11	0.75 ± 0.18	1.4 ± 0.40	1.1 ± 0.12
$k_{cat,rGTP}$ (s^{-1})	0.26 ± 0.07	0.28 ± 0.08	0.38 ± 0.06	0.25 ± 0.03
$k_{cat,dGTP}$ (s^{-1})	0.22 ± 0.02	0.34 ± 0.05	0.32 ± 0.07	0.10 ± 0.02

[a] Values are $\pm$SE for $n = 3$. Kinetic parameters were determined from time points taken at 10 and 20 min, over which incorporation rates were found to be linear.

Effects on the Extension Reaction of Having dNMPs Incorporated in the Transcript. Results from the dinucleotide synthesis reaction revealed that the polymerase discriminates only weakly against using a dNTP as an initiating substrate. Since the initiating substrate occupies the same site as the

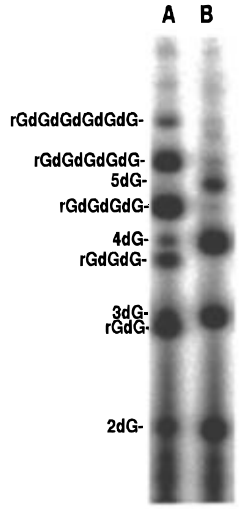


FIGURE 2: Method of determination of percent extension of transcripts of varying rNMP/dNMP composition. Lane A shows reactions run with Y639F and with 0.5 mM dGTP, 0.5 mM rGMP, and [α-^{32}P]dGTP; lane B shows similar reactions run without rGMP. The structure of the products is indicated. The reduced charge on transcripts that are 5′-terminated with rGMP causes them to migrate more slowly than 5′-dGTP-terminated transcripts of similar size. Percent extension of a transcript of length *n* is determined by dividing the molar amount of all transcripts > *n* by all transcripts ≥ *n* [pure oligo(dG) transcripts obtained in lane A are not included in the calculation to determine efficiency of extension of 5′-rGMP-terminated transcripts]. Qualitatively, the levels of extension of the 2dG (lane B) or rGdG (lane A) transcripts appear similar. However, the rGdGdG transcript is clearly extended more efficiently than the 3dG transcript, and the rGdGdGdG transcript is also seen to be extended more efficiently than the 4dG transcript. However, the rGdGdGdGdG transcript is poorly extended, indicating that the single rG in this position no longer enhances transcript extension, possibly because it no longer base-pairs with template (wobble base-pairing with the T at −1 could extend the oligo G hybrid to 4 base pairs).

3′-NMP of the transcript during catalysis (*11*), this could imply that the polymerase does not discriminate against a transcript with a 3′-dNMP. However, it is possible that in the context of a more extended helical structure the presence of a 3′-dNMP in the transcript could be more detrimental to the extension reaction. To evaluate this, we measured the efficiency of extension of transcripts of varying rNMP/dNMP composition generated in situ during initial transcription (see Figure 2 for an illustrative example of this method). These data are presented in Table 5, from which it is clear that, with the wt enzyme, extension reactions involving incorporation of a dNMP are much less efficient than corresponding reactions (identical substrate base and transcript sequence) involving incorporation of an rNMP. It is also clear that the Y639F mutation increases the efficiency of extension in reactions with dNTP substrates. However, in addition to the wt enzyme displaying specificity at the level of substrate incorporation, it is also seen that both the wt and mutant enzymes display specificity for transcript structure in that they extend transcripts containing dNMPs less efficiently than transcripts of identical sequence that are composed entirely of rNMPs. It is found, though, that low to moderate levels of dNMP incorporation in the transcripts give rise to only modest decreases in the efficiency with which those transcripts are extended, nor does the presence of a 3′-dNMP generate a particularly strong barrier to transcript extension. For example, rGrGrGdA transcripts are extended by either the wt or Y639F enzymes with rG, with efficiency similar

Table 5: Percent Extension of Transcripts of Varying rNMP/dNMP Composition with rNTPs or dNTPs[a]

extension reaction	wt	Y639F
rGrG + rG	63−65	57−63
rG**dG** + **dG**	22−25	56−61
dGdG + **dG**	13−27	60−64
rGrGrG + rA	96−97	86−90
rGrGrG + **dA**	1−9	41−57
rG**dGdG** + rA	85−95	50−54
dGdGdG + rA	nd	10−16
rGrGrG + rG	77−81	68−74
rG**dGdG** + **dG**	61−75	88−90
dGdGdG + **dG**	6−14	49−57
rGrGrGrA + rG	93−95	69−76
rGrGrG**dA** + rG	92−98	66−80
rGrGrGrG + rG	69−69	58−61
rG**dGdG**rA + **dG**	3−5	31−36
rG**dGdGdG** + **dG**	2−6	20−30
dGdGdGdG + **dG**	nd	8−13
rGrGrGrArG + rA	97−99	92−94
rGrGrG**dA**rG + **dA**	50−70	82−85
rGrGrGrGrG + rG	73−73	61−64
rG**dGdG**rA**dG** + rA	nd	60−69
rG**dGdGdGdG** + **dG**	nd	4−4
rGrGrGrArGrA + rC	96−99	95−96
rGrGrGrArGrA + **dC**	20−31	92−94
rGrGrG**dA**rG**dA** + rC	77−78	65−72
rGrGrG**dA**rG**dA** + **dC**	11−14	40−41
rG**dGdG**rA**dG**rA + rC	nd	69−72
rGrGrGrArGrArC + rC	96−99	96−98
rGrGrGrArGrA**dC** + **dC**	35−53	88−94
rGrGrG**dA**rG**dA**rC + rC	95−95	91−93
rGrGrG**dA**rG**dAdC** + **dC**	nd	32−34
rG**dGdG**rA**dG**rArC + rC	nd	75−84
rGrGrGrArGrArCrC + rG	90−98	82−85
rGrGrGrArGrA**dCdC** + rG	81−84	71−76
rGrGrG**dA**rG**dA**rCrC + rG	87−94	77−83
rGrGrG**dA**rG**dAdCdC** + rG	nd	15−22

[a] Values given are ranges from two experiments. Since transcripts are generated in situ, during initial transcription, transcripts with multiple deoxyribose substitutions are obtained at low levels with the wt enzyme. In many cases the level of a particular transcript (e.g., dGdGdGdG) generated by the wt enzyme is too low to allow quantitation of its percent extension (nd).

to extension of rGrGrGrA transcripts. Strong decreases in the efficiency of extension that are attributable to transcript structure occur only when (1) the transcript is at least 3 bases in length and (2) it is heavily substituted with dNMPs in the 3−5 bases nearest the 3′-end of the transcript. It is therefore seen that transcripts 3 and 4 bases in length that are 5′-terminated with rGMP but contain dGMP at the internal or 3′-positions are extended with markedly greater efficiency than transcripts of otherwise identical structure but that carry a dGTP at the 5′-position (Table 5 and Figure 2). A similar effect is observed if the relative extension efficiencies of the rGrGrGrArGrAdCdC and rGrGrGdArGdAdCdC transcripts are compared. Thus dNMP substitution at positions up to 2−4 bases away from the 3′-base of the transcript can affect the efficiency with which the transcript is extended. Together with the results from the dinucleotide synthesis reactions, these observations imply (1) that the Y639F mutation enhances dNMP incorporation but does not affect extension of dNMP-substituted transcripts, (2) the presence of a 3′ dNMP or a moderate level of dNMP substitution in the 3′ segment of the transcript only modestly reduces extension efficiency, and (3) a high level of dNMP substitu-

Table 6: Relative Activity of wt and Y639F Polymerases with dNTPs and rNTPs on Different Templates[a]

	poly(dI)·poly(dC)			poly(dA)·poly(dT)			pT75			
	rGTP	dGTP	dGTP + rGMP	rATP	dATP	dATP + rAMP	rGTP	rITP	dGTP	dITP
wt	160	2.0 (0.9−2.5)	4.0 (3.2−4.4)	32 (28−46)	<0.1	<0.1	200	233 (217−250)	1.1 (1.0−1.2)	<0.1
Y639F	160	156 (108−200)	172 (132−232)	10 (8−12)	<0.1	<0.1	200	210 (194−227)	52 (44−60)	<1.1

[a] The templates and NTPs used are as specified in the table. For the pT75 reaction, rATP, rCTP, and rUTP were also present at 0.5 mM. An activity value of 200 in this and all following tables corresponds to incorporation of 52 μmol of NTP/min in reactions with 10^{-7} M pT75 or 0.1 mg/mL homopolymer and 10^{-8} M polymerase. Data for the poly(dI)·poly(dC) template were published previously (*3*) and are presented here for comparison. Values given are mean and ranges from three (homopolymers) or two (pT75) experiments.

tion in the 3−5 bases nearest the 3′-end of the transcript strongly reduces extension efficiency.

One interpretation of the preceding observations is that the conformation of the transcript·template duplex in the active site is an important determinant of the efficiency of the extension reaction. For example, the canonical transcript·template conformation could be an A-form RNA·DNA hybrid and the extension reaction might be compromised if this duplex became B-form as a consequence of extensive dNMP substitution in the transcript. In addition to being influenced by ribose structure, duplex nucleic acid conformation is strongly influenced by water activity, sequence, and base composition (*12*−*16*). In particular, poly(dA)·poly(dT) forms a B-type structure and resists forming an A-like structure even at a relative humidity of 0%. Alternating purine−pyrimidine sequences tend to stabilize B-form DNA (*17*), while guanosine-rich DNA is particularly amenable to forming A-type structures. Poly(dG)·poly(dC) would then be expected to be especially amenable to forming an A-type structure, and in fact this is the only DNA duplex known to be A-form in solution (*18*). We therefore measured the activity of the wt and Y639F enzymes on homopolymeric templates (T7 RNAP efficiently synthesizes polypurine transcripts from polypyrimidine templates in a promoter-independent manner). As shown in Table 6, the Y639F enzyme synthesizes either poly(rG) or poly(dG) with poly(dI)·poly(dC) as a template with essentially indistinguishable activity under these assay conditions. Even the wt enzyme displays significant activity in poly(dG) synthesis on the poly(dC) template. This is remarkable given that Y639F is ≥600-fold less active in synthesis of pure DNA vs pure RNA on a complex sequence template (Table 7). This cannot be attributed simply to the fact that poly(dC) is a homopolymer template since replacement of rATP with dATP in synthesis reactions with the poly(dA)·poly(dT) template completely abolishes the activity of both the wt and Y639F enzymes. This was further evaluated by measuring the relative activity of the wt and Y639F polymerases on supercoiled pT75 in reactions in which rGTP was replaced by rITP, dGTP, or dITP (Table 6). dI·dC base pairs are like dA·dT base pairs in that they strongly favor assumption of B-form conformations and resist transformation to A-form (*14*−*16*). Activity in reactions with rITP was indistinguishable from that in reactions with rGTP, indicating that there was no strong discrimination against incorporation of the inosine base. However, the activity of the Y639F enzyme was reduced by more than 40-fold when dITP replaced dGTP in the reaction, suggesting that the stronger tendency of dI·dC base pairs to form B-type helices may have significantly depressed transcription over the G·C-rich initially transcribed sequence of this promoter.

Solution Conditions That Enhance Extension of Transcripts with Multiple dNMP Substitutions. In light of these results and previous observations that alcohols—through their dehydrating and water-structure perturbing effects—affect helix conformation by destabilizing B-form structures (and thereby favor A-form helices) (*1*, *12*, *13*, *19*) we decided to test the effects of methanol or ethanol addition to transcription reactions with varying combinations of rNTPs and dNTPs (Figure 3, Table 7). Table 7 reveals that addition of 15% methanol to reactions with four rNTPs reduces the activity of both the wt or Y639F polymerases by 4−5-fold. Substitution of rGTP by dGTP in the reaction with the wt enzyme reduces activity by 50−200-fold (depending on the methanol concentration), though activity with the Y639F mutant is reduced only 1−4-fold. Addition of methanol to reactions in which one or two rNTPs were replaced with dNTPs does not significantly increase absolute levels of Y639F activity; however, addition of methanol to such reactions does increase activity relative to reactions carried out with four rNTPs at the same methanol concentration. These results suggest that methanol has at least two effects on the reaction: a general decrease in enzyme activity with increasing methanol concentrations (ethanol was found to depress activity to an even greater degree and was therefore less useful in this regard) is coupled with decreasing sensitivity to replacement of rNTPs with dNTPs in the reaction. The former effect is clearly evidenced in 4 rNTP

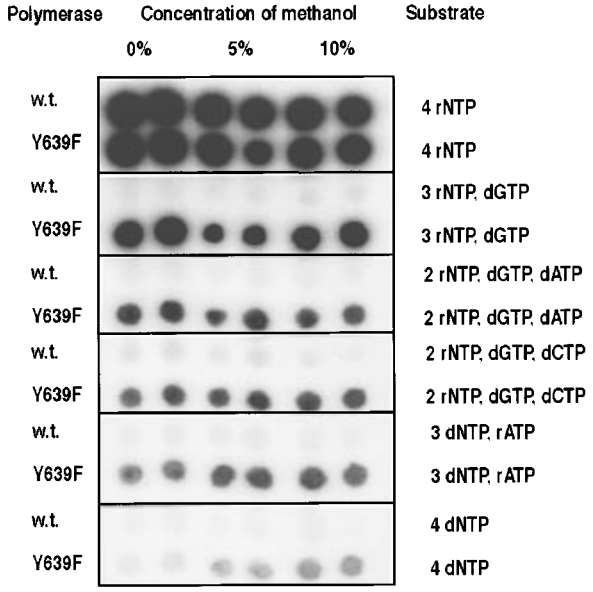


FIGURE 3: Effects of methanol addition on wt and Y639F activity in reactions with different combinations of rNTPs and dNTPs. Transcription reactions carried out with the indicated NTPs, polymerases, and methanol concentrations and supercoiled pT75 as template were carried out for 40 min at 37 °C, spotted onto DE81 filter paper, and washed with 0.5 M Na_2HPO_4 to remove unincorporated radioactivity (either [α-^{32}P]rGTP or dGTP). Methanol addition markedly enhances activity in the 3 dNTP and 4 dNTP reactions.

Table 7: Effect of Methanol and Ethanol on Relative Activity in Reactions with Different Combinations of rNTPs and dNTPs[a]

	wt alcohol (v/v)					Y639F alcohol (v/v)				
NTPs (alcohol)	0%	5%	10%	15%	20%	0%	5%	10%	15%	20%
4 rNTPs (MeOH)	200	167 ± 27	96 ± 15	54 ± 5	42 ± 6	200	138 ± 30	81 ± 10	41 ± 3	25 ± 3
4 rNTPs (EtOH)	200	82 ± 7	52 ± 5	13 ± 2	8.8 ± 1.6	200	80 ± 12	39 ± 4	16 ± 3	8.0 ± 1.2
3 rNTPs, dGTP (MeOH)	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	52 ± 8	54 ± 3	49 ± 3	44 ± 4	30 ± 3
3 rNTPs, dGTP (EtOH)	1.1 ± 0.1	1.5 ± 0.2	1.6 ± 0.2	1.0 ± 0.2	0.42 ± 0.1	52 ± 8	49 ± 7	26 ± 3	14 ± 3	4.5 ± 1.2
2 rNTPs,dA,dG (MeOH)	0.45 ± 0.1	0.36 ± 0.1	0.48 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	14 ± 2	18 ± 3	19 ± 2	21 ± 2	20 ± 3
2 rNTPs,dT,dG (MeOH)	0.33 ± 0.1	0.26 ± 0.1	0.46 ± 0.1	<0.4	<0.4	14 ± 3	16 ± 4	18 ± 5	24 ± 3	22 ± 4
3 dNTPs, rATP (MeOH)	<0.3	<0.3	<0.3	<0.3	<0.3	3.3 ± 1	6.0 ± 0.8	7.1 ± 0.7	8.8 ± 0.8	8.8 ± 1
4 dNTPs (MeOH)	<0.1	<0.1	<0.1	<0.1	<0.1	0.33 ± 0.1	1.1 ± 0.4	2.5 ± 0.4	2.6 ± 0.5	2.4 ± 0.4

[a] Reaction time points were taken at 2.5, 5, 10, 20, and 40 min. Incorporation was linear under all conditions for the first four time points, so only data for the 2.5−20 min time points were used to determine relative activity. Values given are ±SE for $n = 3$.

Table 8: Effect of Methanol on $(k_{cat}/K_m)_{rGTP}/(k_{cat}/K_m)_{dGTP}$[a]

	methanol (v/v)				
	0%	5%	10%	15%	20%
wt	75−83	75−93	77−78	79−98	84−86
Y639F	2.9−3.2	2.5−3.4	2.5−3.2	2.8−3.1	3.1−3.4

[a] Values given are ranges from two experiments.

reactions, while in the 1 dNTP or 2 dNTP reactions the two effects appear balanced and little net effect on absolute activity is obtained due to methanol addition. However, in the 3 dNTP or 4 dNTP reactions the reduced sensitivity to dNTPs dominates, and addition of 15% methanol to the reaction with Y639F leads to substantial (~8-fold) increases in absolute activity. Though this is a substantial net increase, the degree to which methanol addition reduces sensitivity to multiple dNTP replacement in the transcription reaction is actually much greater. For example, in the absence of methanol, replacement of all four rNTPs with dNTPs reduces Y639F activity by ≥600 fold. However, in the presence of 20% methanol activity is only ~10-fold lower in the 4 dNTP reaction than in the 4 rNTP reaction, representing a net 60-fold decrease in the degree to which the reaction is sensitive to replacement of all four rNTPs with dNTPs.

Effect of Methanol on Specificity during Incorporation. Reactions in which one or more rNTPs are substituted with dNTPs may be limited at either the dNMP incorporation or dNMP-substituted transcript extension steps. Since the barrier to Y639F synthesis of transcripts multiply substituted with dNMPs appears to be largely due to transcript structure rather than substrate structure, and since methanol addition had its largest effects in the 3 or 4 dNTP reactions, it appeared likely that methanol enhanced the extension of dNMP-substituted transcripts rather than incorporation of dNMPs. To test this, we evaluated the effect of methanol addition on the relative rates of radiolabeled rGTP or dGTP incorporation in reactions in which unlabeled rGTP was present in large excess. Under such conditions the rate of dGTP incorporation is not limited by effects on transcript structure since the fractional substitution of dGMP into the transcripts is less than ~0.01%. We found that methanol addition had little effect on the rGTP/dGTP specificity ratio in such an assay (Table 8), implying that the methanol enhances extension of dNMP-substituted transcripts rather than incorporation of dNMPs. This was also supported by examination of the transcript patterns obtained with the

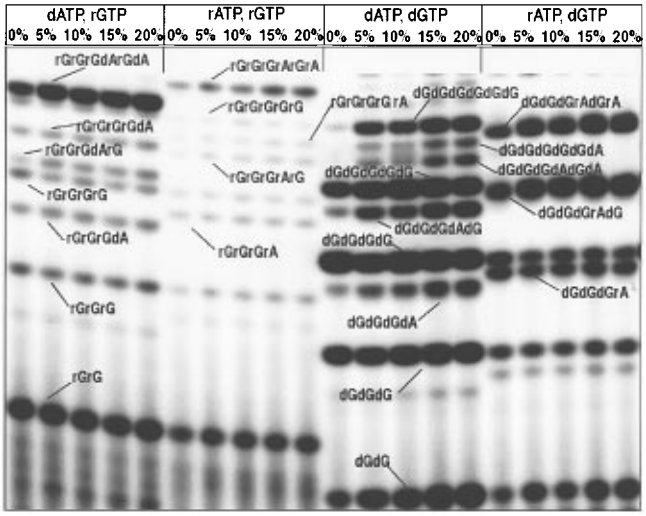


FIGURE 4: Transcription reactions were run with Y639F polymerase and the indicated NTPs on a partially single-stranded synthetic T7 promoter template composed of an 18-base nontemplate strand corresponding to the sequence of the consensus class III T7 promoter from −23 to −5 annealed to a 33-base template strand corresponding to the T7 ϕ10 promoter and ITS (initially transcribed region; from +1 to +6 GGGAGA). [Template] = 10^{-7} M; [pol] = 10^{-6} M; the reaction was run for 15 min at 37 °C before being electrophoresed on 20% acrylamide/4% bisacrylamide/6 M urea gels and visualized by phosphorimaging. Sequence assignments were made by comparison to poly(dG) and poly(rG) oligomers obtained in reactions in which only dGTP or rGTP was present and by comparison of migration patterns of transcripts obtained with the indicated NTPs. The relative migration rates of short transcripts of identical length but varying sequence and ribose composition follows the rule, in order of increasing retardation: dA < rA < dG < rG.

Y639F enzyme in the presence of varying concentrations of methanol and the following combination of NTPs: rATP + rGTP, dATP + rGTP, rATP + dGTP, and dATP + dGTP (Figure 4). The steady-state rate of transcript production in such assays is limited by the rate of transcript release (*20*), and both the incorporation of dNMPs into the transcript and addition of methanol appear to destabilize the complex and therefore lead to net increases in the total amount of transcript obtained. However, in evaluating the effects of methanol on transcript extension or nucleotide incorporation, we focus on the changes in the transcript patterns (processivity) rather than the total transcript levels. The transcript patterns obtained on this promoter are complex: in addition to transcripts corresponding to the sequence of the +1 to +6

region of the promoter (GGGAGA), products with larger numbers of G residues are also obtained due to slippage of the transcript on the initial run of 3 Gs. The G-slippage reaction has invariably been found to be more robust than heterogeneous sequence synthesis: thus, whenever the enzyme is mutated so that it is significantly less active, it has been found that poly-G ladder synthesis predominates during initiation (*21*−*23*). This also occurs when the reaction is less efficient due to noncanonical substrate or transcript structure (*3*); for example, in the 0% methanol, dATP + dGTP reaction in Figure 4, it can be seen that most of the dGdGdG trimer is extended to form larger poly(dG) oligomers rather than the heterogeneous sequence oligomers. Addition of methanol does not affect the transcript pattern in the rATP + rGTP reaction. Nor is there much of an effect on the transcript pattern in the dATP + rGTP reaction. However, in the dGTP + rATP reaction methanol addition has a clear effect: there is an increase in the efficiency of extension of the dGdGdG trimer with rA at the expense of extension to form longer poly(dG) oligomers so that the amount of the 4-dG product decreases while the levels of the longer heterogeneous sequence transcripts increase. In the dATP + dGTP reaction the effect of methanol is even greater: without methanol there is very little production of heterogeneous sequence transcripts and there is little extension of the poly(dG) oligomers beyond 5 bases in length, while addition of increasing concentrations of methanol progressively increases both the levels of heterogeneous sequence transcripts and the efficiency with which the poly(dG) oligomers are extended.

DISCUSSION

Effects of Mn^{2+} on Specificity and Enzyme Activity. Use of Mn^{2+} as a catalytic cofactor has generally been found to cause a reduction in the substrate specificity or fidelity of enzymes that normally use Mg^{2+} to catalyze a phosphoryl transfer reaction. For T7 RNAP we find that the reduced discrimination for incorporation of a 2′-deoxypyrimidine during elongation or incorporation of a 2′-deoxypurine during formation of the first phosphodiester bond is mediated by a reduction in the ratio of the k_{cat} values for rNTP and dNTP substrates, as has also been found for the effect of Mn^{2+} on deoxy/dideoxy discrimination in DNAP I (*24*) and Taq DNAP (*25*). This is a rather critical result in light of our previous conclusion that the K_m difference between these substrates is due to the Y639-OH·ribose 2′-OH interaction. It would have been difficult to understand how Mn^{2+} substitution could influence this interaction. The molecular mechanisms and effects of Mn^{2+} substitution and the Y639F mutation should therefore be complementary and distinct, and this is what is found (Tables 1−3).

Substitution of Mg^{2+} with Mn^{2+} in polymerization reactions leads to a remarkably wide range of effects with many polymerases. In addition to increasing the relative levels of base misincorporation (*26*−*30*), Mn^{2+} increases the relative utilization of substrates modified at different positions on the base, ribose, or phosphate groups (*31*, *32*) as well as relative activity on templates of noncanonical structure (*33*, *34*) or even in the absence of a templating base (*35*). Structural alterations of the polymerase active site also appear to perturb the Mn^{2+}-catalyzed reaction to a lesser degree than the Mg^{2+}-catalyzed reaction. Thus, while substitution of Mn^{2+} for Mg^{2+} markedly reduced the activity of the wt or Y639F enzymes, it either enhanced the activity of the poorly active mutant enzymes bearing nonconservative substitutions at position 639 or it reduced the activity of these mutants to only a small degree (Table 1). Similar observations have been made in other systems (*34*−*37*). Such a broad spectrum of effects is effectively summarized by the statement that the Mn^{2+}-catalyzed reaction is compatible with a wider range of reactive group geometries than is the Mg^{2+}-catalyzed reaction, so that it is less sensitive to changes in reactive group geometries due either to noncanonical substrate structure or to nonconservative active-site mutations. It is as yet unclear what properties of Mn^{2+} relative to Mg^{2+} [greater softness of the Mn^{2+} ion (*38*), faster rates of hydration/dehydration, tighter ligand binding (*39*)] are responsible for its distinctive catalytic properties.

Transcript Structure Effects on Transcript Extension. Before addressing this question, it is important to briefly review the available information on the structures of template·primer duplexes in cocrystals with polymerases and the effects of sequence, ribose structure, and solution conditions on duplex conformation. Crystal structures of polymerase·primer·template complexes have revealed that the duplexes in the active sites form structures that, while varying in details, are recognizably of the A- or B-form families (*39*, *40*). RNA·DNA hybrids favor A-form conformations (*1*, *14*−*16*, *40*, *41*). It has therefore been suggested that the RNA·DNA hybrid in the RNAP active site assumes an A-like conformation (*43*−*45*), and some limited direct evidence for this has been obtained (*46*). DNA·DNA duplexes favor B-form structures (*1*). On the basis of crystal structures it has been suggested that a single rNMP in a ∼10 base DNA·RNA chimeric duplex is sufficient to stabilize an A-form structure, but such studies all involve crystals grown from solutions containing high concentrations of alcohol or 2-methyl-2,4-pentanediol (MPD), conditions that are expected to destabilize B-form helices (*47*−*49*). Indeed, crystals of C·G-rich A-form DNA can be grown from such solutions (*16*). Structural studies of synthetic Okazaki fragments [such as r(ccca)d(AATGA)·d(TCATTTGGG)] in solution are more relevant to the case of an RNAP extending a mixed rNMP/dNMP transcript on a DNA template since they involve hybrids of pure DNA and chimeric RNA/DNA strands. Studies of these chimeras reveal B-type helices in the DNA·DNA segments, a 2−3 base pair junction region, and a hybrid helix form with varying degrees of A-like character in the RNA·DNA segment depending on its length and sequence (*42*, *48*−*50*). Such structures imply that while rNMPs may have a dominant effect on helix conformation, their presence in a hybrid is not overwhelming and RNA/DNA chimeras that are predominantly DNA are typically not A-form in solution. At the level of sequence it is known that dG·dC base pairs are more amenable to forming A-like structures than either dA·dT or dI·dC base pairs (*14*−*16*) and that alternating purine−pyrimidine sequences favor B-form to a greater degree than polypurine·polypyrimidine tracts (*17*). Uniquely amongst pure DNA sequences that have been studied, poly(dG)·poly(dC) is A-form in aqueous solution (*18*). A critical factor in the stabilization of the B-form structure is a highly ordered spine of hydrating water molecules in the minor groove (*15*, *16*, *51*). Disruption of this hydration spine by dehydration or by agents that disrupt water structure has been proposed to be the mechanism through which such environ-

mental conditions destabilize B-form DNA to favor A-form structures (*15*, *16*).

Against this background of information, our observations with T7 RNAP, while essentially correlative, strongly suggest that the barrier to extension of heavily dNMP-substituted transcripts is due to a change in the conformation of the transcript·template hybrid, most probably from an A-like structure to a B-like structure. Specifically we find the following: (1) dGTP is used efficiently as an initiating nucleotide, the presence of a 3′-dNMP in the transcript is not particularly limiting for transcript extension, and a moderate level of dNMP substitution in the transcript only modestly reduces extension efficiency. (2) The efficiency of transcript extension is strongly reduced when the 3−5 bases nearest the 3′-end of the transcript are predominantly deoxyribose. These observations appear inconsistent with any strong, direct discriminatory mechanism against utilization of a 3′-dNMP in the phosphoryl transfer reaction (in contrast to the discrimination observed for utilization of a dNTP vs an rNTP) and do not reveal any strong chemical specificity for a pure RNA transcript. They are consistent with a conformational effect caused by having predominantly deoxyribose content in the region of the transcript which is hybridized to the DNA template. (3) The activity of the Y639F enzyme is ≥600-fold lower for pure DNA than for pure RNA synthesis on a complex sequence template and DNA synthesis is not measurable on poly(dA)·poly(dT), but pure RNA or DNA synthesis activity is nearly identical on poly(dI)·poly(dC). (4) The activity of Y639F on a complex sequence template is much more sensitive to replacement of rGTP with dITP than with dGTP, though activity with rGTP vs rITP is identical. These observations are consistent with the idea of a requirement for an A-like structure in the hybrid and with the amenability with which dG·dC, dA·dT, and dI·dC base pairs form A-DNA. In particular, it appears difficult to account for the remarkably high activity of Y639F in pure DNA synthesis on the poly(dC) template without reference to the unique conformational properties of poly(dG)·poly(dC). (5) The addition of alcohols, and in particular methanol, greatly decreases the sensitivity of Y639F to replacement of multiple rNTPs with dNTPs in the transcription reaction, and this is due to enhanced extension of heavily dNMP-substituted transcripts rather than enhanced utilization of dNTPs. This would be consistent with the ability of such agents, through dehydration and water structure perturbing effects, to destabilize B-form helices, thereby allowing formation of an A-like structure that is a better substrate for extension.

The alcohol concentrations used in these experiments are, in fact, too low to induce a B → A transition in solution for most DNA sequences, and ethanol is actually more effective in inducing such a transition than methanol (*17*). It is therefore probable that the environment of the nucleic acid binding cleft in T7 RNAP also favors destabilization of a B-form conformation, either because the hydration shell of the nucleic acids is partially displaced upon association with the polymerase or because of specific chemical and shape complementarity between the binding cleft and an A-like conformation. Human immunodeficiency virus type I reverse transcriptase induces a DNA·DNA primer·template to assume an A-form conformation (*52*), and DNA polymerase β also causes a DNA·DNA primer·template to take on partial A-like character through interactions with protein side chains that disrupt the hydration spine of the minor groove (*40*).[2] For T7 RNAP, which normally works with an RNA·DNA hybrid, such features of the binding cleft may be inadequate to force an appropriate A-form structure with a DNA·DNA duplex, but together with other factors that also favor A-form conformations but are inadequate in isolation (moderate alcohol concentrations), may allow a DNA·DNA duplex to assume a conformation appropriate for efficient extension. We do not know why methanol is more effective than ethanol for this purpose, but it is possible that it is because methanol is less disruptive of polymerase activity than is ethanol (as assessed in a 4 rNTP reaction; Table 7) or because its smaller size allows it readier access to the transcript·template hybrid in the polymerase binding cleft.

It could be argued that alcohol addition exerts its effects in ways other than an effect on transcript·template conformation; for example, it could disrupt polymerase structure so that the enzyme becomes sloppier in its requirements for canonical substrate or transcript structure. Such structural effects on the enzyme are suggested by the depressing effect of alcohol addition on activity in 4 rNTP reactions. However, we have generally found that situations which lead to reductions in polymerase activity tend to *decrease* the relative utilization of noncanonical substrates even more (*5*). For example, nonconservative substitutions at position 639 that reduce activity typically enhance the rNTP/dNTP specificity of the polymerase relative to the conservative Y639F substitution (Table 1), and rGTP/dGTP specificity is also greater for the relatively inefficient dinucleotide synthesis reaction than it is during elongation (Tables 1 and 3). Such observations are consistent with the idea that the assumption of optimal catalytic geometry of the reactive groups in the active site involves a set of cooperative interactions between polymerase·template, polymerase·substrate, polymerase·primer/transcript, template·substrate, template·primer/transcript, and substrate·primer/transcript. Changes in any one of these interactions via a structural change in template, substrate, or the polymerase tend to affect kinetic parameters (*53*, *54*). Because of the highly nonlinear response of catalytic rates to changes in reactive group geometry, combinations of structural alterations (for example, the combination of a nonconservative active-site mutation and noncanonical substrate structure) may be expected to give rise to more than multiplicative effects on activity, as they are seen to do (Tables 1−3 or see ref *55*). We would therefore expect that any nonspecific disruptive effects of alcohols on enzyme structure that reduce activity in 4 rNTP reactions would tend to reduce activity in reactions with dNTPs to an even greater degree, which is obviously not what is found. In any case, it is clear that such effects on the polymerase, if they occur, do not lead to reduced specificity for canonical substrate structure at the level of nucleotide incorporation (Table 8).

It is possible that, in addition to this conformational mechanism, noncanonical transcript structure or the presence of a transcript binding site on the polymerase with some

[2] In fact, it is possible that most DNA-directed DNA polymerases will favor the formation of A-like structures in their active sites because the A-conformation is structurally more homogeneous with respect to sequence than is B-form DNA (*1*). Such structural homogeneity could be important since appropriate and restricted geometry of the base pairs and reactive groups is expected to be critical for the mechanisms of fidelity and catalysis.

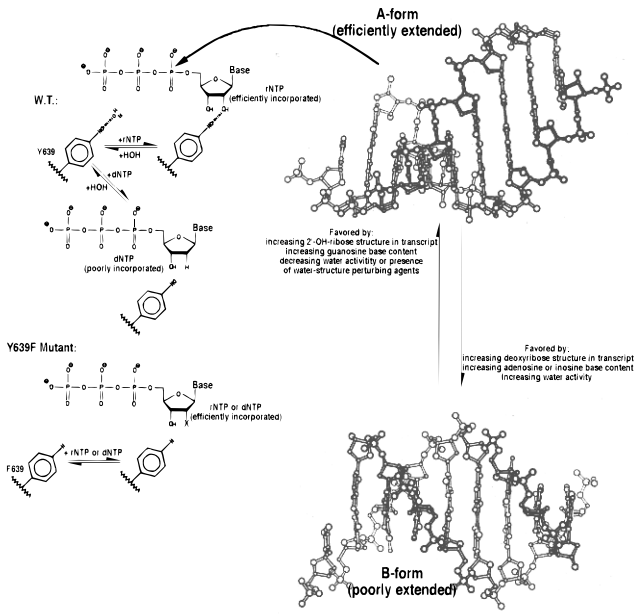


FIGURE 5: Summary of structural mechanisms underlying ribose specificity in RNA polymerization by T7 RNAP. The reaction involves two substrates: NTP and transcript. Specificity for the ribose 2′-group at the level of NTP utilization is mediated by the Y639 side chain as illustrated (*5*), via a mechanism analogous to that used by Y34 in tyrosyl-tRNA synthetase to discriminate tyrosine and phenylalanine substrates (*7*). Transcript structure effects on transcript extension are not confined to the 3′-NMP of the transcript and instead encompass *ca*. 5 bases (or more) at the transcript 3′-end. The correlation between the effects of alcohols, sequence, and ribose/deoxyribose structure on helix conformation (A- vs B-forms) and the effects of these factors on the efficiency of transcript extension imply a requirement for an A-like transcript·template hybrid for efficient extension. [A-form refers to all A-family type structures (A, A′, etc.); B-form refers to all B-family structures (B, C, D, etc.).]

chemical specificity for RNA contribute in other ways to the efficiency with which the transcript is extended. However, because we find that the synthesis of poly(dG) by Y639F is as efficient as poly(rG) synthesis, and because in 20% methanol the activity of Y639F in a 4 dNTP reaction is only ~10-fold less than in a 4 rNTP reaction, we believe that the primary determinant of extension efficiency with regard to the ribose/deoxyribose structure of the transcript is likely to be the helix conformation of the transcript·template hybrid.

The structural mechanisms that have been identified as contributing to substrate and product specificity in T7 RNAP are summarized in Figure 5. The emphasis that Figure 5 places on specific structural mechanisms is not meant to downplay the role played by the poorly processive initiation phase of transcription in limiting activity in reactions with noncanonical substrates. These have been previously discussed at length (*3*, *5*) and include both a kinetic component—a relatively modest elongation rate reduction during poorly processive initial transcription can drastically reduce activity by greatly reducing the fraction of elongation complexes that achieve promoter release—and a structural one—requirements for canonical substrate structure to achieve proper positioning of reactive groups may be more stringent during initial transcription because extended RNA·DNA and RNA·RNAP interactions that can contribute to positioning the 3′-NMP for catalysis are absent, leading, for example, to the greater sensitivity of the dinucleotide synthesis reaction to noncanonical ribose (Tables 6−8) or base (*5*, *56*) structure.

Given our understanding of the mechanisms of ribose discrimination, it should prove possible, for practical applications, to further develop this system for synthesis of nucleic acids with any desired level of 2′-substituted NMPs. For such purposes it is important that the Y639F mutation, Mn^{2+} substitution, and addition of methanol each affect the ribose specificity of the transcription reaction through largely nonoverlapping mechanisms so that these mechanisms can be combined to further enhance incorporation of 2′-substituted NMPs. For example, combination of ~15% methanol and the Y639F substitution allowed us, for the first time, to achieve significant levels of promoter-specific, unprimed DNA synthesis from a complex sequence template. This combination is not yet maximally efficient since methanol significantly reduces overall activity, but our observations suggest that nucleic acids with any desired level of 2′-modified ribose structure could be efficiently synthesized with the Y639F enzyme if solution conditions that destabilize B-form helices without depressing enzyme activity could be identified.

REFERENCES

1. Saenger, W. (1984) *Principles of Nucleic Acid Structure*, Springer-Verlag, New York.
2. Tabor, S., and Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6339−6343.
3. Sousa, R., and Padilla, R. (1995) *EMBO J. 14*, 4609−4621.
4. Gao, G., Orlova, M., Georgiadis, M. M., Hendrickson, W. A., and Goff, S. P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 407−411.
5. Huang, Y., Eckstein, F., Padilla, R., and Sousa, R. (1997) *Biochemistry 36*, 8231−8242.
6. Joyce, C. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1619−1622.
7. Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., and Winter, G. (1985) *Nature 314*, 235−238.
8. Bonner, G., Patra, D., Lafer, E. M., and Sousa, R. (1992) *EMBO J. 11*, 3767−3775.
9. Tabor, S., and Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074−1078.
10. Kostyuk, D. A., Dragan, S. M., Lyakhov, D. L., Rechinsky, V. O., Tunitskaya, V. L., Chernov, B. K., and Kochetkov, S. N. (1995) *FEBS Lett. 369*, 165−168.
11. Martin, C. T., and Coleman, J. E. (1989) *Biochemistry 28*, 2760−2762.
12. Ivanov, V. I., Minchekova, L. E., Minyat, E. E., Frank-Kamenetskii, M. D., and Schyolkina, A. K. (1974) *J. Mol. Biol. 87*, 817−833.
13. Pohl, F. (1976) *Nature 260*, 365−366.
14. Leslie, A. G. W., Arnott, S., Chanrasekaran, R., and Ratliff, R. L. (1980) *J. Mol. Biol. 143*, 49−72.
15. Drew, H. R., and Dickerson, R. E. (1982) *Nature 295*, 294−299.
16. Drew, H. R., and Dickerson, R. E. (1981) *J. Mol. Biol. 151*, 535−556.
17. Arnott, S., Chandrasekaran, R., and Selsing, E. (1975) in *Structure and Conformation of Nucleic Acids and Protein−Nucleic Acid Interactions* (Sundaralingam, M., and Rao, S. T., Eds.) pp 577−596, University Park Press, Baltimore, MD.
18. Sarma, M. H., Gupta, G., and Sarma, R. H. (1986) *Biochemistry 25*, 3659−3665.
19. Ivanov, V. I., Minchekova, L. E., Schyolkina, A. K., and Poletayev, A. I. (1973) *Biopolymers 12*, 89−110.
20. Villemain, J., Guajardo, R., and Sousa, R. (1997) *J. Mol. Biol.* (submitted for publication).
21. Bonner, G., Lafer, E. M., and Sousa, R. (1994a) *J. Biol. Chem. 269*, 25120−25128.
22. Gardener, L. P., Mookhtiar, K. A., and Coleman, J. E. (1997) *Biochemistry 36*, 2908−2918.
23. Mookhtiar, K. A., Peluso, P. S., Muller, D. K., Dunn, J. J., and Coleman, J. E. (1991) *Biochemistry 30*, 6305−6313.

24. El-Deiry, W. S., Downey, K. M., and So, A. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7378−7382.
25. Brandis, J., Edwards, S., and Jonhson, K. (1996) *Biochemistry 35*, 2189−2200.
26. Beckman, R. A., Mildvan, A. S., and Loeb, L. A. (1985) *Biochemistry 24*, 5810−5817.
27. Goodman, M. F., Keener, S., Guidotti, S., and Branscomb, E. W. (1983) *J. Biol. Chem. 258*, 3469−3475.
28. Pezo, V., and Wain-Hobson, S. (1997) *Gene 186*, 67−72.
29. Putrament, A., Baranowska, H., and Prazsmo, W. (1975) *Mol. Gen. Genet. 140*, 339−347.
30. Ripley, L. S. (1975) *Mol. Gen. Genet. 141*, 23−40.
31. Pinto, D., Sarocchi-Landousy, M. T., and Guschlbacher, W. (1979) *Nucleic Acids Res. 6*, 1041−1048.
32. Tabor, S., and Richardson, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4076−4080.
33. Boiteux, S., and Laval, J. (1982) *Biochimie 64*, 637−641.
34. Ide, H., Kow, Y. W., and Wallace, S. S. (1985) *Nucleic Acids Res. 13*, 8035−8052.
35. Pelletier, H., Sawaya, M. R., Wolfle, W., Wilson, S. H., and Kraut, J. (1996) *Biochemistry 35*, 12762−12777.
36. Blasco, M. A., Lazaro, J. M., Bernard, A., Blanco, L., and Salas, M. (1992) *J. Biol. Chem. 267*, 19427−19434.
37. Inokuchi, Y., Kajitani, M., and Hirashima, A. (1994) *J. Biochem. (Tokyo) 116*, 1275−1280.
38. Scott, W. G., and Klug, A. (1996) *Trends Biochem. Sci. 21*, 220−224.
39. Irving, H., and Williams, R. (1953) *J. Am. Chem. Soc. 75*, 3193−3210.
40. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) *Science 264*, 1891−1903.
41. Arnott, S., Chandrasekaran, R., Millane, R. P., and Park, H. S. (1986) *J. Mol. Biol. 188*, 631−640.
42. Selsing, E., Wells, R. D., Early, T. A., and Kearns, D. R. (1978) *Nature 254*, 249−250.
43. Arnott, S., Fuller, W., Hodgson, A., and Prutton, I. (1968) *Nature 220*, 561−564.
44. Florentiev, V. L., and Ivanov, V. I. (1970) *Nature 228*, 519−522.
45. Hamilton, L. D. (1968) *Nature 218*, 633−637.
46. Beabealashvily, R. S., Ivanov, V. I., Minchenkova, L. E., and Savotchikina, L. P. (1972) *Biochim. Biophys. Acta 259*, 35−40.
47. Egli, M., Usman, N., Zhang, S., and Rich, A. (1991) *Proc. Natl. Acad. Sci. U.S.A. 89*, 534−538.
48. Egli, M., Usman, N., and Rich, A. (1993) *Biochemistry 32*, 3221−3237.
49. Wang, A. H. J., Fuji, S., van Boom, J. H., van der Marel, G. A., van Boeckel, S. A. A., and Rich, A. (1982) *Nature 299*, 601−604.
50. Salazar, M., Fedoroff, O. Y., and Reid, B. R. (1996) *Biochemistry 35*, 8126−8135.
51. Conner, B. N., Takano, T., Tanaka, S., Itakura, K., and Dickerson, R. E. (1982) *Nature 295*, 294−299.
52. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6320−6324.
53. Ricchetti, M., and Buc, H. (1993) *EMBO J. 12*, 387−396.
54. Tantillo, C., Ding, J., Jacobo-Molina, A., Nanni, R. G., Boyer, P. L., Hughes, S. H., Pauwels, R., Andries, K., Janssen, P. A. J., and Arnold, E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 243*, 369−387.
55. Polesky, A. H., Steitz, T. A., Grindley, N. D. F., and Joyce, C. M. (1990) *J. Biol. Chem. 265*, 14579−14591.
56. Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783−8798.

BI971609O