Effects of primer–template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies

S.Kwok, D.E.Kellogg, N.McKinney, D.Spasic[1], L.Goda[1], C.Levenson[1] and J.J.Sninsky
Department of Infectious Diseases and [1]Department of Chemistry, Cetus Corporation, Emeryville, CA, USA

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ABSTRACT

We investigated the effects of various primer-template mismatches on DNA amplification of an HIV-1 *gag* region by the polymerase chain reaction (PCR). Single internal mismatches had no significant effect on PCR product yield while those at the 3′-terminal base had varied effects. A:G, G:A, and C:C mismatches reduced overall PCR product yield about 100-fold, A:A mismatches about 20-fold. All other 3′-terminal mismatches were efficiently amplified, although the G:G mismatches appeared to be more sensitive to sequence context and dNTP concentrations than other mismatches. It should be noted that mismatches of T with either G, C, or T had a minimal effect on PCR product yield. Double mismatches within the last four bases of a primer-template duplex where one of the mismatches is at the 3′ terminal nucleotide, in general, reduced PCR product yield dramatically. The presence of a mismatched T at the 3′-terminus, however, allowed significant amplification even when coupled with an adjacent mismatch. Furthermore, even two mismatched Ts at the 3′-terminus allowed efficient amplification.

INTRODUCTION

Viral genomes, particularly those of RNA viruses and retroviruses, contain multiple base alterations, additions, duplications, and deletions. The variability of these viruses has been attributed to the low fidelity and lack of proofreading functions of the polymerases responsible for their replication (1). In addition, the fidelity of RNA polymerase II which plays a critical role in the retroviral life cycle, and of the reverse transcriptase must be taken into consideration. The repeated rounds of replication required for infection further magnify variability. The role that these viral variants play in the natural history of infection is only beginning to be ascertained and appears to vary with each class of virus.

The polymerase chain reaction (PCR)(2–4) has proven to be a sensitive and specific assay for the detection of retroviral sequences (see for example 5–8). Because of the inherent genetic variability of these viruses, detection by PCR requires the identification of primers that will recognize the viral variants. To ensure the efficient amplification and detection of such viruses, we selected primers that amplify regions of viral genomes that contain either conserved amino acid or nucleic acid sequences. In the former case, the selection of conserved regions encoded by amino acids with minimal codon degeneracy reduces the number of possible oligonucleotides required to prime the region (9). In the latter case, the identification of regions conserved in a large number of sequenced isolates will aid in the selection of primer pairs that will amplify most viral variants (Human Retroviruses and AIDS, 1989, Los Alamos National Laboratory).

The ability for an oligonucleotide to serve as a primer for PCR is dependent on several factors, including a) the kinetics of association and dissociation of primer-template duplexes at the annealing and extension temperatures, b) the effects on duplex stability of mismatched bases and their location, and c) the efficiency with which the polymerase can recognize and extend a mismatched duplex. Since single mismatches at or near the terminal 3′ base of a primer are known to affect both oligonucleotide stability and efficiency of polymerase extension, they should effect PCR more dramatically than mismatches at other positions (10).

Several investigators have begun to evaluate the effect of single 3′-terminal mismatches (11–15). In this study, we evaluated the effect on PCR of various primer-template mismatches used for amplification of a region of the human immunodeficiency virus type 1 (HIV-1). The importance of HIV detection (5,6,8 16–22) coupled with the heterogeneity of the HIV genomes (23–26), suggested that primers for this virus would serve as an important model for this study.

MATERIALS AND METHODS

HIV Model System

A 130 bp region of *gag* in HIV-1 (NT 1377–1506 of HIVSF2) was amplified by pairing the upstream primer, SK145 or its derivatives (Table I) with the downstream primer SK150 (5′ TGCTATGTCACTTCCCCTTGGTTCTCTC). Oligonucleotide SK102 (5′ GAGACCATCAATGAGGAAGCTGCAGAATGGGAT) hybridizes to a region within the amplified product and is used as the probe in Southern blot and oligomer hybridization analyses (see below). The oligonucleotides described here also amplify HIV-2.

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The templates used in this study were either a recombinant plasmid that harbors an HIV-1 genome, Z6 (gift of C.Y. Ou and G. Schochetman, CDC), or were templates generated by PCR (see below). The sequence of plasmid Z6 at the primer annealing sites was determined by amplification and cloning into M13 of a 300 bp fragment that spans the 130 bp fragment of interest. Sequence analysis shows that SK145 is homologous to Z6 and SK150 differs from plasmid Z6 at a single nucleotide 22 bases from the 3′ end. The single base alteration in SK150 is to more efficiently provide amplification of HIV-2. The sequence of SK150 therefore is a hybrid sequence varying by a single base from the type 1 and type 2 viruses.

Table I. Sequence of SK145 and its Derivatives.

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
289	----------------------------A	3′
290	----------------------------G	3′
291	----------------------------C	3′
292	---------------------------T-	-1
293	---------------------------G-	-1
294	---------------------------C-	-1
295	--------------------------T--	-2
296	--------------------------G--	-2
297	--------------------------C--	-2
298	-------------------------T---	-3
299	-------------------------G---	-3
300	-------------------------C---	-3

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145.

Oligonucleotides

Two sets of oligonucleotides were synthesized and used for this study. One set of oligonucleotides that differed from SK145 at one of the last 4 bases of the 3′-terminus was used to study the effects of mismatches on amplification (Table I). A comprehensive study of the effects of various primer:template mismatches requires not only primers with base alterations but also templates with various base alterations. We chose to generate templates with the desired change(s) by PCR amplification of plasmid Z6 with a set of 'mutagenic' oligonucleotides. The 'mutagenic' oligonucleotides (SK277 to SK288 and SK312 to SK320) are identical to SK145 at their 5′ ends but have incorporated into their sequence the altered base(s) and are

Table II. Sequence of "Mutagenic" Primers

PRIMER DESIGNATION	PRIMER SEQUENCE	POSITION OF BASE ALTERATION(S)
SK145	AGTGGGGGGACATCAAGCAGCCATGCAAAT	NONE
277	----------------------------Agtta	3′
278	----------------------------Ggtta	3′
279	----------------------------Cgtta	3′
280	---------------------------Ttgtt	-1
281	---------------------------Gtgtt	-1
282	---------------------------Ctgtt	-1
283	--------------------------Tatgt	-2
284	--------------------------Gatgt	-2
285	--------------------------Catgt	-2
286	-------------------------Taatg	-3
287	-------------------------Gaatg	-3
288	-------------------------Caatg	-3
312	---------------------------TCgtta	3′,-1
313	---------------------------CCgtta	3′,-1
314	---------------------------GCgtta	3′,-1
315	---------------------------TAgtta	3′,-1
316	---------------------------CAgtta	3′,-1
317	---------------------------GAgtta	3′,-1
318	---------------------------TGgtta	3′,-1
319	---------------------------CGgtta	3′,-1
320	---------------------------GGgtta	3′,-1

Dashes represent nucleotides that are identical to SK145. Upper case letters denote mismatches relative to SK145. Lower case letters denote bases extended beyond mismatch.

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extended by 4 bases beyond the 'mutagenic' base(s) (Table II). We assumed that the 4 additional complementary bases at the 3′ end would facilitate extension by *Thermus acquaticus* (*Taq*) DNA polymerase. The predicted Tms for SK145 and SK150 are 83°C and 74°C, respectively, in 0.1M NaCl at a concentration of $1X10^{-6}$M using an algorithm proposed by Breslauer (27,28). The internal G:A mismatch between SK150 and plasmid Z6 should have little effect, if any, on the thermostability of the duplex (29).

The oligonucleotides were synthesized on a Model 8750 DNA synthesizer (Milligen/Biosearch, San Rafael, CA) using long-chain alkylamine controlled pore glass supports and beta-cyanoethyl N,N-diisopropyl phosphoramidites (American Bionetics, Hayward, CA). Standard ancillary reagents and synthesis protocols were used (30,31). The oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis which removes contaminating truncated sequences and subsequently desalted by reversed-phase HPLC. Since the oligonucleotides were synthesized 3′ to 5′, 3′ truncated oligonucleotides were not present in the preparations. Base composition analysis was performed to assure that the isolated fragment contained the appropriate number and ratio of nucleosides.

DNA Amplifications

DNAs were amplified in 100 μl reaction volumes with 50 pmoles of each primer, 2 units AmpliTaq DNA polymerase (Perkin-Elmer Cetus Inc.) and either 800, 50, or 6 μM total dNTPs in a buffer containing 10 mM Tris pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$. The concentration of primers used was determined by optical density and evaluated by gel electrophoresis.

All amplifications were performed using either plasmid Z6 or PCR-generated products as templates. To generate the base-altered templates, ten thousand copies of Z6 plasmid were amplified in the absence of human genomic DNA. The amount of base altered templates generated by PCR was estimated by comparing the intensities of the ethidium bromide stained product bands to known amounts of marker DNA. Each template was diluted and normalized to the desired concentration before use. Since each PCR-generated product was diluted by at least 10^6 for use as template, contribution of original Z6 molecules to subsequent amplifications is highly unlikely. To determine the effects on PCR of primer-template mismatches, concentrations of approximately 10,000 and 100 copies of either plasmid Z6 or PCR-generated templates were amplified in the presence of 1 μg human placental DNA. Plasmid DNA concentration was determined by optical density at 260nm.

Samples were amplified by 30 repeated cycles on a DNA Thermal Cycler (Perkin-Elmer Cetus, Inc.) using the following parameters: DNA denaturation, 25 sec at 95°C; primer annealing, 25 sec at 55°C; and primer extension, 1 min. at 72°C. For generation of templates with two consecutive mismatches at the 3′ end, 'mutagenic' oligonucleotides with 2 consecutive mismatches with plasmid Z6 at positions 5 and 6 from the 3′ terminus (SK312 to SK320) were used. In order to achieve more efficient amplifications, a lowering of the annealing temperature to 37°C was necessary. Presumably, these two consecutive mismatches were sufficient to disrupt the stability of the AT-rich 3′ end. All amplifications were performed in triplicate and repeated separately with multiple stock solutions.

Detection

The analysis of two different target concentrations and the dramatic differences in the overall yield of the amplification reactions required the use of different detection schemes. Products of high copy target amplifications were analyzed by NuSieve agarose gel electrophoresis and ethidium bromide staining, and confirmed by Southern blot analysis with a 32P end-labeled SK102 probe (specific activity of 1.5−3μCi/pmole) (32). Oligomer hybridization was used to analyze the products of low copy target amplifications (18). As described here, the PCR product yield from any one reaction varies by no more than 50% from sample to sample and run to run. For simplicity, this variability was not incorporated into Table III.

Table III. Relative amplification efficiencies of 3′-terminal mismatches in the presence of 800 μM dNTPs. Product yields were normalized to the perfect matches (1.0).

		Primer 3′ Base			
		T	C	G	A
Template 3′ Base	T	1.0	1.0	1.0	1.0
	C	1.0	≤.01	1.0	1.0
	G	1.0	1.0	1.0	≤.01
	A	1.0	1.0	≤.01	0.05

RESULTS AND DISCUSSION

Mismatches at the 3′ terminus of a primer

The synthesis of oligonucleotides that differ only at the 3′-terminal base, coupled with templates that contain different bases at the corresponding position, provide a system to test the effect of mismatches on PCR. Although amplification of a subset of these templates and primers would provide information on all mismatches, we chose to generate and amplify each template with the four available primers for two reasons. First, we wanted to determine whether the effects of mismatches on PCR were symmetrical. For example, would a G:T (primer:template) mismatch have the same effect on PCR as a T:G mismatch? Second, an intrinsic 3′ to 5′ exonuclease activity has not been demonstrated for *Taq* polymerase (33,34), therefore, an asymmetric effect of mismatches on PCR may reflect 'context' effects (role of flanking sequences) on the efficiency of extension of a mispaired primer-template.

The results from the 3′ mismatch experiments are summarized in Table III. Under the conditions used, and with 800 μM total dNTPs, most mismatches did not significantly affect amplification. In fact, the presence of a T at the 3′ end of the primer provided efficient amplification irrespective of the corresponding nucleotide in the template. The mean amplification efficiency per cycle of SK145−150 and these mismatched primer pairs is approximately 85% after 30 cycles. Examination of PCR product after 25 cycles suggested that the efficiency of amplification between 25 and 30 cycles was similar to the amplification efficiency of the earlier cycles. The amplification efficiencies were determined as previously described (3). An A:A (primer:template) mismatch resulted in a 20-fold reduction in overall product yield and A:G, G:A and C:C resulted in approximately a 100-fold reduction. The effects of the mismatches on PCR were symmetrical. For example, both A:G and G:A mismatches were equally detrimental to PCR under these conditions. A representative gel and Southern blot analysis of these amplifications is shown in Figure 1 (brackets 1−3).

Variations in the reaction components and annealing temperatures may affect amplification by mismatched primers. In this study, we examined the effect on amplification of lowering the dNTPs to 50 μM and 6 μM. The results from the

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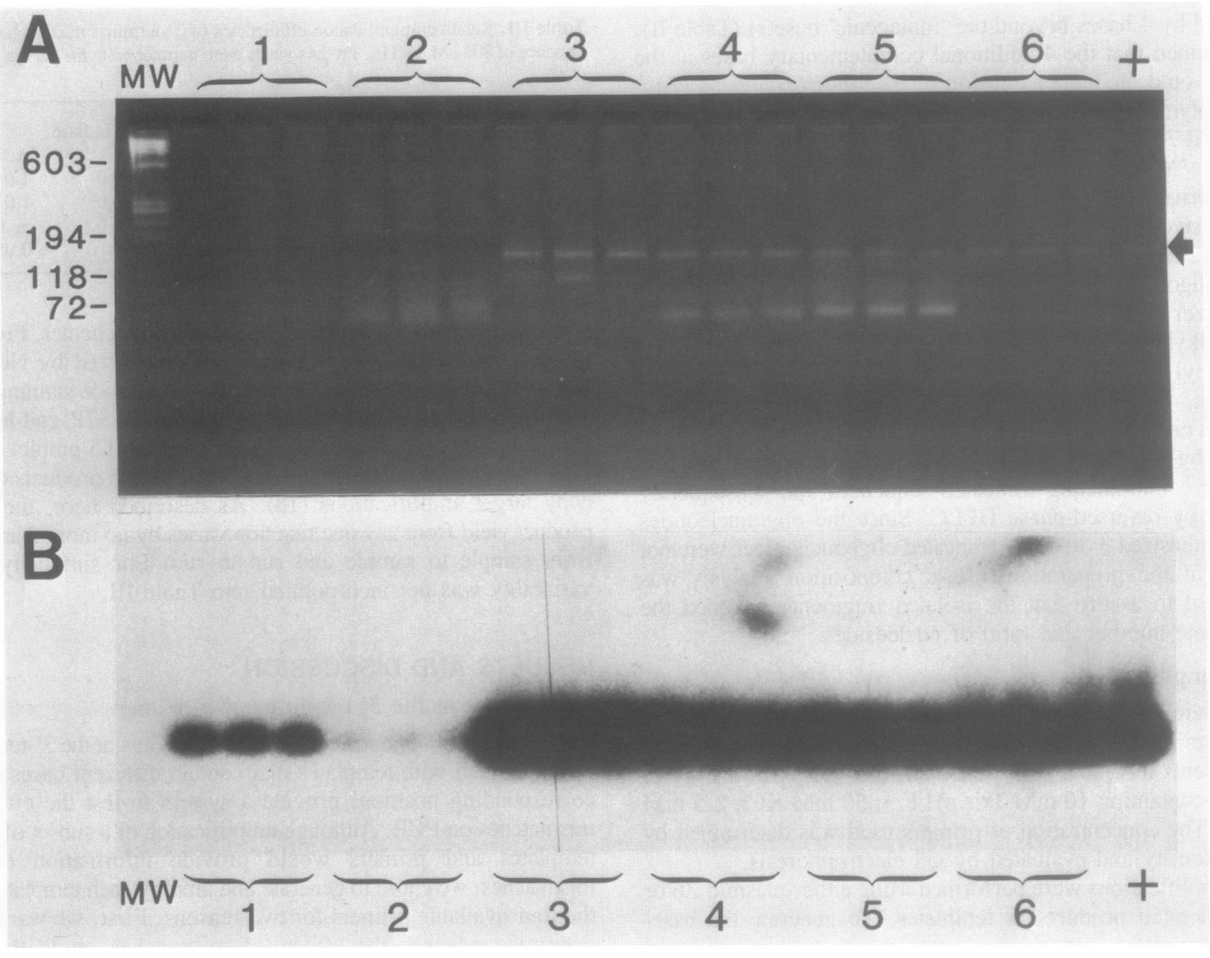

Figure 1. Representative amplifications of plasmid Z6 DNA with primers that were mismatched with template. Samples were amplified in triplicate and visualized on NuSieve agarose gel (Panel A) and analyzed by Southern blot (Panel B). Plasmid Z6 was amplified by coupling SK150 with a primer that had either an A:A, G:A or C:A primer:template mismatch at the terminal 3′ position in 1, 2, and 3, respectively; T:T, G:T or C:T mismatches one nucleotide from the 3′ terminus in 4, 5 and 6, respectively. The positive control (+) represents amplification of plasmid Z6 DNA with a perfectly matched primer. The arrow indicates the position of the product of interest.

amplifications with 50 μM dNTPs were similar to those with 800 μM dNTPs with the exception that the G:G mismatch amplified poorly. In 6 μM dNTPs, only perfectly matched 3′ends were extended with the exception of a T:G mismatch. The overall product yield with this level of dNTPs was at least 10 to 20-fold less than with 800 μM. However, the absence of detectable product may reflect small differences in amplification efficiency rather than complete inhibition of extension. The lower amplification efficiency is more likely a result of lower dNTP concentrations than the higher relative concentration of $MgCl_2$, as this primer pair amplifies efficiently with a broad range of $MgCl_2$.

One of the important factors that affect PCR product yield is the relative efficiency with which the polymerase extends from a mismatched primer-template duplex. Once extension from a mismatched primer occurs, the resultant product and the complement synthesized in subsequent cycles are fully matched with both primers. The molecules with termini defined by both primers accumulate exponentially. On the other hand, products of extensions from the original plasmid template have only one defined terminus and accumulate linearly. Therefore, the contribution of mismatched extension products to total yield, although significant in early cycles, becomes negligible in later cycles. The observed reductions in PCR product reflect dramatic decreases in the efficiency of mismatch extension relative to perfect match extension. If one assumes that mismatch extensions only contribute significantly in the first ten cycles (at which point the mismatch extended templates represent at best 2% of the fully-matched templates) and that the mean efficiency per cycle after cycle ten is 85%, then our studies using 800 μM dNTPs, suggest that an A:A mismatch has an average per cycle efficiency of 50% and the A:G, G:A and C:C mismatches at only an average per cycle efficiency of 16% relative to perfect matches during the first ten cycles. Changes in the reaction conditions such as the concentration of magnesium chloride and annealing temperatures are expected to alter the results described here.

Although the product yields obtained by amplification of a common template with different primers can be directly compared, the products generated from amplification of different templates with common primers may vary somewhat as a result of differences in the amount of PCR-generated template used to seed each reaction. Although the templates were diluted and normalized, the possibility exists that minor differences in product yield merely reflect differences in initial template copy number. Minor differences in template and primer concentrations, however, would not account for the dramatic effects on PCR that

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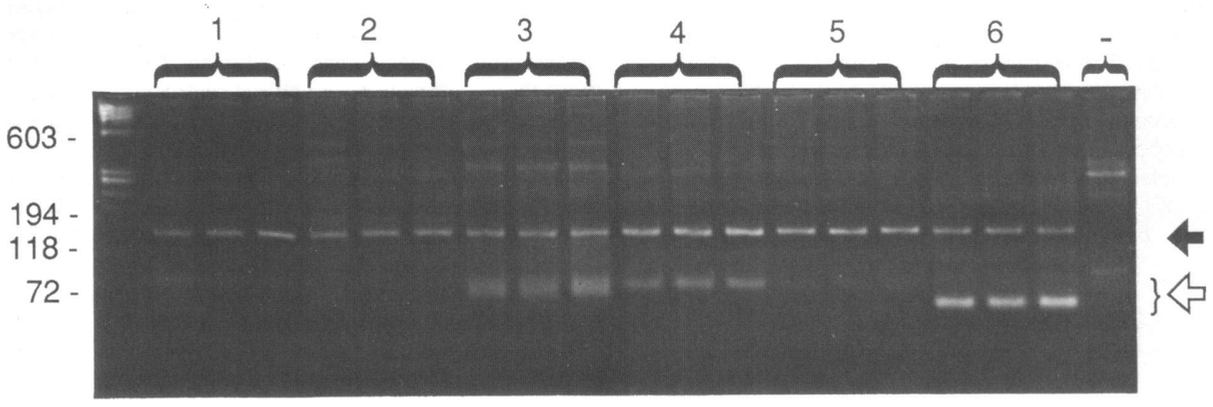

Figure 2. Representative amplifications of plasmid Z6 DNA with primers that differ by a single nucleotide. The plasmid DNA was amplified in triplicate in the presence of 1 ug human placental DNA by coupling SK150 with SK295−300 in (1−6), respectively. Amplification of human placental DNA in the absence of plasmid Z6 is shown in the lane designated (−). The solid arrow shows the position of the desired product; the open arrow shows the positions of the primer-dimers.

were observed with some mismatches. All amplifications were first performed on a high level of target molecules and were repeated on a low level of target. It is expected that effects on PCR yield would be observed in the presence of few targets that may otherwise be masked when amplifying high levels of target molecules. The results from the low target experiments were similar to those obtained from the high target experiments.

Single internal mismatches

In the process of generating templates with altered bases, we demonstrated that a single mismatch 3 residues from the 3′ terminal base of a primer can be efficiently extended without modification of amplification reaction conditions. Similarly, mismatches 1, 2, or 3 bases from the 3′ nucleotide of primers had no apparent affect on overall PCR product yield (for representative amplifications, see figure 1, brackets 4−6). Of particular note are the differences in non-specific amplification products of various molecular weights generated by these reactions. Amplification of the various 'mutated' templates with a common primer pair (SK145−150) resulted in background products that were similar in size. In contrast, amplification of a common template with primers that differ by a single base gave rise to background amplification products that varied dramatically in intensity and size (Figure 2). These data suggest that minor modifications in the primer sequence can dramatically affect the specificity of the amplifications.

In addition, a major product that migrates at approximately the distance expected of the sum of the two primers is often observed in amplification reactions. These products have been cloned and analyzed from several different primer-pair systems (B. Watson, S. Kwok, personal communications). Sequence analysis reveals a fragment bearing the sequence of one of the primers contiguous with the sequence of the complement of the other primer and hence, the product has been termed 'primer-dimer'. Primer-dimers containing a sequence homologous to one of the primers contiguous with its complement have not been identified. It is thought that such a molecule would form a stable hairpin loop structure and would therefore not readily amplify. In some cases, the two primer sequences were separated by 1−10 nucleotides. The mechanism by which these primer:dimers form is not clear. Two alternative explanations can be used to explain these amplification products. First, one of the single stranded primers binds to the polymerase and uses the 3′ end of the second primer as a template for extension. Second, genomic DNA and trace quantities of nucleic acids present in the reactions may contain sequences that are contiguous and similar enough to the primers that they serve as a template to generate these molecules. Given that the downstream primers in these reactions are identical and that the upstream primers differ by only a single base change, it is interesting that amplification of a common template gave rise to not only background products of different sizes and intensities but also primer-dimers of different sizes and intensities (Figure 2).

Multiple mismatches

The effect on PCR of mismatches in a primer:template duplex depends largely on the position and nature of the mismatches. We demonstrated above that some base mismatches (C:T, G:T, T:T, G:G, T:G, T:C, A:C, C:A,) at the 3′-terminus did not have a significant effect on PCR using the conditions described. Although some 3′ mismatches affected amplification, single primer-template mismatches 1 (i.e. penultimate base), 2 or 3 bases from the 3′-nucleotide of a primer did not have a significant affect on PCR product yield. However, when mismatches not involving a T at the 3′ end were coupled with any additional mismatch either 1, 2, or 3 bases from the 3′-nucleotide, PCR product yield was reduced by at least 100-fold (data not shown). However, a T mismatch at the 3′ terminus coupled with an additional mismatch at the penultimate position, resulted in only a 5−10 fold reduction in product yield. Furthermore, the presence of 2 Ts at the 3′ terminus of the primer resulted in overall product yields that were reduced by only 2−5 fold when compared to amplifications by the perfectly matched primers. For these studies, primer pairs SK289−150, SK290−150, SK291−150 or SK292−150 were used to amplify templates generated by amplification of plasmid Z6 with primer pairs SK280−150 through SK288−150 or SK312−150 through SK320−150 (see Tables I and II).

In the generation of templates with two consecutive base alterations, we found that mismatches 5 and 6 residues from the 3′ end had a detrimental effect on amplification when a 55°C annealing temperature was used. However, by lowering the annealing temperature to 37°C, a more efficient amplification was achieved. Because of the AT richness of the 3′ terminus of

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these primers, we speculate that the presence of 2 consecutive mismatches 5 and 6 bases from the 3′ terminus sufficiently destabilized the 3′ terminus such that extension by *Taq* DNA polymerase was very inefficient.

The presence of multiple mismatches at least 8 bases from the 3′ terminus of SK145 does not appear to have a significant effect on PCR under the conditions used. A molecular clone of HIVMAL (35) has five mismatches with SK145 and a single G:A mismatch 21 bases from the 3′-terminus with SK150.

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SK145   AGTGGGGGGACATCAAGCAGCCATGCAAAT
HIVMAL  ---T--A-----C--G-----T--------
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One would predict that the Tm of SK145 on the HIVMAL template might be at least 10°C lower. Despite these mismatches, HIVMAL was efficiently amplified under the conditions used. However, when the annealing temperature was raised to 60°C, PCR product yield was significantly reduced, and at 65°C, amplification was not detected. These results underscore the importance of using less stringent annealing temperatures for the amplification and detection of highly variable targets.

CONCLUSIONS

Several conclusions can be drawn from this study. Certain mismatches at the terminal 3′ position (ie. T:C, T:G, T:T, G:G, and A:C) appear to amplify as efficiently in PCR as the fully complementary primer-template duplex, under these conditions, while an A:A mismatch moderately reduced PCR amplification efficiency per cycle, and A:G and C:C mismatches dramatically reduced PCR amplification efficiency. Although G:G mismatch amplified efficiently at 800 μM dNTPs, the product yield in 50 μM dNTPs was dramatically reduced. Whereas some 3′-terminal mismatches were poorly extended, single base mismatches between the primer and template either one, two or three bases from the 3′ nucleotide of the primer can be extended without a significant effect on overall product yield. However, when coupled with an additional mismatch within the last four bases, overall PCR product yield from a 3′ terminal mismatched primer is drastically reduced. In contrast, oligonucleotides with a T mismatch at the 3′ terminus when coupled with an additional mismatch at the penultimate position served efficiently as primer for amplification. Further, the presence of 2 Ts at the 3′ terminus enabled amplification of templates with mismatches at both positions irrespective of the nucleotides involved.

A priori, the thermodynamic stability of base mismatches cannot explain the results observed here. The efficiency at which polymerase extends from a mismatched base pair depends on a number of complex interactions. First, the overall stability of the primer-template may determine the likelihood that polymerase binds to a duplex. Second, extension by polymerase probably reflects recognition of the base stacking, hydrogen bonding, and overall steric structure of the terminus to be extended. These factors may themselves interact since hydrogen bonds may facilitate stacking by bringing the bases in proximity and vice versa. Although numerous studies have begun to describe the calculated and measured stabilities of the various mismatches alone and relative to sequence context (10,36), the simplest interpretation of our data is that purine-purine mismatches do not extend efficiently while pyrimidine-pyrimidine and purine-pyrimidine mismatches do extend efficiently under these conditions. The G:G and C:C mismatches serve as exceptions to these conclusions. Perhaps the stacking forces of the G residue with the penultimate base and the hydrogen bonded structure proposed by Abou-ela, et al (29) play a role in efficient extension of this mismatch by polymerase. On the other hand, the proposed sugar-phosphate constrained structure with only one hydrogen bond for a C:C mismatch may disrupt the 3′-terminal structure to the extent that efficient extension is not possible (29). Extension of the primer-template duplex from a G:T mismatch perhaps would have been expected since it contributes neither a stabilizing nor destabilizing influence to the nucleic acid duplex (37). The efficient extension of a T:C or T:T mismatch was not, however, expected. Recent studies by M.Goodman and colleagues on the relative extension efficiencies of mismatched termini by AMV reverse transcriptase and DNA polymerase alpha are in general agreement with our results (38). The extent to which one can extrapolate our data to other primer pair-template systems will require additional studies. The extent to which the three contiguous A's near the three terminus contributes to these results is unclear. However, our preliminary experiments with other HIV and HTLV primer pairs support the general applicability of these observations. Included in these experiments are data suggesting that the efficiency of extension of a G:G mismatch may be dependent on sequence context as well as on the concentration of deoxynucleoside triphosphates.

Several investigators have begun to explore the effects of mismatches on the amplification of cellular sequences. A direct comparison of our results with those previously published is difficult due to differences in reaction conditions (buffer, primer concentration, annealing temperature), target copy number, primer length and sequence context. In some studies, the primers were short (12 − 16 bases) and therefore a 3′ mismatch may have more dramatically affected overall primer:template duplex stability as well as the ability for the polymerase to catalyze extension.

Our results are in agreement with those of Ehlen et al. (13) where the amplifications were carried out under similar reaction conditions and with primers of similar length. Ehlen et al. (13) demonstrated that at 200 μM dNTPs, a C:T mismatch can be extended where as at 2 μM dNTPs C:T, A:C and C:C mismatches were not extended. Using similar conditions but with primers that were 16 bases long, Nichols et al. (12) also showed that an A:A mismatch was not extended. Wu et al. (11), again under similar conditions but with primers that were 14 bases in length, presented evidence that A:A and T:T mismatches were not extended at an annealing temperature of 55°C but were extended at annealing temperatures of 44°C and 50°C. The extension of the A:A mismatch at the lower annealing temperature is contrary to our finding. The studies of Okayama et al. (15) were also carried out under similar conditions but with shorter primers. Their results are contrary to all other studies in that 10 of the 12 possible mismatches tested prevented significant amplification; the A:G and C:T mismatched were not evaluated.

Newton et al. (14) demonstrated the use of 3′-terminal mismatched primers for allele-specific amplifications. In their study, G:T, T:G, A:C, and C:A, mismatches were extended by *Taq* DNA polymerase whereas A:A, T:T, and C:T mismatches were refractory to extension. The C:T and T:T results are contrary to our findings and may reflect the substantial differences in the reaction conditions. Consistent with Newton et al., we found that single internal mismatches did not significantly affect

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PCR product yield. Furthermore, the placement of an additional internal mismatch to extendable 3′-terminal mismatches proved refractory to amplification.

Implications of this study extend to multiple areas. First, primers for nucleic acid templates from pathogens known to vary in sequence would benefit from a single T or perhaps double T at the extreme 3′ end so as not to obviate amplification because of mismatched bases at 3′ end. The HIV-1 model system used in this report is of particular importance given the extensive heterogeneity within and among infected individuals. Ideally, the primers used in a diagnostic assay for this virus should not only have the desired sensitivity and specificity on contemporary isolates but should also be engineered to accommodate a degree of divergence expected in future isolates. The observations noted in this study may contribute to the design of primers for the detection of other variable pathogenic viruses such as the human papilloma viruses and RNA viruses. Second, this information will aid in the design of primers used in the search for additional members of known virus groups (7,9). For example, the design of primers with T rather than A or G at the 3′ terminus may increase the likelihood of extension.

On the other hand, there are applications in which primers need to be designed for allele-specific amplifications. The presence of a 3′ terminal A:G or C:C mismatch, and to a lesser extent an A:A mismatch, should bias the amplification to the desired targets. In addition, alterations in the concentration of reaction components (13) and annealing temperature should also effect extension from mismatched primers and therefore should also be exploited.

Finally, we expect that studies similar to those described here will begin to address the specific protein-nucleic acid interactions of polymerases and their substrates so that we may better understand substrate binding and catalysis by this family of enzymes.

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