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Factors affecting fidelity of DNA synthesis during PCR amplification of $d(C\text{-}A)_n{\cdot}d(G\text{-}T)_n$ microsatellite repeats

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ABSTRACT

The susceptibility of microsatellite DNA sequences to insertions and deletions *in vivo* makes them useful for genetic mapping and for detecting genomic instability in tumors. An *in vitro* manifestation of this instability is the production of undesirable frameshift products during amplification of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ microsatellites in the polymerase chain reaction (PCR). These products differ from the primary product by multiples of 2 nucleotides. We have tested the hypothesis that factors known to affect the fidelity of DNA synthesis may affect $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ frameshifting during the PCR. Neither modifications of pH, dNTP concentration, and Mg^{++} concentration using *Amplitaq*, nor the use of thermophilic DNA polymerases including *UlTma*, *Pfu*, *Vent* and *Deep Vent* significantly decreased the production of frameshift products during amplification. However, 3′→5′ exonuclease activity in thermophilic DNA polymerases inhibited the accumulation of PCR products containing non-templated 3′ terminal nucleotides. Most interestingly, extension temperatures of 37°C during amplification using the thermolabile DNA polymerases Sequenase 1.0, Sequenase 2.0, and 3′→5′ exonuclease-deficient Klenow fragment greatly decreased the production of frameshift products. This method can improve the resolution of heterozygous or mutant $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ alleles differing in size by one or two repeat units.

INTRODUCTION

Microsatellites are simple, six base pair or smaller tandem repeats found in all eukaryotic genomes examined to date (1). They are useful as genetic markers because they are widely dispersed in the genome, polymorphic in length, and frequently heterozygous (2). PCR-based genotyping of microsatellite loci is used for genetic mapping (3), kinship studies (4), and studying genomic instability in cancer (5). The most abundant of these sequences in the human genome, having an estimated copy number between 50 000 and 100 000 (6), is the dinucleotide repeat $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$, with the range of n being ~15–30 (2,7). These sequences are primarily found in non-coding DNA, can affect the frequency of recombination (8,9), and may enhance gene expression (10).

The susceptibility of microsatellites to insertions and deletions contributes to the generation of allelic diversity during organismal evolution (11) and makes microsatellites useful for the detection of genomic instability in cancer cells (5). As suggested by the term 'replication errors' used to describe microsatellite instability in cancer (12), the potential role of polymerase errors in this instability makes it important to study factors that may affect the fidelity of DNA polymerases during synthesis of these sequences. The fidelity of DNA synthesis is known to be affected by a variety of factors including polymerase structure, 3′→5′ exonuclease activity, dNTP and divalent cation concentrations, and pH (13,14). Here, we study the effect of some of these factors on the fidelity of synthesis of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ microsatellite sequences during the polymerase chain reaction.

Genotyping $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat loci is typically done by PCR amplification (15,16) of DNA regions containing the repeat, followed by electrophoretic sizing on denaturing polyacrylamide gels and autoradiography. There are two types of undesirable secondary product associated with dinucleotide repeat PCR. The first, mechanistically associated with poor fidelity of synthesis of dinucleotide repeat sequences, consists of frameshift products commonly called 'laddering artifact', or 'shadow bands' (17,18). (For simplicity, we use the term 'frameshift' to indicate *in vitro* insertions and deletions of repeat units even though dinucleotide sequences are generally non-coding.) These products are generally shorter than the primary amplification product (length n) by multiples of 2 nucleotides (nt) (n–2, n–4,...), decrease in intensity with distance from the primary product, and are associated with alterations in the number of repeat units (18). The production of this frameshift product is partially locus dependent. Frameshift products hinder the interpretation of genotypes when two alleles differ in size by only one or two repeat units. Another type of secondary PCR product, not specific to dinucleotide repeats, consists of the non-templated addition of a nucleotide to the 3′ terminus (19–21). This causes the

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amplification product to migrate 1 nt slower than expected, and must be taken into account when cloning PCR products (22).

Two classes of mechanisms for the generation of frameshift product in dinucleotide PCR have been proposed. The first class invokes 'slipped strand mispairing' during DNA synthesis (23,24), in which a non-base-paired DNA loop consisting of one or more repeat units forms within the primer–template structure. A second class of mechanisms involves recombination. Here, the polymerase dissociates prematurely to leave behind a truncated product which can reanneal out of register in subsequent rounds of synthesis (25); alternatively, the polymerase can switch to a different template during synthesis (26). Frameshift products generated in previous cycles may then serve as templates in subsequent rounds of DNA synthesis.

In this study we have tested whether factors known to affect the fidelity of DNA synthesis *in vitro* (13,14) can affect the fidelity of PCR amplification of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ microsatellites. Negligible decreases in frameshift products were obtained by varying 3′→5′ exonuclease activity, pH, [Mg^{++}], [dNTP] processivity, *Escherichia coli* single-stranded binding protein (SSB) and formamide. By comparing proofreading proficient and deficient forms of two polymerases, and by regulating proofreading activity through dNTP concentration, we showed that the accumulation of PCR product with a 3′ non-templated nucleotide was inhibited by 3′→5′ exonuclease activity.

Finally, based upon the well-known denaturing effect of elevated temperatures on DNA, we hypothesized that lower temperatures of DNA synthesis in the PCR would lessen denaturation and thereby decrease loop formation during the extension step of dinucleotide repeat PCR. We have found that amplification using thermolabile DNA polymerases at lower temperatures of DNA synthesis results in significantly less frameshifting during $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat PCR. Our data are consistent with a slipped strand mispairing model of microsatellite frameshifting during the PCR.

MATERIALS AND METHODS

DNA isolation and primers

Human DNA was extracted from formalin-fixed, paraffin-embedded normal tissue after scraping 15 μM thick sections from slides using sterile blades. Deparaffinization and extraction of DNA was performed using a modification of previously described methods (27). Briefly, minced sections were incubated at 48°C in 400 μl extraction buffer containing 500 mM Tris–HCl pH 9.0, 1% SDS, 20 mM EDTA, 10 mM NaCl and 0.5 mg/ml proteinase K for 3 days with occasional mixing. Nucleic acids were then extracted with phenol/chloroform and chloroform, followed by ethanol precipitation and resuspension in TE buffer, pH 8.0. Human Mappair primer set D2S136 from Research Genetics (Huntsville, AL) was used in the amplification reactions at a final concentration of 0.05 μM for each primer. The 5′-end of the 'forward' primer of each primer pair was labelled with γ-^{33}P or γ-^{32}P using T4 polynucleotide kinase. PCR amplification using Stoffel fragment and Δ*Taq* with the primer set for APOC2 was performed under the same reaction conditions (data not shown).

DNA amplification using thermophilic DNA polymerases

Amplification by thermophilic DNA polymerases was achieved using ~200 ng genomic DNA in 10 μl reactions in the presence of 200 μM dNTPs. Additional reaction components specific to each polymerase were used according to each manufacturer's recommendations. *Amplitaq*, Stoffel fragment and *UlTma* were obtained from Perkin-Elmer Cetus (Foster City, CA); *Vent exo*$^+$/*exo*$^-$ and *Deep Vent exo*$^+$/*exo*$^-$ (exo$^+$, 3′→5′ exonuclease proficient; exo$^-$, 3′→5′ exonuclease deficient) from New England Biolabs (Beverly, MA); Δ*Taq* from US Biochemical/Amersham (Arlington Heights, IL); and *Pfu* from Stratagene (La Jolla, CA). PCR was initiated by addition of DNA polymerase after 2 min of an initial 10 min denaturation at 93°C ('hot start'). This was followed by cycling 27 times in a Perkin-Elmer Cetus Model 480 thermal cycler through a program of melting at 93°C for 15 s, annealing at 55°C for 45 s, and extension at 72°C for 30 s. The last step was extension for 5 min at 72°C. An exception to the aforementioned reaction parameters was an extension time of 15 s for reactions containing *Vent exo*$^+$ and *Deep Vent exo*$^+$. Amplification products were mixed 1:1 with 98% formamide and dye, separated by 6% denaturing, polyacrylamide gel electrophoresis, and visualized by autoradiography and/or Molecular Dynamics (Sunnyvale, CA) phosphorimager analysis.

DNA amplification using thermolabile DNA polymerases

Genomic DNA (~2 μg) was amplified in a 100 μl reaction using either Sequenase 1.0, Sequenase 2.0 (28) or the 3′→5′ exonuclease deficient form of Klenow fragment from Amersham (Arlington Heights, IL) (16). Sequenase 1.0 and Sequenase 2.0 are modified forms of the T7 DNA polymerase with deficient and absent 3′→5′ exonuclease activity, respectively (29,30). Sequenase 1.0 and 2.0 amplification reactions contained 100 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 200 μM dNTPs (Pharmacia, Piscataway, NJ) and 0.1 mg/ml acetylated BSA (New England Biolabs, Beverly, MA). Klenow exo$^-$ DNA polymerase amplification reaction contained 20 mM Tris–HCl pH 7.6, 50 mM NaCl, 10 mM $MgCl_2$, 1.5 mM dNTPs and 2 mM DTT. Amplification was achieved by repeating the following four steps 27 times: (i) heat denaturation at 95°C for 1 min, (ii) primer annealing at 37°C for 30 s, (iii) addition of 1 U DNA polymerase, and (iv) DNA synthesis at 37°C for 1 min. The reaction concluded with extension at 37°C for 3 min followed by heat denaturation at 95°C for 3 min. All incubations were performed using water baths to minimize temperature ramping effects. Products were separated and visualized as described above.

RESULTS

DNA polymerase fidelity is dependent on both polymerase characteristics and reaction conditions (31). We tested different polymerases in the PCR in order to identify which determinant(s) might affect the fidelity of synthesis of dinucleotide repeat sequences. By examining the various polymerases, we were able to look at the effects of temperature, 3′→5′ exonuclease activity, inherent polymerase accuracy, and processivity on the fidelity of amplification of dinucleotide repeat loci.

Fidelity of DNA synthesis is greater at 37°C than at 72°C during amplification of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeats

We hypothesized that the high temperatures used during DNA synthesis in the PCR increase denaturation of the 3′ terminus of the nascent strand, which can then misalign during reannealing to produce frameshift products (32). Testing this hypothesis requires extension truly occurring at the lower temperature, and best

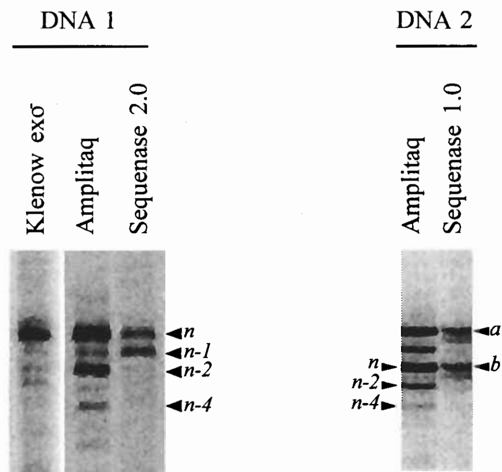

Figure 1. Amplification of a human $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ sequence by thermolabile DNA polymerases at locus D2S136 using genomic DNA. DNA from individual 1 is homozygous at this locus showing a primary allele *n* corresponding to a length of ~95 nt. DNA from individual 2 is heterozygous at this locus and shows the two primary alleles corresponding to lengths of 107 ('*b*') and 111 ('*a*') nt. Amplification by *Amplitaq* was run for comparison to Klenow fragment, Sequenase 2.0 and Sequenase 1.0 as indicated.

excludes the possibility of premature DNA synthesis due to annealing that occurs as the machine ramps from denaturing to 'annealing' and 'extension' temperatures during normal PCR conditions. Thus, to examine the role of reaction temperature with respect to DNA polymerase fidelity, $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ sequences were amplified at 37°C using heat-labile DNA polymerases. Denaturation at each cycle inactivates the thermolabile polymerases, guaranteeing extension at 37°C after new enzyme is added.

DNA samples were amplified using Klenow fragment exo$^-$, Sequenase 1.0 and Sequenase 2.0 (Fig. 1). All three thermolabile polymerases generated an amplification product *n* (the same size as the primary *Amplitaq* product) unaccompanied by significant frameshift product. Klenow fragment exo$^-$ produced a single primary band (*n*) along with some non-specific background in other portions of the lane (not shown). Sequenase 2.0 produced a single primary band (*n*) accompanied by a band that migrates 1 nt (*n–1*) faster than the primary product (*n*). Amplification with Sequenase 2.0 at a total of three loci and with three different DNA samples yielded similar results (data not shown). The relative intensity of *n* to *n–1* product varied slightly in a locus-specific manner.

We attribute the *n–1* and *n* bands to blunt-ended product and product with a 3′ non-templated nucleotide, respectively, for several reasons. First, Sequenase 2.0 product treated with Klenow fragment exo$^-$ [known to add a 3′ non-templated nucleotide (20)] showed a decrease in Sequenase *n–1* product concomitant with an increase in *n* product (data not shown). This is consistent with Klenow fragment addition of a 3′ non-templated nucleotide to a blunt-ended substrate rather than a 3′ overhang. Secondly, the Sequenase *n* band comigrated with the *Amplitaq n* band, which corresponds to a 3′ overhang product. *Amplitaq* polymerase is also known to very efficiently add a 3′ non-templated nucleotide (19). The presence of 3′ non-templated nucleotides was confirmed by the reduction in *Amplitaq n* band upon treatment with *Pfu* DNA polymerase, whose exonuclease is known to remove 3′ non-templated nucleotides (33). Together, these observations suggest that the Sequenase product consists of blunt-ended product (*n–1*) and product with a 3′ non-templated nucleotide added (*n*).

Amplification with Sequenase 1.0 using DNA 2 also produced primary *n* bands accompanied by fainter *n–1* products (DNA 2, Fig. 1). The genotype obtained is much clearer than that obtained using *Amplitaq*. The DNA is heterozygous for this locus, having two alleles *a* and *b* that differ in length by 4 nt. For *Amplitaq*, the band corresponding to the lower '*b*' allele labelled *n* contains primary product contaminated by *n–4* frameshift product from the upper '*a*' allele.

The results for the three thermolabile polymerases are not likely due to pH since the final pHs are within 0.1 U of 7.75 when adjusted for reaction temperature. Furthermore, the fidelity of PCR is not significantly different in this pH range (34).

In an attempt to develop a more practical application of these findings we tried to find conditions that would overcome two problems. The first problem is that Amplitaq activity is poor at low temperatures [~1.5 nt/s at 37°C (35)]. Based upon phosphorimager analysis of a primer extension experiment on a single-stranded template at 37°C, we decided to use a 9 min annealing/extension time and high concentration of enzyme. The second problem is that premature priming and extension can occur during ramping down from denaturing and a low extension temperatures. We used water baths to minimize this effect. Despite these precautions, *Amplitaq* produced an abundance of background bands rather than a clear genotype (data not shown).

3′ non-templated nucleotide additions are removed by 3′→5′ exonuclease activity

The 3′→5′ exonuclease activity of polymerases reduces the accumulation of frameshift and misinsertion mutations (36). In order to determine whether 3′→5′ exonuclease activity can increase the fidelity of synthesis of dinucleotide repeat sequences during the PCR, DNA was amplified using exonuclease-proficient *Pfu* and exo$^+$/exo$^-$ pairs of thermophilic DNA polymerases *Vent* and *Deep Vent*. These polymerases were compared with proofreading-deficient *Amplitaq* DNA polymerase, the enzyme most commonly used in the PCR.

The presence or absence of 3′→5′ exonuclease activity had no effect on the generation of frameshift products in $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ microsatellite PCR (Fig. 2). However, it was notable that *Amplitaq* and other polymerases lacking 3′→5′ exonuclease activity produced primary products (*n*) 1 nt longer than those of the exonuclease competent polymerases. The generation of the longer product (*n*) is attributed to terminal transferase-like activity that adds a non-templated nucleotide to 3′ termini of DNA; conversely, products one nucleotide shorter (*n–1*) reflect removal of the non-templated nucleotide by 3′→5′ exonuclease activity. This phenomenon was previously observed using Klenow polymerase in primer extension assays (20). Here this effect is extended to the PCR using thermophilic DNA polymerases. Thus, a comparison of products generated by the 3′→5′ exonuclease-proficient and deficient forms of *Vent* and *Deep Vent* polymerases (Fig. 2) directly implicates the 3′→5′ exonuclease activity in removing 3′ non-templated nucleotides.

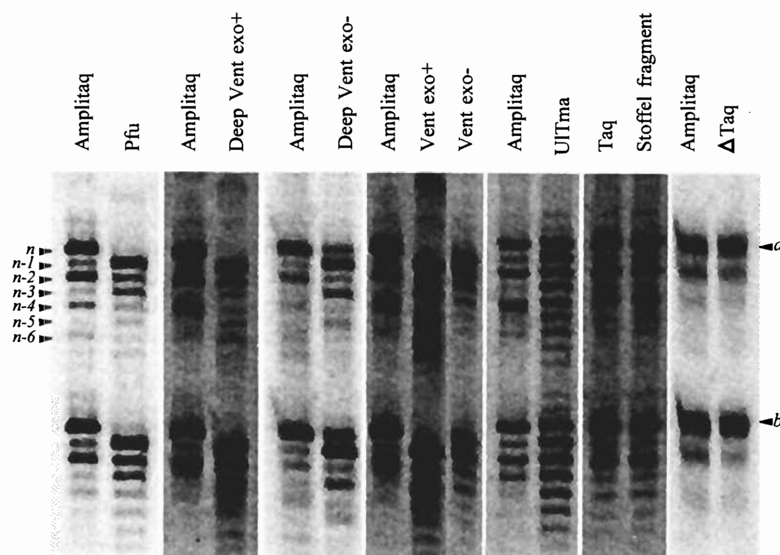

Figure 2. Effects of 3′→5′ exonuclease activity and processivity on 3′ non-templated nucleotide addition and frameshift production during thermostable polymerase PCR. This individual is heterozygous at locus D2S136; two primary alleles corresponding to lengths of ~95 (‘*b*’) and 107 (‘*a*’) nt are present in each lane. The thermophilic DNA polymerases (*Pfu, Deep Vent exo*$^+$, *Deep Vent exo*$^-$, *Vent exo*$^+$, *Vent exo*$^-$, *UlTma*, Δ*Taq* and Stoffel fragment) were used to generate the products shown. *n* designates the longest primary product; the shorter products are designated by the number of nucleotides they are shorter than the *n* product.

Removal of 3′ non-templated nucleotide products by *UlTma* is proportional to dNTP-concentration-dependent 3′→ 5′ exonuclease activity

High levels of dNTPs during DNA synthesis *in vitro* are known to inhibit 3′→5′ exonuclease activity (37). We hypothesized that increasing 3′→5′ exonuclease activity by decreasing dNTP concentrations in the PCR would decrease the accumulation of product with non-templated 3′ nucleotides. To test this hypothesis, we used 3′→5′ exonuclease-proficient *UlTma* DNA polymerase in the PCR over a range of dNTP concentrations (Fig. 3). The *UlTma* PCR products have lengths of *n*, *n–1*, *n–2*, *n–3*, *n–4*, *n–5*, ... The *n* band is the full-length product with an additional 3′ non-templated nucleotide, and the *n–2*, *n–4*, ... bands its frameshift products. The *n–1* band is the full-length, blunt-ended product, and the *n–3*, *n–5*, ... bands its frameshift products. At 200 μM (standard reaction conditions) or 100 μM dNTPs, where the exonuclease activity is partially inhibited, the primary PCR product comigrates with the primary *Amplitaq* PCR product (*n*) corresponding to the presence of a 3′ non-templated nucleotide. At 50, 25, 12 and 6 μM dNTP concentrations where the proofreading activity increases, the proportion of blunt-ended product (*n–1*, *n–3*, ...) increases, with a concomitant decrease in product containing 3′ non-templated nucleotides (*n*, *n–2*, ...). At 3 μM dNTP concentrations and below, predominantly blunt-ended products (*n–1*, *n–3*, ...) were obtained. These data indicate that the generation of blunt-ended PCR product by *UlTma* is inversely related to dNTP concentration, which in turn is consistent with inhibition of the 3′→5′ exonuclease activity by higher dNTP concentrations.

Further evidence to support our interpretation of the band pattern was provided by treatment of *UlTma*, *Pfu* and *Amplitaq* PCR products with Klenow fragment exo$^-$ as described in the previous section. The results showed that only those products thought to be blunt-ended (*n–1*, *n–3*, ...) had a non-templated nucleotide efficiently added to the 3′ terminus (data not shown).

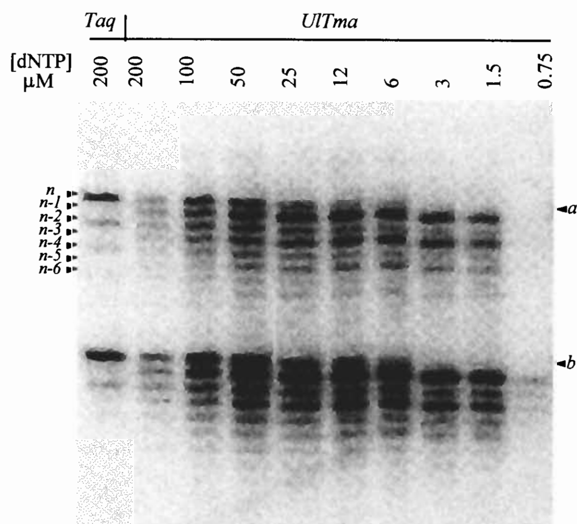

Figure 3. Effect of dNTP concentration on 3′→5′ exonuclease activity of *UlTma* DNA polymerase in the PCR. This individual is heterozygous at this locus; two primary alleles corresponding to lengths of ~95 (‘*b*’) and 107 (‘*a*’) nt are present in each lane. *Amplitaq* was run for size comparison. dNTP concentrations for *UlTma* range from 0.75 to 200 μM as indicated.

Decreased processivity does not affect the fidelity of dinucleotide repeat sequence amplification

In order to examine the potential role of processivity in the fidelity of PCR amplification of dinucleotide repeats, products generated by *Amplitaq* were compared to those of Stoffel fragment and Δ*Taq* DNA polymerases (Fig. 2). Stoffel fragment and Δ*Taq* (*KlenTaq*) are truncated forms of *Amplitaq* which are deleted for 5′→3′ exonuclease activity. The deletion of 5′→3′ activity in the Stoffel fragment results in a 10-fold decrease in processivity

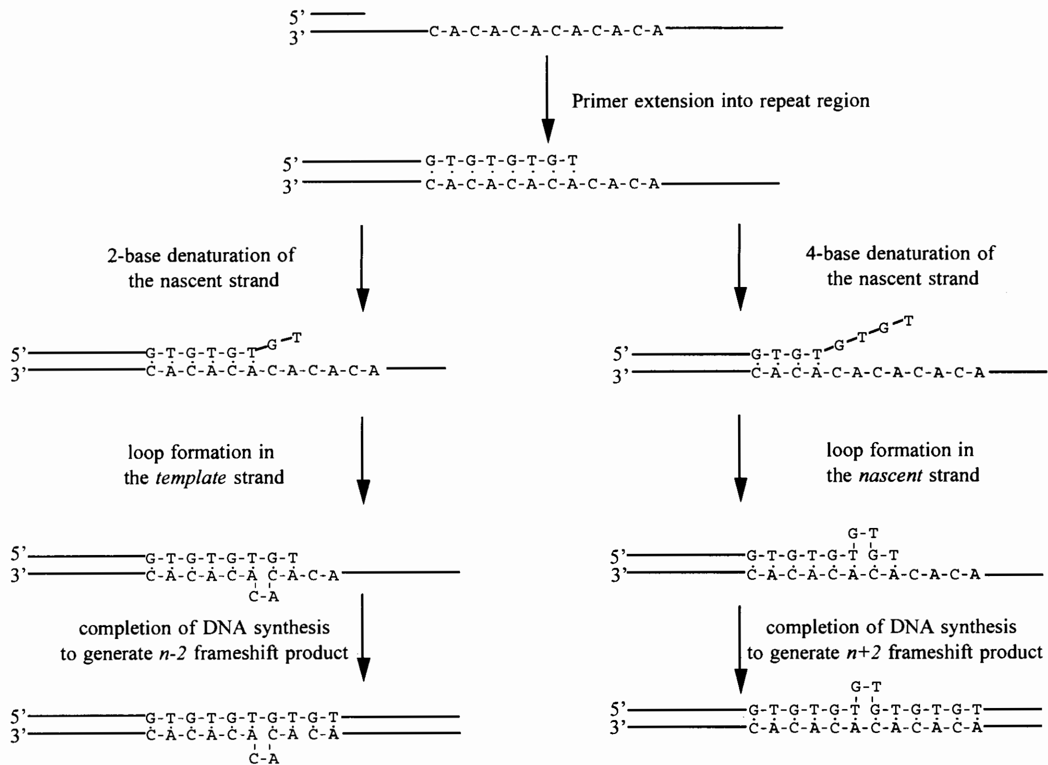

Figure 4. A model for slipped strand mispairing during the PCR showing why *n–2* frameshift products are favored over *n+2* products. Upon extension of the nascent strand into the repeat region, temperature-dependent dissociation of the 3′-end occurs. The nascent strand then reanneals out of register. As shown on the left versus the right, denaturation of only two bases is required to produce *n–2* frameshift product, while denaturation of four bases is required to produce *n+2* products. For simplicity, only a subset of the usual number of repeat units are shown.

during DNA synthesis (38). We found that amplification using the Stoffel fragment and Δ*Taq* yielded primary and secondary products identical to *Amplitaq*, indicating that decreased processivity does not significantly affect the generation of frameshift products. At other loci, Δ*Taq* showed product with lengths of *n*, *n–1*, *n–2*, *n–3*, *n–4*, ..., which may be due to less efficient 3′ terminal non-templated nucleotide addition (data not shown).

DISCUSSION

The fidelity of DNA synthesis is affected by the polymerase, 3′→5′ exonuclease activity, and other factors (13,31). The production of frameshift products associated with poor fidelity during the widely-used PCR amplification of dinucleotide repeat sequences can be a significant problem in determining heterozygous or mutant genotypes where alleles are of similar size. This problem led us to test different DNA polymerases and reaction conditions for their effect on the fidelity of amplification of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ sequences.

Our most striking reduction in frameshift products was obtained using thermolabile polymerases under amplification conditions where DNA synthesis occurred at 37°C rather than 72°C as typically used for thermophilic polymerases (Fig. 1). This improvement in polymerase fidelity during DNA synthesis of dinucleotide repeat sequences may be attributed either to the lower temperatures during synthesis or to the identity of the polymerase. Our favored explanation is the temperature of extension rather than the identity of the polymerase for two reasons. First, the tendency of duplex DNA to partially denature is known to increase with temperature, which in turn facilitates the formation of loops with misaligned 3′ termini to generate frameshift products. Second, *Amplitaq* and Klenow fragment exo$^-$ yielded discordant patterns of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ repeat amplification products despite their strong structural and functional similarities. The high degree of amino acid homology and identity within the polymerase domains of these enzymes (39) has recently been shown to translate into nearly identical three-dimensional crystallographic structures of the apoproteins (40,41). This structural overlap includes residues believed to be directly involved in catalysis. Functionally, the two polymerases have equivalent error rates for minus-one-base frameshift errors (~1/20 000) at a monotonous repeat sequence at their respective reaction temperatures (42). It thus appears reasonable to believe that the high temperature of DNA synthesis during PCR adversely affects the DNA structure within nascent dinucleotide repeat sequences, causing an increase in the proportion of misaligned primer–template DNA structures available to the polymerase. Our model, showing how polymerase fidelity is dependent upon the temperature of synthesis, is a slipped strand mispairing model (24,43; Fig. 4). We do not consider recombination models here since they predict the formation of intermediate size alleles in normal heterozygotes, which is not seen.

There are two potential explanations of the curious fact that frameshift products are typically shorter, not longer, than the expected size. First, one can argue that the full length of the template strand gives that strand more opportunity to buckle and

productively misalign in the repeat region. As synthesis of the repeat region proceeds during each cycle, the time-averaged length of the nascent strand is of course shorter than full-length, giving it less opportunity to buckle and misalign to yield longer product. A thermodynamic explanation is that in dinucleotide sequences, insertion frameshifts require two more base pairs to be disrupted than deletion frameshifts, illustrated in Figure 4. This explanation is an extension of a model proposed by Kunkel to explain the 10-fold higher incidence of minus-one-base frameshifting over plus-one-base frameshifting in mononucleotide runs (44).

Despite the general principle that polymerases possessing 3′→5′ exonuclease activity greatly increase the fidelity of DNA synthesis (45), the characteristic secondary products of dinucleotide repeat amplification did not appear to be affected by the presence or absence of 3′→5′ exonuclease activity. This result was particularly surprising in light of the profound effect of 3′→5′ exonuclease activity on single base frameshift fidelity (36). A variety of other conditions including pH, dNTP concentration, and Mg^{++} concentration are known to affect the fidelity of DNA synthesis; however, optimization of these parameters did not significantly affect the proportion of amplification frameshift products.

Previous studies using primer extension reactions have shown that DNA polymerases lacking 3′→5′ exonuclease activity frequently add 3′ non-templated nucleotides (19–21). Our experiments extend these findings to the PCR. First, two exonuclease-proficient and deficient forms of two thermostable DNA polymerases were used to show that 3′→5′ exonucleolytic proofreading is associated with a lesser accumulation of non-templated nucleotide addition products during the PCR. Secondly, we showed that the level of 3′→5′ exonuclease activity modified by dNTP concentration was inversely proportional to the accumulation of non-templated terminal nucleotide addition products. These results support the hypothesis that 3′→5′ exonuclease activity removes 3′ non-templated nucleotides during the PCR.

We have found that the maximum fidelity of DNA synthesis is obtained by amplification of $(dC\text{-}dA)_n \cdot (dG\text{-}dT)_n$ by thermolabile DNA polymerases at 37°C. This application may be used in instances where it is important to resolve alleles that differ in size by only one or two repeat units. The potential benefits may apply in mapping studies and in the study of microsatellite instability (5). The use of this method is limited by the extra time and cost of using thermolabile, rather than thermostable enzymes, but is less time-consuming than methods involving linear amplification (17). Related investigations may yield other ways to increase the fidelity of microsatellite and other DNA amplification during the PCR.

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