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Fidelity of DNA synthesis catalyzed by human DNA polymerase α and HIV-1 reverse transcriptase: effect of reaction pH

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ABSTRACT

The accuracy of DNA synthesis catalyzed by the Thermus aquaticus DNA polymerase and the 3′→5′ exonuclease-deficient Klenow fragment of *Escherichia coli* DNA polymerase I varies as a function of reaction pH (Eckert,K.A. and Kunkel,T.A. (1990) *Nucleic Acids Res.* 18, 3739–3744; Eckert,K.A. and Kunkel,T.A. (1993) *J. Biol. Chem.* 268, 13462–13471). In the current study, we demonstrate that the fidelity of human DNA polymerase α increases 10-fold when the pH of the *in vitro* synthesis reaction is lowered from pH 8.6 to pH 6.1 (37°C), as determined using a base substitution reversion assay to score polymerase errors within the *lacZα* gene of bacteriophage M13mp2. Similarly, the base substitution fidelity of DNA-dependent DNA synthesis by the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) was improved nine-fold at pH 6.5 relative to pH 8.0 (37°C). A detailed comparison of HIV-1 RT error specificity at neutral and low pH in a *lacZα* forward mutation assay revealed that low pH suppresses both mispairing-mediated and misalignment-mediated mutations; however, the characteristic HIV-1 RT pattern of mutational hotspots at homopolymeric sequences is retained at the lower pH. Consistent with the presumption that these mutations result, in part, from increased termination of DNA synthesis within the hotspot sequences relative to other homopolymeric sequences, the HIV-1 RT termination pattern during processive DNA synthesis is not altered by low pH. The HIV-1 RT results are in agreement with our previous hypothesis that the observed increase in polymerase fidelity at low pH results from a decreased efficiency of continuing DNA synthesis from premutational DNA intermediates.

INTRODUCTION

Just as DNA polymerases differ enzymatically with regard to specific steps in the reaction scheme for DNA synthesis, these enzymes generate unique mutational spectra with respect to both the type and frequency of errors produced during DNA synthesis *in vitro* (1, 2). Recent amino acid sequence alignments of more than 50 DNA polymerase genes have been used to classify these enzymes into four distinct groups: family A (Pol I-like), family B (α-like), family C (Pol III-like), and Family× (β-like) (3). The viral reverse transcriptases which catalyze DNA-dependent DNA synthesis comprise a distinct family of DNA polymerizing enzymes. Despite the varied degree of phylogenetic relatedness of the polymerase genes, three highly conserved amino acid motifs have been deduced among the protein sequences which transcend the major families, and which may define functionally important domains of the enzymes (4). Recent X-ray crystallographic studies have revealed a remarkable structural similarity between the catalytic subdomains of the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT), an asymmetric heterodimer, and the Klenow fragment of *E.coli* DNA polymerase I, a single polypeptide (5). The recent construction of mutant forms of human DNA polymerase α (6) also provides strong evidence that the conserved catalytic motif identified by amino acid alignment (4) can be functionally extended to eukaryotic DNA polymerases.

During an investigation of the parameters that affect the accuracy of DNA polymerases in the absence of exonucleolytic proofreading, we found that the fidelity for both base substitution and frameshift errors produced by the Thermus aquaticus (Taq) DNA polymerase (7) and the 3′→5′ proofreading exonuclease-deficient Klenow polymerase (8) are significantly improved when the *in vitro* reaction pH is lowered to pH 6–6.5, relative to neutral pH. The precise mechanism by which low pH improves polymerase accuracy is unknown. We have demonstrated for the exonuclease-deficient Klenow polymerase that the primary effect of low pH on base substitution fidelity is to increase polymerase discrimination against DNA synthesis from terminally mispaired template-primers, rather than to decrease the frequency of nucleotide misinsertion (8). Concomitant with decreases in base substitution and frameshift error rates, we observed an enhanced processivity at specific template sequences at low pH, presumably reflecting a pH-dependent alteration in specific interactions between the DNA polymerase and the template-primer which suppresses errors (8).

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We wished to test whether the observed effects of pH on fidelity is general for all DNA polymerases or unique to the class A polymerases. We have examined the *in vitro* fidelity of human DNA polymerase α, a class B polymerase, and the HIV-1 RT as a function of reaction pH. In addition to being phylogenetically distinct from the Klenow polymerase, these polymerases generate very different types of errors within the bacteriophage M13mp2 *lacZ*α mutational target gene (9, 10). In particular, the HIV-1 RT generates a high frequency of mutations resulting from template-primer misalignments in homopolymeric sequences (10). These characteristic mutational hotspots presumably result from an increased frequency of formation and/or utilization of misaligned template-primers during DNA synthesis. The observed high probability of HIV-1 RT termination within hotspot sequences has been interpreted to mean that misalignments may be increased during the dissociation−re-initiation phases of the synthesis reaction (11). In this report, we analyze the effects of low pH on the fidelity and processivity of the HIV-1 RT, and compare these results to those previously reported for the Klenow polymerase.

METHODS

Enzymes

The recombinant form of HIV-1 reverse transcriptase from Genetics Institute (Cambridge, MA) was obtained using an *E.coli* expression plasmid that contains the coding sequence of HIV-1 RT with the carboxyl-terminal Leu omitted, and is >90 percent pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (12). The preparation displays two major bands of equal intensity, 66 and 51 kilodaltons, and is devoid of 3′→5′ exonuclease activity (13). The specific activity of the enzyme is 1000 nmol dTMP incorporated/min/mg at 37°C using a poly rA−oligo dT substrate. Human DNA polymerase α, purified as described (6) from insect cells expressing the catalytic subunit, was a gift from William Copeland and Teresa Wang, (Stanford, CA). After separation on a polyacrylamide gel, this preparation appears >95% pure as visualized by Coomassie staining, and contains two human DNA polymerase α bands: p180 and p165. The specific activity of the enzyme is 32,700 units/mg, where one unit is defined as the amount of enzyme that incorporates 1 nmole dTTP/hour at 37°C using optimally gapped calf thymus DNA substrate.

M13mp2 fidelity assays

Polymerase fidelity was measured using M13mp2 DNA substrates, constructed as described (14), which contain a 390 nucleotide gap opposite the *lacZ*α gene. DNA polymerase errors produced during *in vitro* gap-filling DNA synthesis are measured after transfection of *Escherichia coli* strain MC1061 with the synthesis products, as described previously (15, 16). In the forward mutation assay (15), mutations are scored as light blue or colorless plaques resulting from loss of β-galactosidase activity, whereas in the opal codon reversion assay (14), mutations are scored as blue revertant plaques resulting from complete or partial restoration of *lacZ* activity after one of eight possible polymerase base substitution errors at the TGA codon. Procedures for scoring mutants and DNA sequence analysis of the mutant M13mp2 viral DNA have been described previously (16). In both assays, the mutant frequency is defined as the proportion of mutant plaques to the total number of plaques scored. The polymerase error rate per detectable nucleotide (ER) can be calculated from the mutant frequency (MF) as follows: ER = 1/[(number of mutations observed/total number of independent mutations) (observed MF − background MF/0.6)÷no. detectable sites], where 0.6 is the frequency of expression of the minus strand in *E.coli* (14).

Polymerase reaction conditions

All polymerase reactions contained 20 mM buffer, 2 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM each of dATP, dGTP, dCTP and dTTP, and approximately 1 nM gapped DNA substrate. The buffers used in this study were chosen for each polymerase based on the buffer pK_a value and the relative activity of the enzyme in the buffer. (Specific buffers are indicated in the Tables and Figure legends.) The reaction mixtures were prewarmed to 37°C for three min, followed by the addition of the polymerase. After incubation at 37°C for 10−60 min, the reactions were stopped by the addition of EDTA to a final concentration of 15 mM. In all subsequent discussions, the reaction pH presented is the pH of the buffer plus enzyme at 37°C (calculated from room temperature measurements and ΔpH/°C) in order to equalize for the differing temperature gradients among buffers. Each polymerase preparation differs in specific activity and storage buffer; therefore, the volume of polymerase added to the reaction and the pH of the storage or dilution buffer changes the pH of the final gap-filling reaction to differing extents. For reactions reported here, 0.06 volume of the HIV-RT (50 nM in 20 mM Tris−HCl, pH 7.8) and 0.1 volume of the human DNA polymerase α (1.83 U/μl reaction in 50 mM Tris−HCl, pH 8.0) were added to the reactions. Samples of each reaction were analyzed for the extent of gap-filling synthesis by electrophoresis through a 0.8% agarose gel. In all experiments reported here, polymerization yielded reaction products that migrated as a discrete band coincident with a double-stranded nicked (RFII) DNA standard. Under the conditions of electrophoresis used in these experiments, resolution of a gapped DNA molecule from the RFII standard can be made to within approximately 50 nucleotides. Note that synthesis need proceed for only 250 of the 390 nucleotides in the gap in order to completely copy the target (15).

Termination probability analysis

Measurements of processivity as a function of reaction pH used M13mp2 ssDNA templates primed with a series of [γ^{32}P]ATP 5′-end-labeled DNA oligonucleotides complementary to positions 152−166 (lac 152), 105−119 (lac 105), 59−65 (lac 59), and 10−27 (lac10) of the *lacZ*α coding sequence. Preparation of the DNA template-primers was as previously described (8). DNA synthesis reaction conditions were identical to those used for the M13mp2 fidelity assays, except that the DNA substrate (150 fmol) was present in a molar excess over HIV-1 reverse transcriptase (50 fmol). The reactions (50 μl) were incubated at 37°C for 5−30 min. Aliquots of the reactions were stopped at various time points by mixing with an equal volume of stop dye solution (99% formamide, 5 mM EDTA, 0.1% xylene cylanol, 0.1% bromophenol blue). The amount of radioactivity in each DNA product band was quantitated using a Molecular Dynamics PhosphorImager (Sunnyvale, CA) after separation on a 20% denaturing polyacrylamide gel, using DNA markers generated by dideoxy sequencing from the same template-primer. Termination probability at a specific site is defined as the ratio of the number of product molecules at a given chain length divided by this number plus the number of all longer products (17). The values for termination probability given in the Figures

and Tables were determined from 30 min synthesis reactions, and are generally reproducible to within a factor of two; however, occasionally an outlying value is obtained (data not shown; see reference 12).

These experiments do not employ an exogeneous trap to prevent polymerase reinitiations on the substrate template-primers. We believe that the majority of synthesis is limited to a single cycle of processive elongation based on the following observations. First, under the conditions of template:enzyme molar ratios used in these experiments (3:1), the relative termination probability at specific bands is constant with respect to reaction time. Deviation from processive conditions, i.e. reinitiation of DNA synthesis on previously extended DNA template-primer molecules, was observed at higher concentrations of enzyme as a chasing of radioactivity from smaller DNA products into larger DNA molecules with increasing reaction time (data not shown). Second, less total DNA product is formed (ranging from 8–40 fmol) than the amount of polymerase added to the reactions (50 fmol), and typically less than 35% of the DNA substrate is extended. In addition, the intensity of individual bands does not significantly increase between five and 30 minutes, suggesting that the amount of DNA synthesis initiation on unused template-primer molecules is negligible. Thus, the amount of reinitiation on previously extended template-primer molecules relative to the total pool of product DNA is not expected to be significant enough to alter the calculated termination probability.

RESULTS

Polymerase fidelity in the M13mp2 gap-filling assays

Using an M13mp2 opal codon reversion assay to detect base substitution errors, we have observed previously that the frequency of mutants produced by the exonuclease-deficient Klenow polymerase increased as the pH of the *in vitro* gap-filling reaction was raised (8; see Table 1). We now show that the fidelities of other classes of polymerases respond in a similar manner to changes in reaction pH. The base substitution error rate of human DNA polymerase α increases approximately 10-fold as the pH is raised from pH 6.1 (1/42,000) through pH 8.6 (1/3,900) (Table 1). Similarly, the HIV-1 RT base substitution error rate is 10-fold higher at pH 8.0 (1/3,700) relative to pH 6.5 (1/38,000) (Table 1).

Our previous results concerning the fidelities of the Taq and exonuclease-deficient Klenow polymerases demonstrated that lowering the reaction pH improved both minus-one-base deletion error rates as well as base substitution error rates, as measured using single-site reversion assays (7, 8). To more rigorously test the extent of the pH effect on errors produced by different mutational pathways, HIV-1 RT fidelity was examined in the M13mp2 forward mutation assay as a function of reaction pH. This assay allows the detection of base substitution mutations at 125 sites and one-base frameshift mutations at 150 sites, as well as large deletions and complex mutations. As shown in Table 2, HIV-1 RT fidelity is eight-fold lower at pH 8.0 (1.1×10^{-1}), relative to pH 6.2 (1.40×10^{-2}). However, even under reaction conditions which increase fidelity, the HIV-RT continues to be less accurate than the AMV-RT under standard buffer conditions in the forward mutation assay (Table 2).

Specificity of HIV-1 RT errors at neutral and low pH

We have compared the types of mutations produced during gap-filling synthesis in the forward mutation assay by the HIV-1 RT under our standard buffer conditions (Hepes, pH 7.5) with those obtained under high fidelity buffer conditions (Bis–Tris, pH 6.2). Table 3 summarizes the results after DNA sequence analysis of approximately 100 mutants generated at each pH. The specific sequence changes of the mutants are given in Figure 1 (single mutants) and Table 4 (multiple mutants). Consistent with the error

Table 1. Effect of reaction pH on polymerase fidelity in the opal codon reversion assay

Polymerase	Buffer	pH (37°C)[a]	Reversion Frequency ($\times10^6$)[b]		Error Rate per nt
D355A, E357A	Klenow[c]				
	Pipes	6.2	16	(6.1)	1/150,000
	Hepes	7.6	200	(51)	1/10,000
	Ches	8.6	720	(170)	1/2,500
Human pol α					
	Bis–Tris	6.1	47	(1.0)	1/42,000
	Tris	7.8	280	(45)	1/6,500
	Ches	8.6	470	(10)	1/3,900
HIV-1 RT					
	Tris	6.5	52	(10)	1/38,000
	Tris	8.0	490	(10)	1/3,700

[a]pH at reaction temperature calculated from the pH of a mock reaction at room temperature.
[b]Mean (standard deviation) of two or more independent experiments.
[c]Data taken from reference (8).

Table 2. Effect of reaction pH on polymerase fidelity in the forward mutation assay

Polymerase	Buffer	pH (37°C)[a]	Mutant Frequency ($\times10^4$)[b]	
none[c]			6.6	(0.78)
HIV-1 RT				
	Bis–Tris	6.2	140	(26)
	Tris	6.5	250	
	Hepes	7.5	770	(93)
	Bis–Tris	7.7	850	(70)
	Tris	8.0	1100	
AMV-RT[d]				
	Hepes	7.4	42	(18)

[a]pH at reaction temperature calculated from the pH of a mock reaction at room temperature.
[b]Mean (standard deviation) for at least two independent experiments.
[c]Mutant frequency determined for ssDNA used as a template for the gap-filling reactions.
[d]Data taken from reference (23).

Table 3. Fidelity of HIV-1 reverse transcriptase *versus* pH, by mutational class

Class of mutation	Mutation Frequency $\times10^4$[a]	
	pH 6.2	pH 7.5
Single base substitution	69 (49)	220 (28)
Single base deletion	45 (32)	210 (27)
Single base addition	7.1 (5)	79 (10)
Multiple mutations	18 (13)	250 (32)
Other		7.9 (1)[b]
Total	140 (99)	770 (98)

[a]Mutant frequencies were determined from M13mp2 gap-filling assays using Bis–Tris (pH 6.2) and Hepes (pH 7.5) reaction buffers. The number of mutants sequenced from each collection is shown in parentheses.
[b]Mutant contains DNA sequence changes in the region 88–102 which cannot be assigned unambiguously. Net result of the mutation is the loss of three bases.

Table 4. List of HIV-1 RT mutants containing multiple mutations

pH 6.2				pH 7.5			
Position	Mutation	Position	Mutation	Position	Mutation	Position	Mutation
A. Double mutations							
Events separated by >10 nucleotides:							
69	G→T*	−68	G→A*	−21	T→*C	−36	T→C*
70	△T*	−22	T→C*	−1	△G*	−34	△T*
91	▲A*	47	G→T*	29	G→A*	−36	T→C*
106	△C*	−29	T→C*	103	T→C*	46	T→G*
122	T→C*	81	C→G*	112	T→C*	106	△C*
137	△T*	101	C→T*	112	△T*	82	G→A*
				121	T→C*	57	T→C*
				136	C→T*	91	△A*
				137	△T*	−1	△G*
				137	△T*	11	G→T*
				137	△T*	24	A→T*
				137	△T*	57	T→C*
				137	△T*	76	△A*
				137	▲T*	106	C→A*
				137	▲T*	106	△C*
				137	▲T*	90	G→A*
				137	▲T*	−29	T→C*
				141	G→C*	106	△C*
				144	△A*	88	△G*
				162	G→A*	−24	G→T*
Adjacent or closely spaced events:							
−50	T→C	−52	▲C	−36	T→C	−29	T→C
58	△C	59	△A	−38	G→C	−41	△C
−34	△T	−35	△T	−55	△C	−58	T→C
70	△T	71	△T	88	△G	89	△G
91	△A	95	C→T				
123	G→T	121	T→C				
B. Triple mutations							
137	T→C	131	T→C	153	△A*	−21	T→C*
		130	A→C			−71	A→T*
				88	△G*	105	A→T
						106	C→A
				70	▲T*	−1	△G*
						−73	T→C*
				−36	T→C*	104	▲T
						106	△C
				132	▲C	127	△C
						128	△A
C. Quadruple mutations							
				21	T→C*	113	T→C
				109	△A	106	△C
				−36	T→C*	3	T→A
				4	T→A	5	G→T
D. Quintuple mutation							
				91	▲A*		
				30	G→T	36	G→A
				4	△G	−1	G→A

*Mutation is considered as an independent occurrence for determination of error rates in subsequent tables.
▲, one base addition; △, one base deletion.

spectrum previously reported for the HIV-1 RT (10), approximately equal frequencies of single base substitution and one-base deletion errors are generated at pH 7.5, as well as a high frequency of one-base addition errors (Table 3). Gap-filling DNA synthesis at low pH by the HIV-1 RT resulted in the suppression of all classes of mutations, but to differing extents. For example, while the frequency of single base substitution mutations is decreased three-fold at pH 6.2 relative to pH 7.5, the frequency of one-base addition errors is decreased 10-fold at the lower pH (Table 3).

Effect of pH on misalignment-mediated errors. The most striking result of the DNA sequence analyses can be seen by comparing the spectra of single base mutations produced at the two pH conditions (Figure 1). The mutational hotspots in homopolymeric *lacZ* template sequences characteristic of DNA-dependent DNA synthesis by the HIV-1 RT (10) continue to be present in the low pH spectrum. These mutational hotspots share in common error production via template-primer misalignments. Such DNA slippage events can result in three distinct types of mutations (18). If the extra base is present in the DNA template strand of a

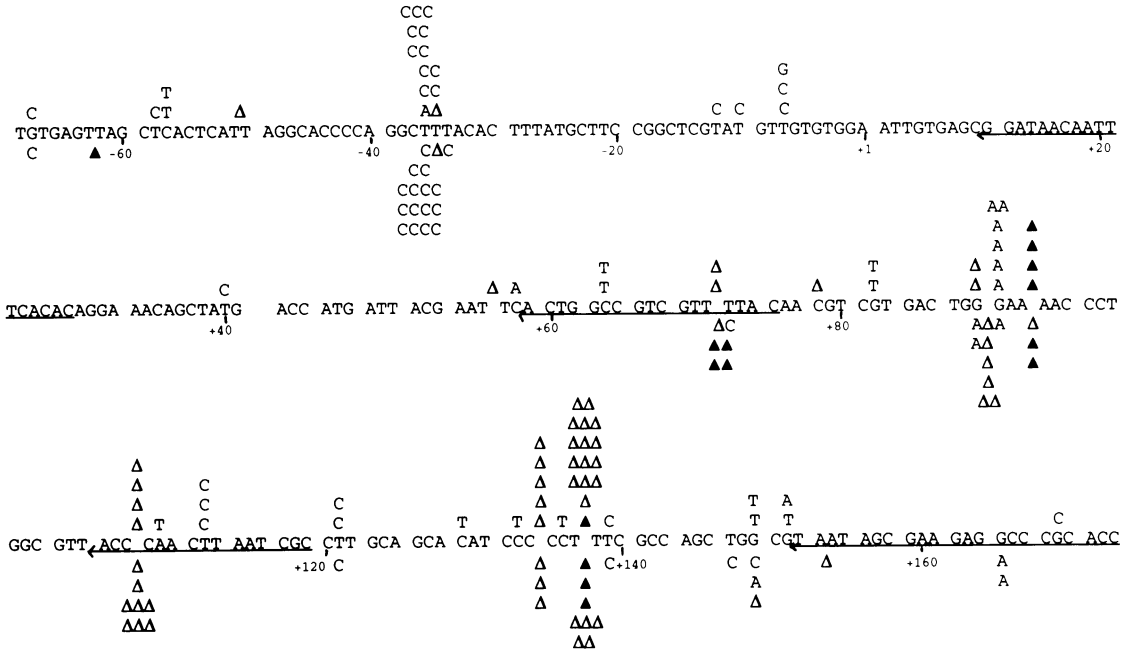

Figure 1. HIV-1 RT mutational spectra at neutral and low pH. The line of DNA sequence is the *lacZα* mutational target, presented in the 5′→3′ direction, with single base mutants obtained from forward gap-filling assays shown as changes above (Bis−Tris pH 6.2 buffer) and below (Hepes pH 7.5 buffer) the line. When a one base frameshift error occurs in a homopolymeric run, the precise base added or lost is unknown, and the symbol (▲, addition; △, deletion) is centered above or below the sequence. Underlined sequences indicate positions of oligonucleotide primers used in termination probability analyses, with the 3′-OH shown as an arrow head.

Table 5. Comparative error rates for HIV hotspots

Template Sequence	Position	Error rate per nucleotide[a] pH 6.2		pH 7.5		Ratio 7.5/6.2
Dislocation errors						
TTT	−34 to −36[b]	1/500	(9)	1/48	(20)	10
AAAA	91−94[c]	1/750	(6)	1/480	(2)	1.6
CCC	106−108[d]	<1/4500		1/480	(2)	>9.4
−1 Frameshifts						
TTTT	70−73	1/6000	(3)	1/3800	(1)	1.6
GGG	88−90	1/6800	(2)	1/360	(8)	19
AAAA	91−94	<1/18,000		1/1900	(2)	>9.5
CCC	106−108	1/2700	(5)	1/260	(11)	10
CCCCC	132−136	1/4500	(5)	1/1600	(3)	2.8
TTT	137−139	1/840	(16)	1/290	(10)	2.9
+1 Frameshifts						
TTTT	70−73	<1/20,000		1/770	(5)	>26
AAAA	91−94	1/3600	(5)	1/1300	(3)	2.8
TTT	137−139	1/14,000	(1)	1/410	(7)	34

[a]Error rates per detectable nucleotide were calculated using 105 (pH 6.2) and 123 (pH 7.5) total independent occurrences. Number of occurrences of each mutational event is given in parentheses.
[b]Error scored is T→C at −36 (see Figure 1).
[c]Error scored is G→A at 90 (see Figure 1).
[d]Error scored is C→A at 106 (see Figure 1).

reiterated sequence, a minus-one-base frameshift error results; however, if the extra base is present in the DNA primer strand, a plus-one-base frameshift error results. Realignment of a slipped template or primer strand following a correct incorporation event produces a single base substitution mutation by a dislocation mechanism. Such errors are exemplified by the mutational hotspots at positions −36 (T→C) and 90 (G→A) (see Figure 1), which result from an extra base present in the template strand or primer strand, respectively (11).

In order to accurately compare the HIV-1 RT error rates at neutral and low pH, we have calculated an error rate per detectable nucleotide for each mutational hotspot (Table 5). This error rate includes hotspot mutations detected both in phage containing single DNA sequence chances (Figure 1) and in phage

Table 6. Effect of pH on HIV-1 RT mispairing-mediated base substitution error rates

Base Change	Mispair	Error rate per nucleotide[a] pH 6.2		pH 7.5		Ratio 7.5/6.2
G→A	G·T	1/50,000	(2)	1/3,000	(7)	17
A→T	A·A	1/104,000	(1)	1/11,000	(2)	9.4
G→C	G·G	1/43,000	(2)	1/4,600	(4)	9.3
T→G	T·C	1/104,000	(1)	1/22,000	(1)	4.7
T→C	T·G	1/7,600	(16)	1/2,200	(12)	3.4
G→T	G·A	1/16,000	(7)	1/12,000	(2)	1.3
C→T	C·A	1/12,000	(9)	1/24,000	(1)	0.50
overall		1/14,000		1/4,200		3.3

[a]Error rates per detectable nucleotide were calculated using 105 (pH 6.2) and 123 (pH 7.5) total independent occurrences. Number of occurrences of each mutational event is given in parentheses.

containing multiple DNA sequence changes (Table 4). DNA sequence changes in multiple mutants were considered as discrete mutational events if the errors were separated by more than ten nucleotides, and are indicated with asterisks in Table 4. This comparison demonstrates that polymerase error rates for minus-one-base frameshift errors, plus-one-base frameshift mutations, and dislocation errors are two- to 20-fold lower at pH 6.2 relative to pH 7.5 within homopolymeric runs of template A, T, G, or C (Table 5).

The magnitude of the pH suppression does not appear to be determined solely by DNA sequence, but is influenced also by the nature of the premutational DNA intermediate. For example, at the TTTT sequence at positions 70–73, the frequency of plus-one-base frameshift mutations is greatly reduced at low pH (>20-fold), relative to neutral pH, while the effect of low pH on the frequency of minus-one-base errors at the same site is negligible (<2-fold). A similar bias was measured for frameshift mutations occurring at the TTT sequence at positions 137–139 (Table 5). An interesting pH effect on misalignment-mediated mutations also is observable for mutations occurring at the AAAA run at positions 91–94. The error rate for minus-one-base frameshift errors is decreased >10-fold at this site, relative to neutral pH (Table 5). The production of this error requires the formation of a premutational intermediate containing an extra A base in the template strand. In contrast, a negligible pH effect is observed for the production of either plus-one-base frameshift errors within the AAAA run or G→A dislocation errors at position 90. Both of these events require a premutational intermediate that contains an extra T base in the DNA primer strand within this reiterated sequence.

A 10-fold decrease in the error rate for T→C mutations at position −36 was observed at low pH relative to neutral pH (Table 5). The production of this mutational hotspot by the HIV-1 RT has been suggested previously to result from a dislocation mechanism (10), wherein the error at position −36 is templated by the nucleotide at position −37. Template engineering of the M13mp2 viral DNA to change position −37 to an A residue revealed that the −36 hotspot for the HIV-1 RT is actually a composite of ~25% mispairing-mediated (T→C) mutations and ~75% dislocation-mediated (T→A) mutations (11). If low pH suppressed only the formation of mispairing-mediated errors, we can estimate an error rate of 1/200 for T→C mutations at pH 6.2 (e.g., 25% of the pH 7.5 error rate in Table 5), assuming such a bias in the types of error at position −36 also occurs with a C residue at position −37. Instead, we have observed an even lower error rate at pH 6.2 (1/500), consistent with the suppression of some dislocation errors at low pH.

Effect of pH on mispairing-mediated errors. We have also analyzed the effect of lowering reaction pH on the production of direct miscoding base substitution errors by the HIV-1 RT. In this analysis, those base substitution mutations previously identified (10) as resulting from template-primer dislocation (T→C at −36, T→C at −29, G→A at 90, C→A at 106, T→C at 121 in Figure 1 and Table 4) are excluded from the error rate calculations. At pH 7.5, the most prevalent types of base substitution errors produced by the HIV-1 RT are T→C and G→A transition mutations which result from T·G and G·T (template·primer) mispairing intermediates, respectively (Table 6). Among transversion errors, G→C mutations dominate at neutral pH. At low pH, a significant variation in the degree of suppression of specific types of base substitution errors was observed. The error rates for T→C and G→A transitions are three- and 17-fold lower, respectively, at pH 6.2 relative to pH 7.5, and approximately a 10-fold suppression in G→C and A→T transversion error rates were observed at the lower pH (Table 6). Low pH had no effect on the error rate of G→T and C→T mutations.

Effect of pH on HIV-1 RT termination probability during processive DNA synthesis

As a means of ascertaining whether pH affects the interaction of the HIV-1 RT with the DNA template-primer, we have analyzed the site-specific patterns of termination as a function of reaction pH along the M13mp2 DNA template. Figure 2 shows a time course of the DNA products resulting from synthesis reactions initiating from *lacZ* position 152 (panel A) or 105 (panel C). Using a molar excess of DNA template over enzyme, we observe a pattern of synthesis termination that is constant with respect to reaction time. At neutral pH, the DNA synthesis products produced by the HIV-1 RT ranged in size from 1 to ≥ 100 nucleotides in length, and the amount of synthesis termination is highly sequence-specific along the single-stranded template (Figure 2A and 2C, lanes 4–6). In order to describe these effects quantitatively we determined the termination probability for each site along the M13mp2 template DNA (Figure 2B and 2D). Termination probability measures the chance that the polymerase terminated DNA synthesis at a specific template site *versus* continued DNA synthesis beyond that site. At neutral pH, the termination probability per nucleotide varies 30-fold, from the strongest termination site (0.15 ± 0.094 at position 70) to one of the weakest termination sites (0.005 ± 0.004 at position 96).

Lowering the reaction pH had no effect on processive DNA synthesis by the HIV-1 RT, visualized as changes in either the

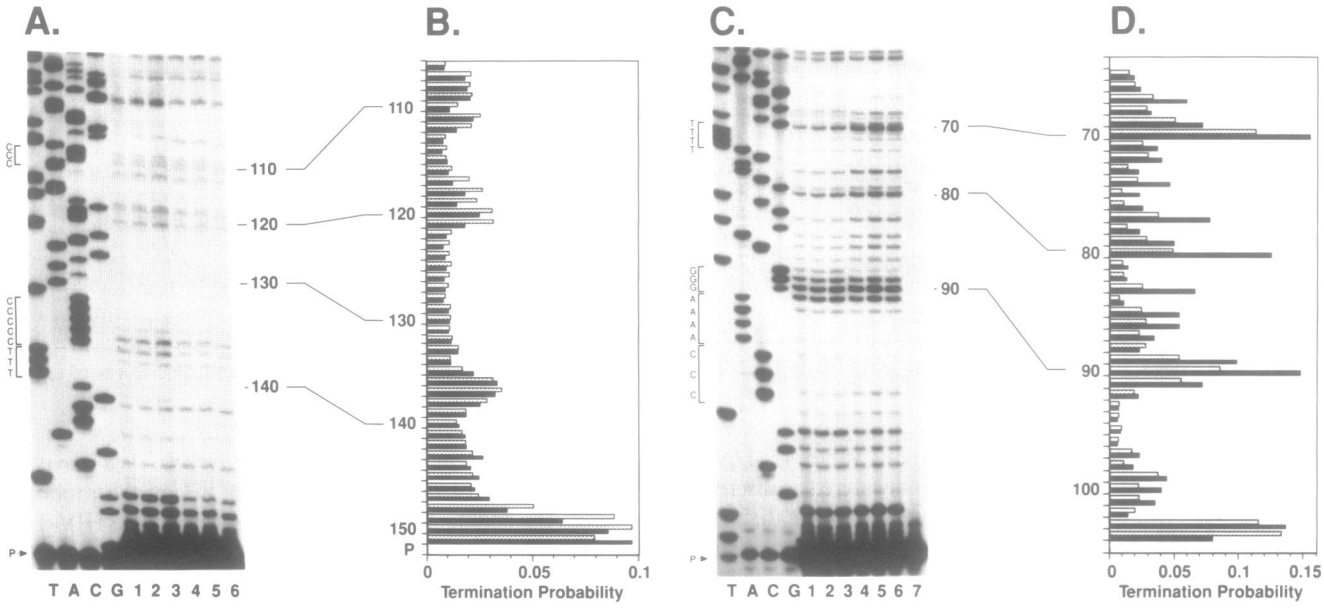

Figure 2. Effect of pH on processivity. **A** and **C**: Representative autoradiograms of the products of processive DNA synthesis on a primed M13mp2 single-strand template, separated by electrophoresis in a 20% denaturing polyacrylamide gel. Panel (A) shows synthesis reactions initiated from position 152; Panel (C) shows synthesis reactions initiated from position 105. Lanes labeled TACG are DNA size markers generated by dideoxy sequencing from the same template-primer. Lanes 1−3: Bis−Tris, pH 6.2 buffer, 5 min, 15 min and 30 min reaction times, respectively; lanes 4−6: Hepes, pH 7.5 buffer, 5 min, 15 min and 30 min reaction times, respectively; lane 7: zero enzyme control reaction Sequences noted along the left of the gel are mutational hotspots as determined in M13mp2 fidelity assays. Numbers along the right side of the gel indicate nucleotide positions along the *lacZ* gene. **B** and **D**: Quantitation of the termination probability per nucleotide for each template position, calculated from 30 min processivity reactions. Panel B shows the mean values of two independent reactions initiated from position 152; Panel D shows the mean of four independent synthesis reactions for each pH initiated from position 105. Solid bars: Hepes, pH 7.5; Stippled bars: Bis−Tris, pH 6.2.

overall length of products synthesized (Figure 2A and 2C, lanes 1−3) or the sequence specific termination pattern (Figure 2B and 2D). For example, under both pH conditions, similar strong termination sites were observed in the region *lac* 70−90 while little termination was detectable in the regions *lac* 121−134 or *lac* 93−96 (Figure 2). While a greater variability in termination probabilities between the two pH conditions was measured for synthesis initiated from *lacZ* position 105 (Figure 2D) than from *lacZ* position 152 (Figure 2B), no difference in termination probability greater than two-fold was reproducible in independent experiments at any of the 187 template sites examined at neutral and low pH (Figure 2, and data not shown).

Our laboratory previously has observed concomitant changes in HIV-1 RT error rates and in the probability that the polymerase terminated processive DNA synthesis within specific hotspot sequences (11). Therefore, we asked whether the observed pH-dependent decreases in HIV-1 RT error rates at these mutational hotspots result from pH-dependent alterations in termination patterns during processive synthesis. The distinguishing feature of the major HIV-1 RT frameshift sites at neutral pH is the high degree of termination within those target sequences relative to termination at other sites within the target gene (10). For example, at the GGG and AAAA mutational hotspots (positions 88−90 and 91−94, respectively), strong termination sites are observed for at least one nucleotide position within the homopolymeric run, whilst little termination is observed in the adjacent mutational coldspot (CCC run at positions 95−97) (Figures 1 and 2C).

We have measured no significant effect of reaction pH on the relative termination pattern of the HIV-1 RT at six frameshift hotspot sequences (GGG, 88−90; TTTT, 70−73; CCCCC, 132−136; CCC, 106−108; AAAA, 91−94; and TTT, 137−139). For example, at low pH, sites 88−90 and 91−94 continue to be sites of strong termination, whereas weak termination is observed at the CCC sequence at position 95−97 (Figure 2). In order to compare termination probabilities with frameshift error rates within homopolymeric sequences, we have summed the termination values for each nucleotide position where termination would allow formation of a frameshift intermediate with at least one correct 3′-terminal basepair. For example, at positions 70−73, termination at nucleotide 70 is not included in the summation for minus-one-base frameshifts. Comparing the qualitative ranking of hotspot sites from those of highest to those of lowest termination probability demonstrates that low pH does not alter the relationship between termination probability and frameshift error rates that was described previously for neutral pH (10, 11). Thus, at both pH conditions, the site of greatest termination was within the GGG sequence at positions 88−90 with regard to minus-one-base frameshift errors (0.14 ± 0.026 at pH 6.2; 0.25 ± 0.18 at pH 7.5) and within the TTTT sequence at positions 70−73 with regard to plus-one-base frameshift errors (0.17 ± 0.05 at pH 6.2; 0. 23 ± 0.16 at pH 7.5).

DISCUSSION

We have characterized an increase in the fidelity of DNA synthesis catalyzed by various polymerases for several types of errors at low reaction pH (pH 6−6.5). A pH-dependent increase in base substitution fidelity at low pH, relative to neutral pH, has been measured for three distinct families of DNA polymerases: Family A, Taq polymerase (7) and exonuclease-

deficient Klenow polymerase (8); Family B, catalytic form of human DNA polymerase α; and the reverse transcriptase family, HIV-1 RT (Table 1). The significant improvement in the HIV-1 RT fidelity in the forward mutation assay (Table 2) afforded us the opportunity to study the generality of the pH effect on specific types of mutations in more detail. A decrease in the error rate for HIV-1 RT mutations produced by three distinct pathways for errors during DNA synthesis was observed at low pH at several template positions: base substitutions initiated by misincorporation (direct miscoding) (Table 6), base substitutions initiated by template-primer misalignment (dislocation), and one-base addition or deletion errors initiated by template-primer slippage (frameshifts) (Table 5).

The absolute magnitude of the pH suppression of errors is dependent upon the precise DNA sequence and the type of error considered. The most dramatic demonstration of this can be seen by comparing HIV-1 RT error rates for mutations at the homopolymeric run at positions 91−94 with those at positions 70−73 (Table 5). At both sites, a large (10-fold) decrease in error rate at low pH was measured for mutations involving a misaligned template-primer containing an extra base in the A strand (minus-one frameshift at 91−94 or plus-one frameshift at 70−73), while a negligible effect was observed when the mutations involved an extra base in the T strand (plus-one frameshift at 91−94, G→A at 90, or minus-one frameshift at 70−73). While the numbers of mutants in this collection is relatively small, the comparison provides a starting point from which to model the effect of local DNA sequence context and structure on polymerase error rates at low pH.

Sequence specificity of the pH effect may also operate on mispairing-mediated base substitution mutations. We suggested previously for the exonuclease-deficient Klenow polymerase that G·A and A·C mispairs might be stabilized by protonation of the adenine at low pH to account for the lack of a pH effect on the misinsertion frequency of G·dATP and A·dCTP mispairs (8). Protonation of adenine residues at low pH (19) might also explain the similar HIV-1 RT error rates for G→T and C→T mutations observed at pH 7.5 and pH 6.2 (Table 6). Alternatively, the lack of a pH effect on these two mutations may be an experimental artifact, resulting from damage to the single-stranded DNA template used in the gap-filling reactions. The experimental protocol used to construct the gapped template molecules is designed to minimize the potential for depurination and deamination events (14). Thus, while the possibility of unintentional DNA damage cannot be ruled out by the current data, the HIV-1 RT base substitution data also are consistent with protonation of G·A and C·A mispairs at pH 6.2 counteracting a general increase in polymerase fidelity at lower pH, the net result of which is similar G→T and C→T error rates at neutral and low pH. The differences in the magnitude of the pH-dependent suppression of base substitution error rates among the various polymerases studied (Table 1) may result from differences in the types of errors produced by the individual polymerases, together with the sequence specificity of the pH effect.

Our laboratory previously has described a correlation between the position of HIV-1 RT mutational hotspots and the occurrence of strong termination sites for DNA synthesis within that homopolymeric sequence (10). This correlation was strengthened by the observation that changes in DNA sequence flanking the homopolymeric runs altered termination probabilities (12) and were accompanied by changes in the error rates for frameshift mutations within those sequences (11). One model for the production of mutations via template-primer misalignments is that the termination of DNA synthesis within a homopolymeric sequence is correlated with the probability that a misaligned, premutational intermediate will be formed within that sequence. Once formed, the detection of a phenotypically selectable mutation requires the resolution of this premutational intermediate by continued DNA synthesis (extension) by the polymerase from the misaligned 3′-primer-terminus. We have analyzed our data to determine whether pH is affecting HIV-1 RT error rates by changing termination patterns during processive DNA synthesis. We have observed no change in the probability that the HIV-1 RT terminates synthesis at six specific homopolymeric sequences as a function of pH (Figure 2), nor have we observed a difference in the distribution of HIV-1 RT mutations (i.e., presence and location of 'hotspots') at low pH relative to neutral pH (Figure 1). Therefore, we suggest that pH is not lowering HIV-1 RT frameshift error rates by decreasing the probability that a misalignment will occur at a specific DNA sequence. Instead, we suggest that low pH is reducing the frequency with which the misaligned premutational intermediates are fixed into mutational events, either by decreasing the stability of the misaligned DNA structure or by decreasing HIV-1 RT extension synthesis from a misaligned premutational intermediate. This model can be tested experimentally by determinations of the relative $K_{D(DNA)}$ values for the HIV-1 RT, as well as by a direct measurement of the steady-state efficiency of HIV-1 RT extension synthesis from a correctly paired *versus* misaligned DNA template as a function of pH. Using a steady-state approach, we have demonstrated previously that the ability of the exonuclease-deficient Klenow polymerase to bind to and/or continue DNA synthesis from 3′-terminal mispairs is dramatically reduced at low pH, accounting for the increased base substitution fidelity of the enzyme at low pH (8).

Our analysis of the pH effects on the termination patterns of the HIV-1 RT differ from those previously observed for the exonuclease-deficient Klenow polymerase, in that the processivity of the Klenow polymerase was enhanced at specific template sequences at low pH (8). One explanation for the observed difference between the two enzymes is that the pH-dependent changes in HIV-1 RT termination probability are too subtle to be detected in our polyacrylamide gel assay. This would be particularly true for a pH effect which lowers termination at sites where the termination probability at neutral pH is already low (less that 2%). Alternatively, the parameters which dictate whether the polymerases will remain bound or dissociate during a single cycle of DNA synthesis may be distinct for the two enzymes (21, 22), and/or the proteins may have different biochemical sensitivities to changes in reaction pH at a critical step in the pathway.

What is the significance of our observed pH affect on purified polymerases *in vitro* to the accuracy of DNA replication *in vivo*? The intracellular pH of eukaryotic cells is not constant (24). Activation of the transmembrane Na^+/H^+ antiport by various growth regulatory pathways can increase the intracellular cytoplasmic pH by 0.1−0.2 pH units (25, 26), and changes in pH may be envisioned to occur in the nucleus during DNA replication. Thus, the dynamic intracellular environment could play a role in the regulation of polymerase error rates. In addition, the effects of low pH on fidelity *in vitro* may mimic a state of the polymerase induced by assembly of a macromolecular replication complex *in vivo*.

We propose that pH affects an aspect of the DNA−protein

interaction common among polymerases, due to the significant suppression of errors which arise by different premutational intermediates and of errors resulting from distinctly different classes of polymerases. The Klenow polymerase, human DNA polymerase α, and the HIV-1 RT have homologous catalytic domains, as defined structurally or functionally (4–6). Other such small, conserved structures which are important in correct positioning of a dNTP substrate within the polymerase active site or binding of template DNA may become apparent as the crystal structures of available polymerases are refined and new polymerase structures are solved.

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