3′−5′ Exonucleolytic Activity of DNA Polymerases: Structural Features That Allow Kinetic Discrimination between Ribo- and Deoxyribonucleotide Residues†

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ABSTRACT: We have determined rates for the excision of nucleotides from the 3′ termini of chimeric DNA−RNA oligonucleotides using the Klenow fragment (KF) and two other DNA polymerases, from phages T4 and T7. For these studies, we synthesized DNA−RNA chimeric oligonucleotides with RNA residues in defined positions. When a ribonucleotide residue was placed at the 3′ terminus, all three DNA polymerases removed it at the same rate as they did for substrates composed solely of deoxynucleotide residues. There was a decrease in the excision rate, however, when a ribonucleotide residue was located at the second or third position from the 3′ terminus. When both the second and third positions were occupied by ribonucleotide residues, the excision rate for the 3′ terminal nucleotide was reduced even further and was almost identical to the rate observed when the DNA polymerases encountered single-stranded RNA. The magnitude of the effect of ribonucleotide residues on the excision rate was lower when Mn^{2+} replaced Mg^{2+} as the essential divalent cation. Two KF mutations, Y423A and N420A, selectively affected the excision rates for the chimeric substrates. Specifically, Y423A totally abolished the rate reduction when there was a single ribonucleotide residue immediately preceding the 3′ terminus, whereas N420A diminished, but did not eliminate, the rate reduction relative to that of wild-type KF when the single ribonucleotide residue occupied either the second or third position from the 3′ terminus. These results are consistent with the structure of a KF−ss DNA complex from which it can be deduced, by modeling, that a 2′ OH group on the second sugar from the 3′ terminus would sterically clash with the Tyr 423 side chain, and a 2′ OH group on the third sugar would clash with the side chain of Asn 420. The corresponding mutations in T4 DNA polymerase did not affect the rate of hydrolysis of the chimeric oligonucleotides. Thus, there appears to be a major difference in the kinetic behavior of KF and T4 DNA polymerase with respect to the exonuclease reaction. These results are discussed with respect to their possible biological relevance to DNA replication.

Many DNA polymerases have an intrinsic 3′−5′ exonuclease activity that serves to correct errors caused by incorporation of mismatched nucleotide residues (for reviews, see refs *1*−*3*). While the editing mechanism for misincorporated bases has been studied extensively, little attention has been given to the possibility that DNA polymerases may encounter ribonucleotide residues that are introduced into the primer strand. This situation rarely arises during replication of the leading strand or during DNA repair because DNA polymerases are easily able to discriminate between ribo- and deoxyribonucleoside triphosphates during polymerization (*4*, *5*). The situation is different for lagging strand synthesis, which is initiated by RNA primers, resulting in chimeric RNA−DNA oligomers (Okazaki fragments) formed by extension of the RNA primers (*6*, *7*). The observation that ssRNA was degraded much more slowly than ssDNA was first made by Kurosawa and Okazaki in 1979 (*7*) when they digested Okazaki fragments with T4 DNA polymerase; however, the kinetic and structural basis for this result was not investigated further. To better understand how DNA polymerases degrade these chimeric substrates, we prepared DNA−RNA chimeric oligonucleotides with RNA residues located at defined positions proximal to the 3′ terminus and then determined the 3′−5′ nucleotide excision rate with three DNA polymerases: the Klenow fragment (KF),[1] an A family DNA repair polymerase from *Escherichia coli*, T4 DNA polymerase, a B family replicative polymerase, and T7 DNA polymerase, an A family replicative polymerase. We have attempted to interpret our kinetic data within the framework of the known structural features of the exonuclease active sites of KF (*8*, *9*) and RB69 DNA polymerase (*10*, *11*) [a close relative of T4 DNA polymerase (*12*)].

EXPERIMENTAL PROCEDURES

Materials

The expression plasmid for wild-type T7 DNA polymerase was a generous gift from S. Tabor. Restriction endonucleases

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[1] Abbreviations: KF, Klenow fragment; TBAF, tetrabutylammonium fluoride; R, ribocytidyl; C, deoxycytidyl; rU, ribouridyl; M, 2′-*O*-methyl; dN, any one of the four deoxynucleotide residues (dA, dG, dC, and dT); rN, any one of the four ribonucleotide residues (rA, rG, rC, and rU); ssDNA, single-stranded DNA.

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and T4 polynucleotide kinase were obtained from New England Biolabs. [γ-^{32}P]ATP was from Amersham Pharmacia Biotech. Chromatography media were from Amersham Pharmacia Biotech. Electrophoresis reagents were from Bio-Rad. Oligonucleotide synthesis and DNA sequencing services were provided by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). Magnesium chloride and manganese acetate were obtained from Fluka. All other chemicals that were used were analytical grade.

Methods

Preparation of DNA Polymerases and Their Mutants. Wild-type KF, T4, and T7 DNA polymerases were prepared from high expression vectors in *E. coli* BL21 DE3 cells and purified according to established procedures (*13*−*15*). With T7 DNA polymerase, *E. coli* thioredoxin was added to the crude lysate before purification. The T7 DNA polymerase used for the kinetic studies was a 1:1 complex with thioredoxin. The KF mutants Y423A and N420A were constructed, expressed, and purified using procedures previously described (*16*). The T4 DNA polymerase mutants N214A and F218A were prepared according to the procedures reported by Sattar et al. (*17*).

Preparation of Substrates. RNA−DNA chimeric oligonucleotides, obtained from the W. M. Keck laboratory, were deprotected as follows. One micromole of the lyophilized crude oligonucleotide was incubated at 25 °C for 16 h in 0.5 mL of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran; the reaction was quenched with 0.5 mL of deionized water, and then the mixture was desalted using a column of G-50 superfine Sephadex. The desalted oligonucleotide (in 0.01 M NH_4Ac) was vacuum-dried and redissolved in deionized water before being used. Labeling of substrates with [γ-^{32}P]ATP was carried out as described by Lin et al. (*13*).

Exonuclease Assays. Exonuclease assays were performed under steady state conditions as described previously by Lin et al. (*13*). A series of tubes containing 100 μM oligonucleotide substrate in 66 mM Tris-HCl buffer (pH 8.8) containing 6 mM $MgCl_2$ were incubated for 10 min at 30 °C with different concentrations of enzymes. The reactions were quenched with EDTA (final concentration, 20 mM) and the mixtures fractionated on 20% polyacrylamide gels in 8 M urea. The gels were dried and the intensities of the bands determined by densitometry after the gels were exposed to Royal X-OMAT AR Kodak X-ray film at −70 °C. The relative excision rates were determined by estimating the enzyme concentration (E_{50}) required to degrade 50% of the substrate in the 10 min incubation period. For each substrate, the E_{50} value was calculated by interpolation between the two enzyme concentrations that bracketed the 50% substrate degradation level. The E_{50} for the standard substrate $p(dC)_{10}$ was then divided by the value for the chimeric substrate, giving a measure of the relative degradation rate.

RESULTS

We began this study using a 5′-^{32}P-labeled 16mer chimeric DNA−RNA oligonucleotide with a single embedded ribouridyl (rU) residue to examine the effect that an rU residue would have on the ability of the Klenow fragment (KF) and two other DNA polymerases, from phages T4 and T7, to degrade this substrate. All three polymerases produced similar digestion patterns with the chimeric oligonucleotide ^{32}P-[G·C·G·A·$(T)_5$·rU·$(T)_6$], namely, an accumulation of ^{32}P-[G·C·G·A·$(T)_5$·rU·T·T] and ^{32}P-[G·C·G·A·$(T)_5$·rU·T] as shown in Figure 1 for T4 DNA pol. Prolonged incubation with each of these DNA polymerases, however, resulted in nearly complete digestion of the substrate to tri- and dinucleotides. From this "exo scanning" experiment, we inferred that the 3′−5′ exo activities of all three DNA polymerases were impeded by a ribouridyl (rU) residue in the second or third position from the 3′ terminus.[2] The lack of any effect of an R residue located more than two positions upstream from the 3′ terminus was verified by another exo scanning experiment, using p[T·rU·$(T)_{22}$] where no accumulation of any oligonucleotide longer than p[T·rU·T·T] was found (data not shown).

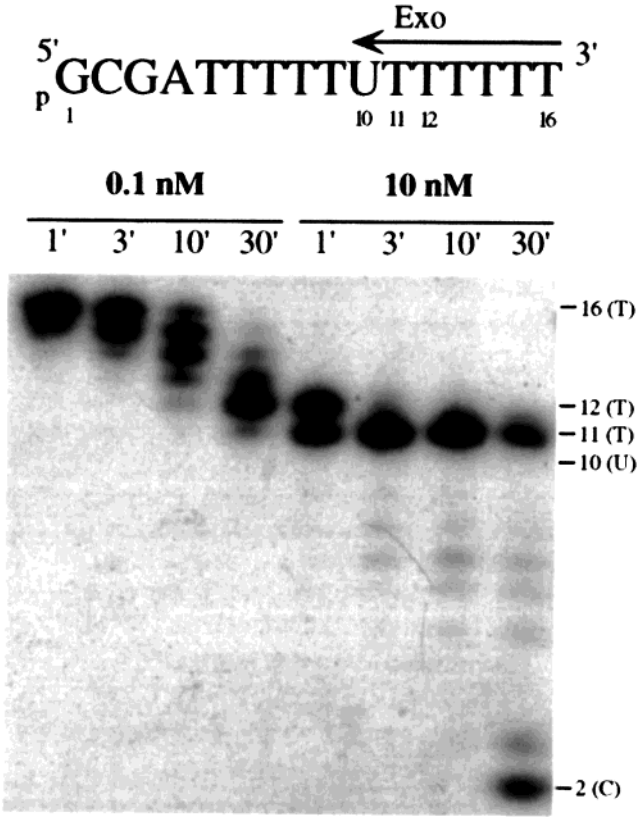

FIGURE 1: Effect of a single ribo-U residue on 3′−5′ exonucleolytic digestion by T4 DNA polymerase. The chimeric 16mer oligonucleotide was 5′-^{32}P-labeled and contained a single ribo-U residue at the indicated position. Two concentrations of T4 DNA pol were used: 0.1 and 10 nM. Aliquots for each digest were removed at 1, 3, 10, and 30 min (as shown above each of the lanes) and run on a 20%, 8 M urea−acrylamide gel which was then dried and autoradiographed. The lengths of several of the important bands along with the 3′ residue of each band are indicated on the right side of the autoradiogram (in parentheses).

To quantify the reduction in excision rates caused by the presence of ribonucleotide residues and to find out whether the rate decrease caused by an rU substitution was due to the base or the sugar, we synthesized and tested a set of 5′-^{32}P-labeled rU/T chimeric oligonucleotides and a matched set of rC/dC chimeric decanucleotides (hereafter, we shall use R to represent rC and C to represent dC) for the decamer

[2] To avoid repetition, we will use the term second or third position always in reference to the 3′ terminus which we call position 1.

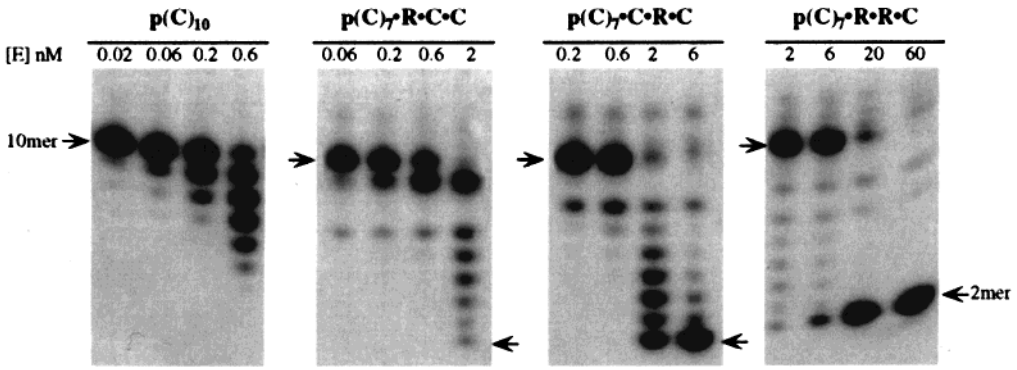

FIGURE 2: 3′−5′ exonucleolytic digestion patterns produced by T4 DNA polymerase acting on chimeric DNA−RNA decamers. These patterns were used to determine the relative rates of excision of the 3′ terminal residue. The 5′-^{32}P-labeled oligonucleotides were incubated with varying amounts of enzyme, indicated above each lane, for 10 min. The samples were then run on a 20%, 8 M urea−acrylamide gel which was dried and autoradiographed. The area and intensity of each spot were determined by densitometry and the E_{50} values estimated by interpolation. The oligonucleotides that were used are given above each panel. C is dC, and R is rC.

Table 1: Relative Rates for Excision of 3′ Terminal Nucleotide Residues from Chimeric DNA−RNA Cytidine Oligomers[a]

	DNA polymerase (Mg^{2+})[c]			DNA polymerase (Mn^{2+})[d]		
substrate[b]	T4	T7	KF	T4	T7	KF
p(C)$_6$•C•C•C•C	1	1	1	1	1	1
p(C)$_6$•C•C•C•R	1	1	1	1	1	1
p(C)$_6$•R•C•C•C	1	1	1	1	1	1
p(C)$_6$•C•R•C•C	0.2	0.5	0.07	0.3	0.5	0.3
p(C)$_6$•C•C•R•C	0.03	0.2	4×10^{-3}	0.2	0.3	0.07
p(C)$_6$•C•R•R•C	2×10^{-3}	0.02	2×10^{-4}	0.03	0.1	2×10^{-3}

[a] Essentially identical results were obtained for substrates having a single rU residue embedded within dT residues (data not shown). [b] R is rC, and C is dC. Precision, ±30%. [c] The Mg^{2+} concentrations were 10 mM for T4 DNA pol and T7 DNA pol and 20 mM for KF. [d] The Mn^{2+} concentrations were 12 mM for T4 DNA pol and T7 DNA pol and 10 mM for KF.

Table 2: Relative Rates for Excision of 3′ Terminal Nucleotide Residues from Short Deoxycytidine Oligomers[a]

DNA polymerase	p(dC)$_4$	p(dC)$_3$	p(dC)$_2$
T4	1	0.07	3×10^{-5}
T7	1	0.5	2×10^{-3}
KF	1	0.07	2×10^{-5}

[a] Precision, ±20%.

Table 3: Steady State Kinetic Parameters for the Excision of 3′ Terminal Nucleotide Residues from Chimeric DNA−RNA Oligonucleotides by the Klenow Fragment

substrate[a]	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m
p(C)$_6$•C•C•C•C	13	2.4	0.2
p(C)$_6$•C•R•C•C	100	1.7	1.7×10^{-2}
p(C)$_6$•C•C•R•C	40	0.1	2.5×10^{-3}
p(C)$_6$•C•R•R•C	180	7×10^{-4}	3.9×10^{-6}

[a] R is rC, and C is dC. Precision, ±20%.

substrates. Within each set of oligonucleotides, ribonucleotide residues were systematically inserted at different positions starting at the 3′ terminus. We then estimated the relative rates of removal of the 3′ terminal nucleotide residue from these chimeric substrates by the three DNA polymerases and normalized the values to those obtained with p(C)$_{10}$ which were arbitrarily set to 1 for each polymerase. Essentially the same results were obtained in the rU versus T and the R versus C comparisons, indicating that the sugar determined the excision rates and that substitution of U for T had little or no effect. A typical example of the data used to estimate the relative rates for the R versus C set is shown in Figure 2, and a summary of the relative excision rates for the RNA−DNA chimeras is presented in Table 1. When the 3′ terminus was occupied by a ribonucleotide residue (R), there was no decrease in excision rate relative to that of p(C)$_{10}$. This was true for all three DNA polymerases. When an R residue was located at the fourth position, or even further upstream from the 3′ terminus, there was also no reduction in the excision rate of the 3′ terminal residue (Table 1) (see below). There was a substantial rate reduction when a single R residue was adjacent to the 3′ terminus, [p(C)$_6$-C•C•R•C] (denoted hereafter as -C•C•R•C). A smaller, but still clear-cut, rate reduction was observed when R was the third residue from the 3′ terminus [p(C)$_6$-C•R•C•C] (denoted hereafter as -C•R•C•C). Finally, when R residues occupied both the second and third positions, [p(C)$_6$-C•R•R•C] (denoted hereafter as -C•R•R•C), the drop in excision rate was the most pronounced (Table 1). Substitution of Mn^{2+} for Mg^{2+} attenuated the effect of R residues on the decrease in excision rates, but the overall pattern of rate reduction remained the same as that found with Mg^{2+}.

On the basis of the results described above, we believe that there are significant interactions of ssDNA with the exonuclease domains of all three DNA polymerases that involve the sugar moieties of the three nucleotide residues at the 3′ terminus of the substrate. If this is the case, then the rate of removal of nucleotide residues from the 3′ terminus should be approximately the same for a tetra- and a trinucleotide, whereas hydrolysis of a dinucleotide would be much slower. When the rates of cleavage of the 3′ terminal nucleotide from p(dC)$_4$, p(dC)$_3$, and p(dC)$_2$ were compared (Table 2), the difference between the rates for p(dC)$_4$ and p(dC)$_3$ was 15-fold when using T4 DNA pol and KF, and only 2-fold when using T7 DNA pol. In contrast, the cleavage rate of p(dC)$_4$ relative to that of p(dC)$_2$ was 500-fold greater for T7 DNA pol, 30000-fold greater for T4 DNA pol, and 50000-fold greater for KF.

Steady State Kinetic Parameters for Excision by KF. The steady state kinetic parameters for excision of the 3′ terminal C from -C•C•C•C, -C•R•C•C, -C•C•R•C, and -C•R•R•C by KF were also determined. The results, shown in Table 3, indicate that the decrease in excision rates for the chimeric substrates is due to both an increase in K_m and a decrease in

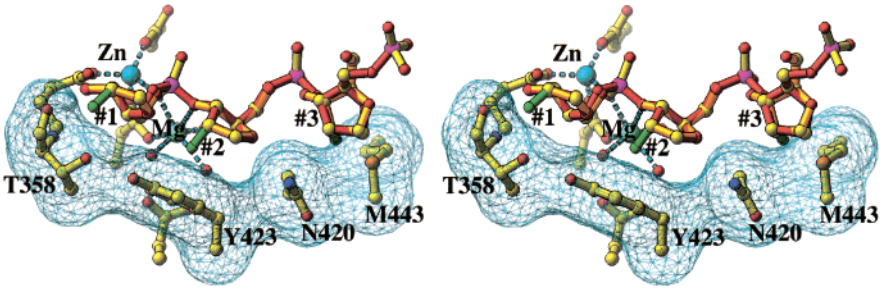

FIGURE 3: Stereodiagram of a ball-and-stick representation of part of the KF exonuclease active site, modeled with three ribonucleotide residues designated 1−3. Residue 1 is at the 3′ terminus of the oligonucleotide. The bases were omitted for clarity in presentation. The 2′ OH groups of the ribosyl moieties are bright green. The 2′ OH group of ribonucleotide residue 3 is pointing away from the viewing plane. The amino acid residues that contact the ribosyl 2′ OH groups (M443, N420, Y423, and T358) are shown, along with the electron densities of the atoms in the side chains that are closest to the 2′ OH groups. The distances between the 2′ OH groups and the protein are as follows: (1) 2′ OH and T358 (backbone carboxyl oxygen, 1.5 Å; (2) 2′ OH and Y423 (phenyl ring), 2.1 Å; and (3) 2′ OH and M443 (side chain sulfur), 1.7 Å. The coordinates are from Brautigam and Steitz (*18*) and modeled according to the method of Horton and Finzel (*31*).

Table 4: Relative Rates for Excision of 3′ Nucleotide Residues by KF and T4 DNA Polymerase and Their Mutants[a]

DNA polymerases and their mutants	$p(C)_6$·C·C·C·C	$p(C)_6$·C·R·C·C	$p(C)_6$·C·C·R·C	$p(C)_6$·C·R·R·C
KF	1	0.07	4×10^{-3}	2×10^{-4}
KF N420A[b]	1	0.5	0.1	0.02
KF Y423A[b]	1	0.03	1	0.03
T4 DNA pol	1	0.2	0.03	2×10^{-3}
T4 DNA pol N214A	1	0.2	0.03	2×10^{-3}
T4 DNA pol F218A	1	0.2	0.03	2×10^{-3}

[a] R is rC, and C is dC. Precision, ±30%. All reactions were carried out in the presence of 10 mM Mg^{2+}. [b] Both mutant proteins exhibit substantially lower excision rates on $p(dC)_{10}$ than wild-type KF.

k_{cat} relative to those of -C·C·C·C. The K_m increase accounts for most of the difference between the rates of -C·R·C·C and -C·C·C·C. With -C·C·R·C, the main effect is a decrease in k_{cat}. Both the K_m and k_{cat} values are dramatically altered when the substrate is -C·R·R·C.

Correlation of Structure with Rates: Models and Predictions. To obtain a more complete understanding of the structural basis of the rate decrease when the DNA polymerases encounter ribonucleotide residues, we examined the crystal structures of RB69 DNA pol (whose sequence is 77% identical with that of T4 DNA pol) (*12*) and KF complexed with deoxyoligonucleotides (*18*) to see if there would be a steric clash when a ribonucleotide residue was modeled at the second and/or third position of the oligonucleotide (we could not do this for T7 DNA pol since the crystal structure was determined using a mutant lacking an intact exonuclease active center). For KF, Y423 is close to a 2′ hydroxyl group on the penultimate nucleotide and N420 is close to a 2′ hydroxyl on the third nucleotide residue from the 3′ end (Figure 3) (*18*). We therefore tested the effect of mutations that decrease the size of these two side chains (Table 4). The KF Y423A mutation eliminated the inhibitory effect of the ribosyl residue in -C·C·R·C on the excision rate, and the inhibitory effect of the two ribosyl residues in -C·R·R·C was reduced to the same level observed for -C·R·C·C. The N420A mutation reduced the effect on the excision rate of ribonucleotides in either the second or third position (or both). In the case of T4 DNA pol, the residues most likely to be at the interface with the oligonucleotide, and with the potential to clash sterically with the ribosyl 2′ OH groups, are N214 (equivalent to KF N420) and F218 (equivalent to KF Y423), respectively (*10*). Unexpectedly, the T4 DNA pol mutations N214A and F218A had no effect on the excision rates with the three chimeric substrates (Table 4).

The structure of the KF oligonucleotide complex (*18*) predicts that steric clashes would occur between the 2′ OH of the ribosyl moieties in the oligonucleotide substrate and several amino acid side chains in KF (see Figure 3 and the Discussion). If this prediction is correct, then substitution of CH_3 for H on the ribosyl 2′ oxygens should increase the steric interference. To test this, we prepared a set of chimeric oligonucleotides with 2′-*O*-methyl groups (hereafter termed M) on the first, second, and third residues and used them as substrates for (i) KF and the two KF N420A and Y423A mutants, (ii) T4 DNA pol and the T4 N214A and F218A mutants, and (iii) T7 DNA pol. As can be seen from the results in the upper half of Table 5, where the assays were carried out in the presence of Mg^{2+}, the presence of the 2′-*O*-methyl group on the ribosyl moiety in the third position decreased the rate of 3′ nucleotide excision for all three DNA polymerases by 10−30-fold compared to that of -C·C·C·C; this is similar to the effect observed when there is a ribose group at the same position (Table 4). Moving the 2′-*O*-methyl group to the ribosyl moiety in the second position caused a dramatic reduction in the excision rate for all three DNA polymerases; in each case, the effect was substantially greater than for a ribose substituent at the second position (Table 4). When the ribosyl 2′-*O*-methyl group was in the first position (at the 3′ terminus), the effect on the excision rates was most pronounced for KF (a 2000-fold drop); for T4 and T7 DNA pol, there was a 50-fold reduction, compared to hydrolysis of -C·C·C·C. When Mn^{2+} was used in place of Mg^{2+}, there was a smaller rate reduction with all of the DNA polymerases (lower half of Table 5).

The KF N420A mutation did not affect the excision rate with -C·M·C·C (lower half of Table 5), whereas with -C·R·C·C, it alleviated the effect of the R substitution on the excision rate (Table 4). When -C·C·M·C was the

Table 5: Relative Rates for Excision of 3′ Nucleotide Residues from Chimeric DNA−RNA Oligonucleotides Containing 2′-*O*-Methylcytidine (M) Residues[a]

metal[b]	enzyme	p(C)$_6$·C·C·C·C	p(C)$_6$·C·M·C·C·C	p(C)$_6$·C·C·C·M·C	p(C)$_6$·C·C·C·M
Mg^{2+}	KF	1	0.03	3×10^{-5}	5×10^{-4}
	T4 DNA pol	1	0.1	3×10^{-4}	0.02
	T4 DNA pol N214A	1	0.1	3×10^{-4}	0.02
	T4 DNA pol F218A	1	0.1	2×10^{-3}	0.02
	T7 DNA pol	1	0.03	1×10^{-4}	0.02
Mn^{2+}	KF	1	0.1	1×10^{-5}	5×10^{-3}
	KF N420A	1	0.1	3×10^{-3}	$\ll 1.0 \times 10^{-4}$
	KF Y423A	1	0.1	1	$\ll 1.0 \times 10^{-4}$
	T4 DNA pol	1	0.3	0.02	0.1
	T4 DNA pol N214A	1	0.3	0.02	0.1
	T4 DNA pol F218A	1	0.3	0.07	0.1
	T7 DNA pol	1	0.3	0.01	0.2

[a] M is 2′-*O*-methylribocytidyl, and C is dC. Precision, ±30%. [b] All experiments were performed with 10 mM Mn^{2+} or 10 mM Mg^{2+}. Reactions were carried out at pH 8.5 and 37 °C.

substrate, the dramatic decrease in the excision rate observed with wild-type KF could be partially overcome by using the N420A mutant and was totally eliminated by using the KF Y423A mutant (Table 5). The situation was very different when the 3′ terminal ribose had a 2′-*O*-methyl group (-C·C·C·M). In this case, the two KF mutants were even more sensitive than wild-type KF to the 2′-*O*-methyl substitution. It is important to note that all the experiments with the KF mutants and substrates containing 2′-*O*-methylcytidine residues were carried out using Mn^{2+} since no excision could be observed for -C·C·M·C or -C·C·C·M in the presence of Mg^{2+}. We extended our investigation of the effect of 2′-*O*-methyl groups, using the same set of substrates, to T4 DNA pol and two of its mutants. The results in Table 5 show that the N214A mutation in T4 DNA pol does not affect the excision rate for any of the three chimeric substrates, -C·M·C·C, -C·C·M·C, or -C·C·C·M. On the other hand, the F218A mutation partially alleviated the rate reduction with -C·C·M·C.

DISCUSSION

Using single-stranded DNA−RNA chimeric oligonucleotides, we have shown that all three DNA polymerases used in this study have slower 3′-5′ exonuclease excision rates when a ribosyl group is substituted for a deoxyribosyl group in the second or third position from the 3′ terminus. The largest reduction was observed when both the second and third positions were occupied by ribonucleotide residues.

Our results can account for a previously puzzling observation, namely, that treatment of an Okazaki fragment with T4 DNA polymerase produced an RNA−DNA chimera with a single deoxyriboncleotide residue at the 3′ terminus as the major product (*7*). This is exactly what would be predicted on the basis of our findings with synthetic DNA−RNA chimeras where the largest rate reduction for removing dN from an Okazaki fragment should occur when it terminates in rN·rN·dN (Table 1). It is also consistent with an early finding by Lehman and Richardson, who reported that a 3′−5′ exonuclease associated with *E. coli* pol I excised ribonucleotide residues from an DNA−RNA copolymer at $^1/_7$ of the rate found for a polymer composed solely of DNA (*19*).

Three nucleotide residues at the 3′ terminus have extensive interactions with the exo domain of the three DNA polymerases. We have previously reported that, using T4 DNA pol to digest oligo dT of varying lengths (from 32 to 3 nucleotides), the excision rate gradually decreased with decreasing substrate length until the dinucleotide stage when degradation virtually ceased (*13*). We also showed that this rate decrease was due almost entirely to an increase in K_m (*13*). Here we have demonstrated that, with all three DNA polymerases, the cleavage rate of p(dC)$_2$ is drastically reduced compared to the 3′−5′ exo rates observed with p(dC)$_4$ and p(dC)$_3$. In the cocrystal structures of T4 DNA pol and KF with single-stranded oligonucleotides at the 3′−5′ exonuclease site (*9*, *10*, *18*, *20*), only three nucleotide residues, and perhaps part of a fourth, could be identified in electron density maps, even when larger deoxyoligonucleotides such as p(dT)$_{16}$ were used (*10*). Thus, kinetic and crystallographic results suggest that the DNA polymerases contact the three nucleotide residues at the 3′ terminus and that the interactions with the sugar moieties of these residues have a significant influence on the rate at which the DNA polymerases hydrolyze their substrates.

Correlation of the Kinetic Data on KF with the Structure of the KF−ssDNA Complex. To understand the structural basis for the rate reductions just described, we examined KF and its mutants in more detail. Since there is a high-resolution crystal structure of a KF complex with a heptameric oligonucleotide GCTTACG (*18*), we used this structural information as a basis for modeling in a trimeric ribonucleotide to provide the structural diagrams shown in Figure 3. Because the sugars of the two terminal nucleotide residues in the crystal had a 3′ endo conformation, they did not have to be altered when substituting ribosyl for deoxyribosyl groups in the complex with KF. The conformation of the third sugar in the crystal, however, was not clearly defined (*18*). This model was used to estimate distances from the 2′ oxygens of the ribosyl moieties to the atoms on the nearby side chains of Y423, N420, and M443 and to examine the potential for steric clashes between the 2′ hydroxyls from the second and third ribosyl groups with the atoms of the side chains as indicated in Figure 3.

When a 2′ oxygen in the ribosyl moiety of the 3′ terminal residue is modeled into the KF−oligonucleotide complex, there is the potential for hydrogen bonding between the 2′ OH and the hydroxyl group of T358 as well as the T358 main chain carbonyl oxygen. Even though there appears to be a steric clash between the 2′ OH and the side chain of

the T358 residue, there are not many interactions between the protein and the first base or sugar that would prevent rotation around the first phosphodiester bond, thus avoiding steric interference. This is consistent with our observation that hydrolysis of a 3′ terminal ribonucleotide residue proceeds at a rate similar to the rate of hydrolysis of a DNA substrate. However, a 2′-*O*-methyl group on the 3′ terminal ribose causes a 2000-fold decrease in the excision rate with wild-type KF. Presumably, the bulkier methyl group prevents rearrangement of the enzyme−substrate complex to avoid a steric clash. When a 2′ OH on the second sugar was modeled into the KF−oligonucleotide complex, there appears to be steric interference between the 2′ OH group and three carbon atoms of the aromatic ring of Y423 in KF as shown in Figure 3. It may not be possible to alleviate this steric interference by a conformational adjustment of the enzyme−substrate complex because a hydrogen bond between the hydroxyls of Y423 and S360 could impede movement of the aromatic side chain. In addition, the ribosyl group has very little freedom to rotate since it is fixed in position by flanking phosphate groups. Our observation that replacement of Y423 with Ala completely eliminates the rate reduction associated with ribose substitution at the second position strongly supports the idea that there is steric interference between the 2′ OH group on the ribose and the tyrosine ring. The steric interference is even more pronounced when a 2′-*O*-methyl group is in the second position (-C·C·M·C), but the rate reduction can still be reversed completely by the Y423A mutation, providing further support for the notion that a steric clash is responsible for the rate reduction. Additionally, the N420A mutation partially alleviates the effect of a 2′ OH or a 2′-*O*-methyl group in the second position. Presumably, the loss of the N420 side chain relieves the steric clash by allowing some flexibility in the complex.

When a 2′ OH substituent on the third sugar from the 3′ terminus was modeled into the KF−oligonucleotide complex, steric interference would be expected to occur between this ribosyl OH group and (i) the sulfur of M433, (ii) the β-amide nitrogen of N420, and (iii) the β-carbon of the N420 side chain. This steric interference cannot be relieved by repositioning the N420 side chain because the amide oxygen is hydrogen bonded to the ϵ-amino group of K422, fixing the position of the N420 side chain. The third sugar also cannot alter its position easily because it is fixed by two flanking phosphates. The N420A mutant only partially relieves the rate reduction, perhaps because some remaining steric hindrance with the sulfur of M443 or with the β-carbon of residue 420 still hinders effective binding. When the 2′ OH group of the third sugar is methylated (-C·M·C·C), then the N420A mutation is unable to alleviate the rate reduction.

Clearly, when there is steric interference, the interface between the oligonucleotide substrate and the polymerase must be altered if binding and hydrolysis is to occur. In the case of -C·R·C·C, the very small decrease in k_{cat} (Table 3) suggests that the interface readjustment did not perturb the geometry of the reaction center relative to the scissile phosphate; nonetheless, the steric clash caused the K_m to increase significantly (7−8-fold). With the ribosyl group in the second position, in -C·C·R·C, the k_{cat} was reduced dramatically but the K_m was only moderately increased, suggesting that although rearrangment could preserve favorable ground state binding, it perturbed the transition state geometry. This is not surprising since the sugar in the second position is directly linked to the scissile phosphate. As might be expected, combining both substitutions (-C·R·R·C) results in a further increase in K_m and decrease in k_{cat}.

Attempts To Correlate the Kinetic Data on the Exonuclease Activity of T4 DNA Pol with Its Structure. On the basis of the similarity of their exo domain structures, residues N214 and F218 of T4 DNA pol correspond to N420 and Y423 of KF (*11*, *18*). These two residues in T4 DNA pol would therefore be expected to have the closest contact with the second and third sugars in the oligonucleotide substrate. In contrast to the situation with KF, replacement of N214 and F218 with Ala did not alter the excision rates for the ribonucleotide-containing substrates. The results from single-turnover experiments with wild-type T4 DNA pol and the F218A and N214A mutants are consistent with the possibility that the hydrolytic step is not rate-limiting (C. X. Wang et al., unpublished results). This notion is supported by previous studies on T4 DNA pol (*21*, *22*). In contrast, studies on KF indicate that the rate-determining step is either chemistry or enzyme−DNA binding (*23*). If a step other than chemistry is rate-limiting for T4 DNA pol, this could explain why the rate is less sensitive to ribonucleotide substitution (compared with KF), and why the effect of N214A and F218A mutations is so slight. A 2′-*O*-methyl substituent on the sugar preceding the 3′ terminus caused a dramatic reduction in the steady state excision rates, even for T4 DNA pol, and this rate reduction could be alleviated somewhat when F218 was replaced with Ala. Our interpretation is that the bulkier 2′-*O*-methyl substituent in this position has caused chemistry to become at least partially rate-limiting, revealing evidence of the steric clash between the 2′ sugar substituent and the aromatic ring of F218. The difference in our results for KF and T4 DNA pol can readily be rationalized in kinetic terms: T4 DNA pol has a much more active exonuclease, and therefore, the rate is more likely to be limited by a step other than chemical catalysis. Nevertheless, it remains unclear why two enzymes with extremely similar active sites catalyze the exonuclease reaction at such vastly different rates.

Biological Rationale for the Exonucleolytic Activity of DNA Polymerases Acting on DNA−RNA Chimeric Substrates. Although our studies were carried out mostly with ssDNA, similar results would be expected when a DNA polymerase encounters a primer−template complex since at least three residues from the 3′ terminus of the primer strand have to be frayed for the 3′ terminal nucleotide residue to access the exonuclease active site (*24*−*27*). Our observation that a 3′ terminal ribonucleotide residue can be removed as easily as a deoxyriboncleotide residue indicates that the 2′ OH group does not contact the polymerase and has no influence on the excision rate. This is consistent with the crystal structures of KF complexed with single-stranded oligonucleotides (*18*, *25*). The implication of this result for DNA replication is that a primer terminating in -dN-dN-rN, which would result from infrequent misincorporation of an rN residue, would have the terminal ribonucleotide residue removed as easily as a deoxyribonucleotide before the primer is extended further. By contrast, -rN·rN·rN or -rN·rN·dN, present at the 5′ end of Okazaki fragments, will not be degraded during the time that it takes to replicate the entire bacterial or phage DNA. It should be noted, however, that the major discrimination mechanism used by the polymerases

to prevent rN incorporation occurs during the nucleotidyl transfer reaction rather than during editing (*4*, *28*−*30*).

There is a plausible biological rationale for the finding that the exonuclease reaction catalyzed by KF is more adversely affected by the presence of ribonucleotide residues than the exonucleases of the T7 and T4 phage DNA polymerases. Both of the phage DNA polymerases are replicative enzymes and have fewer copies per cell than the host DNA pol I which is a repair enzyme (*5*). The phage DNA polymerases are components of a replisome and must have potent exonuclease activity acting both on ribonucleotide residues that are incorporated by mistake or on mismatched deoxynucleotide residues to keep the overall error frequency at a very low level. By contrast, a DNA polymerase present in high copy number, which is not sequestered as part of the replisome, would have greater potential to degrade RNA in vivo. To preserve the integrity of the cellular RNA, a high-copy number DNA polymerase would need to have very low exonucleolytic activity toward RNA.

In summary, it is possible that the exo domains evolved so that they could efficiently remove infrequently misincorporated ribonucleotide residues as well as mismatched bases from an elongating primer without degrading mRNA or RNA primers that initiate lagging strand synthesis.

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