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Extension of base mispairs by *Taq* DNA polymerase: implications for single nucleotide discrimination in PCR

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ABSTRACT

***Thermus aquaticus* (*Taq*) DNA polymerase was used to measure the extension efficiency for all configurations of matched and mismatched base pairs at template-primer 3′-termini. The transition mispairs, A(primer)·C, C·A, G·T, and T·G were extended 10^{-3} to 10^{-4}-fold less efficiently than their correctly paired counterparts. Relative efficiencies for extending transversion mispairs were 10^{-4} to 10^{-5} for T·C and T·T, about 10^{-6} for A·A, and less than 10^{-6} for G·A, A·G, G·G and C·C. The transversion mispair C(primer)·T was extended with high efficiency, about 10^{-2} compared to a correct A·T basepair. The unexpected ease of extending the C·T mismatch was not likely to have been caused by primer-template misalignment. *Taq* polymerase was observed to bind with similar affinities to each of the correctly paired and mispaired primer-template 3′-ends. Thus, the failure of *Taq* polymerase to extend mismatches efficiently appears to be an intrinsic property of the enzyme and not due to an inability to bind to 3′-terminal mispairs. For almost all of the mispairs, C·T being the exception, *Taq* polymerase exhibits about 100 to 1000-fold greater discrimination against mismatch extension compared to avian myeloblastosis reverse transcriptase and HIV-1 reverse transcriptase which extend most mismatched basepairs permissively. Relative mismatch extension efficiencies for *Taq* polymerase were measured at 45°C, 55°C and 70°C and found to be independent of temperature. The mispair extension data should be important in designing experiments using PCR to distinguish between sequences that vary by a single nucleotide.**

INTRODUCTION

The polymerase chain reaction (1–3) has great specificity, and a single gene present in a complex genome can be uniquely amplified. With appropriate design of primers, alleles of the same gene differing by one or a few nucleotide substitutions can be distinguished, and only one of the two alleles amplified (reviewed in Ref. 4). Thus, discrimination at the level of single nucleotides, by a technique referred to as allele-specific PCR, can be applied to disease diagnosis, polymorphism analysis, sequencing strategies for DNA mixtures and detection of rare mutations (4–12).

Techniques for selective amplification of sequences differing by a single nucleotide make use of the property that primer template complexes containing a single mismatch have lower melting temperatures than perfectly matched complexes. PCR at the appropriate temperature will therefore promote selective amplification since amplification is carried out with much greater efficiency using stably annealed compared to partially melted primer-templates. Specificity is further enhanced if a mismatched base is placed at the 3′-end of the two primers since DNA polymerases extend mismatches much less efficiently than correct matches (13–19). This feature of DNA polymerases may be caused by either a higher affinity of the enzyme for a template that is perfectly matched, an inherent difficulty in extending a primer terminal mismatch or a combination of both factors.

Recently, a detailed study of avian myeloblastosis reverse transcriptase (AMV RT) has shown that inefficient mismatch extension by this enzyme is caused by a kinetic block to elongation rather than by a difference in binding affinity to matched versus mismatched primer-template termini (15,16). We have used steady-state enzyme kinetic analysis in conjunction with an equilibrium competition binding assay to study mismatch extension by *Taq* polymerase. *Taq* polymerase is thermally stable and can serve as a model to investigate the effects of elevated temprature on the relative efficiencies of extending matched and mismatched primer termini. In this paper, *Taq* polymerase is used to analyze binding and extension for the 4 matched and 12 mismatched primer 3′-termini involving the common nucleotides. The results also should have practical application in experiments designed to distinguish between sequences that vary by a single nucleotide, an example being allele-specific PCR.

MATERIALS AND METHODS

Materials

Purified *Taq* polymerase (lot number ANC 402) lacking detectable 3′-exonuclease activity was purchased from BRL. *Taq* polymerase has a specific activity of 200,000 units/mg (J.

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Campbell, BRL-LTI, personal communication) and a molecular weight of 94 kDa (20; J. Campbell, BRL-LTI, personal communication); one unit is defined as incorporation of 10 nmol deoxyribonucleotide into acid-precipitable material in 30 minutes at 72°C. The optimum polymerization activity is at 75°C. In several kinetics experiments purified *Taq* polymerase from Perkin−Elmer Cetus, a gift from D.H.Gelfand and J.J.Sninsky, Cetus Corporation, was used. The *Taq* polymerase from Cetus had a specific activity of 200,000 units/mg and a molecular weight 94 kDa (21). Apparent second order rate constants for extension of A(primer)·C, T·C, and C·C mismatches measured using polymerases obtained from both BRL and Cetus gave indistinguishable results. Based on the stoichiometry between polymerase concentration and primer template concentration, a measurement of the % of input annealed primers extended in the presence of an unlabeled DNA trap (to limit interactions between enzyme and 5′-^{32}P-labeled primer-template molecules to a single encounter), allowed us to estimate that greater than 50% of the *Taq* polymerase molecules were active in the reaction. We have documented the absence of 3′-exonuclease activity in the preparations of *Taq* polymerase used here by the absence of polyacrylamide gel bands corresponding to degradation of 5′-^{32}P-labeled primer molecules (data not shown). T4 polynucleotide kinase was purchased from U.S Biochemical Corp. Restriction enzymes *MboI*, *DdeI* and *HinfI*, Exonuclease III, calf thymus DNA, heparin (sodium salt, grade I) and purified dNTP substrates were purchased from Pharmacia LKB Biotechnology, Inc. Radioactive [γ-^{32}P]ATP was purchased from ICN Radiochemicals, Inc. Four oligonucleotide primers varying by only one base at the 3′-end and four complementary templates varying by one base at the site opposite the primers 3′- end were synthesized using an Applied Biosciences DNA Synthesizer by L.Williams (Comprehensive Cancer Center, University of Southern California, Los Angeles) and used after gel purification. The sequences used were: primer (35 mer), 5′-GCGACTGAGAGCGTAGCTGACCATGACTGTGAAC-N-3′(N=A,T,G,C) ; template (50 mer) 3′-CGCTGACTCTC-GCATCGACTGGTACTGACACTTGN′ATACTCCTATCA-TCT-5′ (N′=A,T,G,C). Thus, these 8 oligomers can form all 16 possible primer-template termini combinations at the 3′-end of primers.

Methods

The velocity of extending a preformed matched or mismatched primer-template terminus using a polyacrylamide gel assay to measure steady-state primer elongation kinetics was described previously(13,15,16). A Phosphorimager (Molecular Dynamics) was used to quantify gel band intensities corresponding to primer molecules of different lengths. The primer elongation velocity is defined as the percent primer extension/minute. Plotting v *versus* [dNTP] fits a Michaelis−Menten equation, and the apparent second order rate constant, V_{max}/K_m for each primer terminus was determined by non-linear least squares fit to a Michaelis−Menten curve. The relative extension efficiency, f^0_{ext}, given by the ratio of V_{max}/K_m values for mismatched compared to matched primer termini (see Eq. 1) measures the relative rates of nucleotide addition from mismatched compared to correctly matched primer termini. f^0_{ext} represents the relative probability that a polymerase will bind to and extend either of the two primer-templates, present at equimolar concentrations, and at low [dNTP] (15).

Reaction conditions for Kinetics Measurements. Procedures for primer 5′-end labeling, annealing, electrophoresis, autoradiography and data analysis were described in detail previously (16). We verified that under our experimental conditions, >95% of primers were annealed to template DNA (data not shown). Procedures for measuring absolute and relative values of K_D, the polymerase-DNA equilibrium binding constant were carried out as described (16, see also Results, Figure 3). Briefly, all reactions took place in *Taq* reaction buffer containing 50 mM KCl, 10mM Tris-HCl (pH 8.3), 2.5mM $MgCl_2$, 0.1 mg/ml gelatin and 1.2 nM *Taq* polymerase, 33 nM DNA and variable concentrations of next correct nucleotide (dTTP). Prior to carrying out kinetics measurements for single nucleotide enlongation of primers, a time course was run for each paired and mispaired terminus to determine the linear reaction range. In the linear range, primer usage was typically less than about 20%. Based on the time course data, reaction times used for different primer-template constructs varied between 7 seconds to 30 mimutes. In the protocol for carrying out steady-state kinetics measurements, a mixture of 3 μl of enzyme-DNA and 3 μl of dNTP in reaction buffer were preincubated separately at 70°C for 1 minute to allow equilibration. The two solutions were combined and incubated for designated reaction times. The reactions were quenched by addition of 20 μl of 96% formamide, 0.02 M EDTA.

Table I. Determination of the Relative Binding Affinity of *Taq* DNA Polymerase for All of the 16 Possible Primer Terminus·Template Combinations Using Equilibrium Competition

Ternimus	G*•C	T•C	A•C	C•C
% extension	20.4	24.1	23.4	23.8
K_D(rel)	1.00	0.69	0.74	0.71
Ternimus	G•A	T*•A	A•A	C•A
% extension	19.0	17.4	24.1	20.8
K_D(rel)	0.83	1.00	0.44	0.67
Ternimus	G•T	T•T	A*•T	C•T
% extension	18.6	20.5	17.0	17.2
K_D(rel)	0.83	0.66	1.00	0.97
Ternimus	G•G	T•G	A•G	C*•G
% extension	21.3	22.0	20.2	14.2
K_D(rel)	0.33	0.29	0.41	1.00

For primer terminus·template combinations, the notation G*·C denotes a primer with G as the 3′-terminal base and C as the base opposing it on the template where the * denotes a 5′-^{32}P labeled primer strand. Each box of the table represents a reaction where the terminus given represents the identity of the unlabeled competitor DNA while the labeled DNA is given by the asterisked terminus in the box in the same row. Thus, in the first row, second column, for the box T·C, the labeled DNA is G·C, while the competitor DNA is T·C. % extension is the percentage of labeled primer G·C extended during the time of reaction. Repetitive experiments gave an estimate of 20% error on the value of % extension. K_D(rel) values for each primer-template combination are computed using $K_D(rel) = K_D(mis)/K_D(corr) = Ex_{mis}/(2Ex_{corr} - Ex_{mis})$—see METHODS. K_D (rel) is the relative binding affinity of *Taq* polymerase for mismatched compared to correctly matched primer termini in each row of the Table.

Equilibrium binding of Taq polymerase to matched and mismatched primer termini. The conditions for equilibrium binding experiments were essentially the same as described previously (16), except 8 nM of labeled primer-template DNA and 8 nM unlabeled challenge DNA at equal volume were pre-mixed and incubated with *Taq* polymerase (1.0 nM) on ice for 10 minutes to allow the enzyme to partition freely between the different species of DNA. The unlabeled challenge DNA was used as a source of competing primer-template for enzyme binding. It competes equally with radioactively-labeled primer for extension, and it is identical to the labeled template-primer DNA in volume, concentration and solvent composition, and, where appropriate, contains either matched or mismatched primer 3′-termini. Before initiation of the reaction, solution A containing primer-template-enzyme and a solution B containing dNTP and trap mix in 2× typical *Taq* reaction buffer were equilibrated at 70°C for 1 min. The restriction enzyme digested calf tymus DNA-heparin trap mix (16) was used to insure that the enzyme encounters either labeled template-primer or unlabeled challenge DNA only once during the course of an experiment. The reaction was begun by adding equal volumes of solution A and solution B together. Reactions then were quenched after 7 seconds by adding 20 μl formamide/EDTA.

The data shown in Table I were obtained and analyzed in the following manner. First, a trapping experiment was done using labeled DNA containing a correctly paired 3′-terminus and the challenge DNA also has a correctly paired 3′-terminus. The fraction of initial labeled primer extended is denoted as Ex_{corr}. A second experiment is performed where the unlabeled challenge DNA contains a mismatched primer 3′-terminus, and the fraction of the labeled DNA extended is denoted Ex_{mis}. Using these two extension percentages, the relative ratio of the dissociation constants of the enzyme for matched and mismatched challenge DNA can be computed using: $K_D(rel) = K_D(mis)/K_D(corr) = Ex_{mis}/(2Ex_{corr} - Ex_{mis})$—see Ref. 16, equation (A7). The Phosphorimager was used to measure the fraction of primer extended.

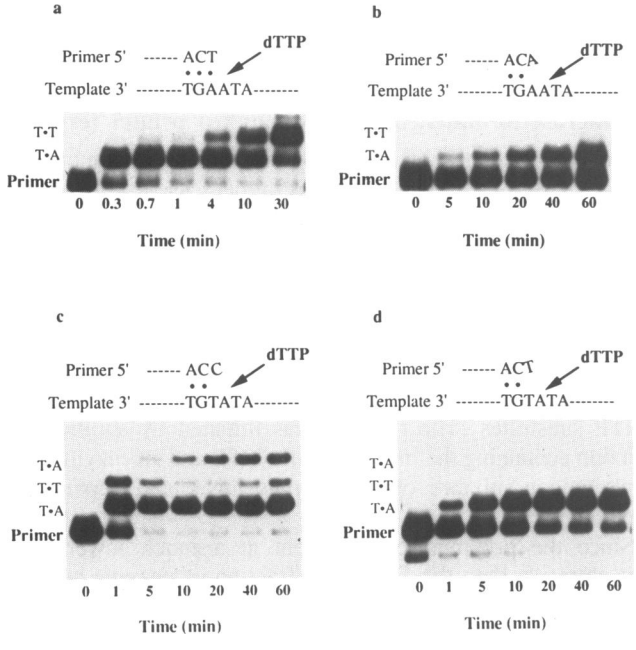

Figure 1. *Taq* polymerase extension of matched and mismatched primer termini as a function of time. (a) Addition of T onto matched T(primer)·A terminus. (b) Addition of T onto mismatched A·A terminus. (c) Addition of T onto mismatched C·T terminus. (d) Addition of T onto mismatched T·T terminus. Primer molecules labeled with ^{32}P at their 5′-ends were separated by PEG electrophoresis, see Methods. The T·T bands indicated in panel a arose from the misincorporation of T opposite T for incubation times exceeding 4 min. The T·T band and upper T·A band indicated in panel c arose from the misincorporation of T opposite T and subsequent correct incorporation of T opposite A. The dTTP concentration was 25 μM in panel a, 1.5 mM in panel b, 1.1 mM in panel c, and 1.4 mM in panel d. The primer is a 35-mer, indicated by the shorter series of dashed lines, and the template is a 50-mer, indicated by the longer series of dashed lines.

RESULTS

Mismatched basepairs at primer 3′-termini have different stabilities depending on the identity of the mispair and on the stability of neighboring base pairs. The rates at which 3′ primer-template mismatches are enlongated depend on the ability of polymerases to bind and extend different terminal mismatches. In this paper, we used a polyacrylamide gel assay (13,15,16,19) to measure extension efficiencies and relative binding affinities of *Taq* polymerase at all sixteen possible primer-template 3′ end combinations, 4 matched and 12 mismatched.

The extension of a pre-formed matched or mismatched terminus by an enzyme behaves as an ordered reaction in which the enzyme first binds to a primer-template terminus, followed by the binding of dNTP to the DNA-enzyme complex and ending with nucleotide incorporation into DNA (22,23). A reduced rate of extending mismatched compared to matched primer termini could be caused either by a reduction in the polymerase-DNA binding constant, K_D, or a smaller intrinsic mismatch extension rate by a bound enzyme, or by a combination of both factors.

Extension Kinetics

All steady-state kinetics measurements were carried out under initial rate conditions with less than 20% of the primer-templates extended during the reaction. A time course to extend a T(primer)·A base pair and A·A, C·T and T·T mispairs is shown in Figure 1. Typically, incubations for extension of correctly paired termini were about 7 to 10 seconds while incubation times on the order of 10 s to 60 min were used for mispaired termini. At the first time point shown in Figure 1a (0.3 min), greater than 90% of the T·A pairs were extended by *Taq* polymerase to form a T·A base pair. Note that for longer incubation periods (>4 min, with [dTTP] = 25 μM), formation of a T·T mispair also occurred at a template site two bases downstream from the primer terminus. In contrast to the rapid extension of the correct pair, no extension of the A·A mispair was detected for incubation periods of less than 5 min, [dTTP] = 1.5 mM (Figure 1b); roughly one-half of the primers containing an A·A terminus were extended during a 60 min incubation.

Extension of both C·T and T·T mispairs occurred in less than 1 min (Figures 1c and 1d). Following addition of the next correct T·A base pair onto the C·T mispair, a T·T mispair was formed, followed by another T·A base pair (Figure 1c; [dTTP] = 1.1 mM). However, subsequent formation of a T·T mispair was not observed after addition of a T·A base pair onto either an A·A mispair (Figure 1b; [dTTP] = 1.5 mM) or T·T mispair (Figure 1d; [dTTP] = 1.4 mM). *Taq* polymerase has been reported to be devoid of associated 3′-exonuclease activity (24).

The preparations of *Taq* polymerase used here contained no detectible 3′-exonuclease activity as determined by the absence of degradation of 5′-^{32}P-labeled primer molecules (data not shown).

The mismatch extension efficiency, f_{ext}, is given by the ratio of velocities to extend a mismatched (w) compared to a correctly matched (r) primer terminus (15). As shown in Ref. 15, f_{ext} is depends explicitly on: (i) the absolute concentration of next correct nucleotide [dNTP], (ii) polymerase processivity, and (iii) the ratio of equilibrium binding constants to mismatched and matched primer termini, $K_{D,w}/K_{D,r}$. The **maximum** possible descrimination for extending correctly paired termni in the presence of equimolar concentrations of mismatched termini, which we designate as f^0_{ext}, occurs when the next correct dNTP concentration is small, and is equal to the ratio of apparent second order rate constants (15),

$$f^0_{ext} = (V_{max}/K_m)_w/(V_{max}/K_m)_r \quad (1)$$

The apparent V_{max}/K_m ratios are equal to the 'true' V_{max}/K_m values (obtained by extrapolation to infinite [DNA]) multiplied by the ratio of the equilibrium binding constants, $(K_{D,r}/K_{D,w})$, (see Ref.15 , Eq. 2b). Thus, the polymerase factors that determine the **maximum** possible descrimination between elongating matched *versus* mismatched primer termini are the ratio of 'standard' elongation rates governing extension of mismatched and matched primer termini and ratio of the equilibrium binding constants for the two types of termini. The V_{max}/K_m ratios for extension of all combinations of mismatched *versus* correctly matched termini (Eq. 1) are given in Figure 2.

The values of the transition mispairs, A(primer)·C, C·A, G·T, and T·G are in the range of 10^{-3} to 10^{-4} (Figure 2). Thus, extension of these mispairs by bound polymerase is from 1,000 to10,000 times harder then extension of the respective G(primer)·C, T·A, A·T, and C·G basepairs. Extension of the transversion mispairs, T(primer)·C, T·T, and A·A were from 5-fold to at least 100-fold less efficient than the transition mismatches. Four transversion mismatches, C·C, G·G, A·G and G·A were poorly extended, even after an hour incubation.

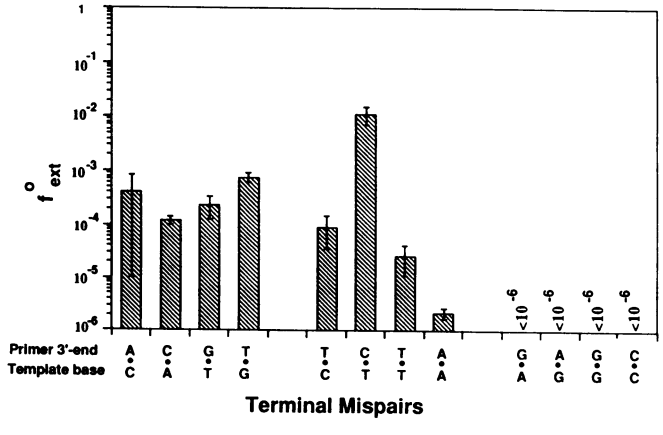

Figure 2. Standard extension efficiencies of terminal mispairs by *Taq* DNA polymerase. Extension efficiencies calculated according to equation 1—see text, are grouped from left to right for Pur·Pyr and Pyr·Pur mismatches causing transitions, both Pyr·Pyr and Pur·Pur mismatches causing transversions. Extension of the four transversion mispairs at the right hand side of the figure were below the detection sensitivity of the assay, $f^0_{ext} < 10^{-6}$.

Based on the sensitivity of the gel assay, we estimate that these mispairs were extended with at least a million-fold lower efficiency compared to the corresponding correct pairs.

Surprisingly, the C(primer)·T transversion mispair was extended by *Taq* polymerase with exceptionally high efficiency, roughly 1/50 the rate for extension of the corresponding correct A·T basepair. The unexpected ease of extending the C·T mismatch was not likely to have been caused by primer-template misalignment (17). Efficient extension by primer-template misalignment would most likely require the presence of G on the template, located 5′ to the C(primer)·T mismatch; however, A is the base immediately downstream from the mismatch (see Methods). A second possibility exists for C on the primer to pair opposite G, located 3′ to the mismatch site. However, efficient extension of the misaligned C(primer)·G base pair would require formation of another mispair involving insertion of T opposite the original template T site. The possibility that rapid extension of C·T might be caused by the contamination of the primer terminus with the correct base A has been ruled out by time course experiments showing that essentially 100% of the C-terminated primer can be extended by the next nucleotide [dTTP] ~1100 μM in a 1 min incubation (Figure 1c). If efficient extension had been caused by the presence of primers ending with A, then $f^0_{ext} \approx 1$; instead we find that $f^0_{ext} = 2\times10^{-2}$. Therefore, we conclude that efficient extension of the C·T mispair is caused by 'anomalous' enzyme behavior rather than by either localized structural deformation in the primer-template at the site of the mispair or by contamination of C·T mismatched primer-template termini with A·T.

Equilibrium competition to measure binding of *Taq* polymerase to matched and mismatched primer termini

Since reduced rates of extending base mispairs compared to correct base pairs could be caused by decreased affinity of the enzyme for melted primer termini, we measured the relative binding affinities of *Taq* polymerase for each of the paired and mispaired termini (16). To measure relative binding affinities, equal volumes at equimolar concentrations of 5′-labled primer-template and unlabeled challenge primer-template were pre-incubated with *Taq* polymerase to allow partitioning of the enzyme between labeled and unlabeled DNA in the absence of dNTP substrates. The reaction was initiated by addition of a solution containing the 'next correct' dNTP and an enzyme 'trap' containing a mixture of calf-thymus DNA and heparin (see Methods).

Since the polymerase is present at a much lower molar concentration then the DNA, the fraction of enzyme bound to each species of DNA, i.e., labeled or unlabeled matched or mismatched termini, is therefore directly proportional to the binding affinity of the enzyme to each. The addition of a high concentration of an enzyme trap simultanously with the dNTP substrate insures that the primer extension reaction is taking place under 'single turnover' conditions. Under these conditions, it is straightforward to calculate the **relative** affinities of the enzyme for paired and mispaired termini by measuring the extent to which unlabeled challenge DNA containing a mispaired primer terminus is able to inhibit elongation of a correctly paired terminus on ^{32}P-labeled primer DNA (see e.g., Ref. 16). In the preincubation period, the bound enzymes equilibrate between matched and mismatched primer termini. The subsequent reaction period must then be long enough to allow the bound polymerases to add a nucleotide or dissociate and become trapped, but not

long enough that trapping becomes ineffective, i.e., where the enzyme is released from the trap and can reinitiate synthesis on labeled primer-templates.

Data used to calculate the ratios of equilibrium binding constants of *Taq* polymerase to A(primer)·T basepairs compared to G·T, T·T, and C·T mispairs are shown in Figure 3. In lane 2 of the gel, trapping solution is added prior to the enzyme, and after a 2 minute preincubation, dTTP is added to form the downstream T·A base pair. Extension of a normal A·T terminus is shown in lane 3. Lane 3 is similar to lane 2 except no trap was present during pre-incubation; instead, trap was added together with dNTP to initiate extension. Since extension has not occurred in the reaction shown in lane 2, the trap was effective throughout the incubation period. In lanes 4–7, labeled DNA containing a matched A(primer)·T terminus was preincubated in the presence of unlabeled competitor DNA, containing either a matched A·T terminus (lane 6) or mismatched terminus, G·T (lane 4), T·T (lane 5), and C·T (lane 7). An approximate 2-fold reduction in reaction in lanes 4–7, compared to lane 3 in which 1× dilution buffer was used instead of any competitor DNAs, indicates that the binding of *Taq* polymerase is roughly similar to matched and mismatched primer 3′-termini, in agreement with recent observations using other DNA polymerases (16).

The same general conclusion can be arrived at regarding binding of *Taq* polymerase to all of the matched and mismatched primer termini (data not shown). The results are presented in Table II. Since the relative values K_D are all similar to within a factor of 3, we conclude that *Taq* polymerase exhibits similar affinities for binding matched and mismatched primer termini, and we also conclude that the 103 to greater than 106-fold reduction in the enzyme's ability to extend mismatched compared with correctly matched primer termini is attributable primarily to a lowered intrinsic efficiency to extend unstable primers and not to an inability of the enzyme to bind mismatched termini.

Using a standard steady-state enzyme kinetic method, we also measured absolute K_D values for *Taq* polymerase binding to two mismatches, G(primer)·T and C·T, see e.g., Ref. 16. K_D values obtained were in the range of 6 to 8 nM (data not shown), which were similar to values obtained with AMV RT (16) and HIV-1 RT (19).

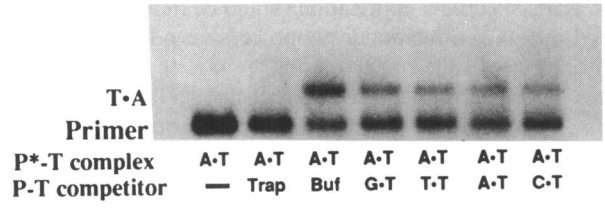

Figure 3. Equilibrium binding assay to measure relative K_D values. Left lane contains labeled primer-template in the absence of enzyme; lane 2 represents a control reaction containing trapping solution (activated DNA and heparin) present prior to addition of *Taq* polymerase; lane 3 represents a reaction in which labeled primer-template is preincubated with polymerase in the absence of competitor DNA. A volume of dilution buffer, 'Buf', was added equal to the volume used for addition of competitor DNA in lanes 4–7. The reaction is initiated by addition of a solution containing the 'next correct' dNTP in the presence of trap; lanes 4–7 are identical to lane 3 except that the preincubation mix contains unlabeled competitor DNA consisting of a G·T mismatch (lane 4), T·T mismatch (lane 5), A·T correct match (lane 6), C·T mismatch (lane 7). Primer molecules were separated by PEG electrophoresis as shown in Figure 1. P*-T complex represents 5′-^{32}P-labeled primer-template DNA complex; P-T competitor represents unlabeled P-T complex termini.

Effect of temperature on mismatch extension efficiencies

For almost all of the mismatches examined, the standard mismatch extension efficiency (f^0_{ext}, Eq. 1) of *Taq* polymerase is more than 100-fold lower (extension fidelity higher) than avian myeloblastosis reverse transcriptase (AMV RT) (Ref.16). Since the reaction temperatures for *Taq* polymerase are at 70°C compared to 37°C for AMV RT, it is important to determine if the large f^0_{ext} values exhibited by *Taq* polymerase are the direct result of the enzyme having excellent intrinsic discrimination. Alternatively, the higher reaction temperature might be responsible for high discrimination by causing destabilization of mismatched termini to a greater extent than correctly matched termini. To distinguish between these two possibilties, we measured V_{max}/K_m ratios for matched and mismatched primer extensions and determined f^0_{ext} as a function of temperature using one matched terminus, G(primer)·C and two mismatched primer termini T(primer)·C and A·C. As expected, the extension efficiencies for both matched and mismatched primer termini increased nonlinearly as the temperature was increased from 45°C to 70°C (Figure 4a). However, the mismatch extension efficiency, f^0_{ext}, remained **constant** as a function of temperature (Figure 4b). Thus, the increased primer extension rates with increased temperatures were similar for both matched and mismatched primer termini. We

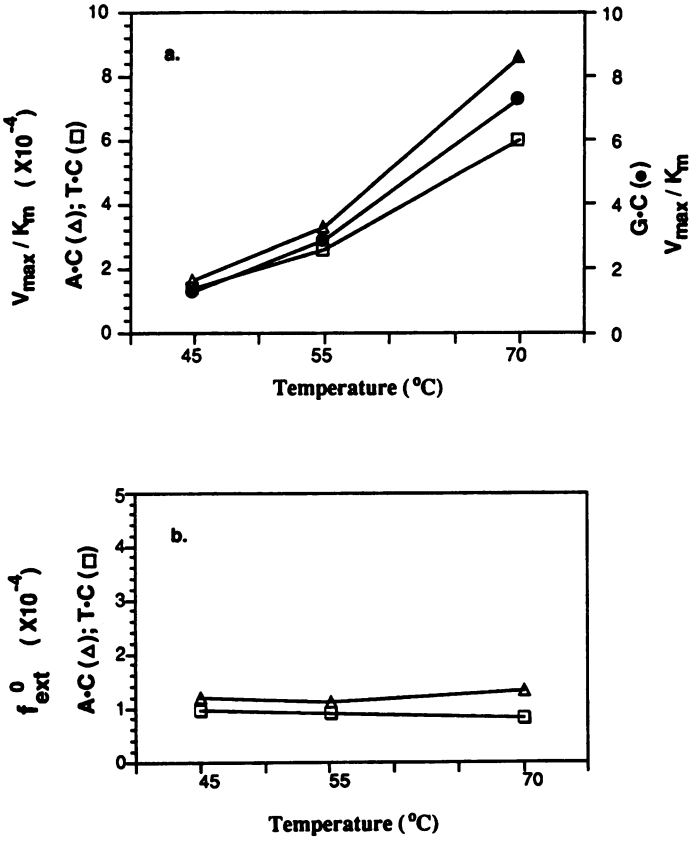

Figure 4. Extension efficiency of matched and mismatched termini as a function of temperature. (a) Extension efficiencies of A·C (open triangles) and T·C (open squares) mispairs, left hand scale, and G·C base pairs, (solid circles), right hand scale. (b) Standard mismatch extension efficiency, f^0_{ext} for A·C mispair compared to G·C base pair (open triangles) and T·C mispair compared to G·C base pair (open squares). V_{max}/K_m values are defined in arbitrary units of sec-1; see Ref. 16.

conclude that the inefficient extension of mismatched primer termini by *Taq* polymerase seems to be an intrinsic feature of the enzyme and is not caused by a differential destabilization of matched and mismatched primer termini caused by the elevation in temperature.

DISCUSSION

We have measured the extension of mismatched compared to correctly matched primer-termplate termini using *Taq* DNA polymerase, an enzyme devoid of measurable 3′ to 5′ exonuclease activity. Two aspects of the extension reaction were investigated separately: (i) the relative efficiencies of extending the 12 terminal base mispairs relative to their 4 correct counterparts (Figure 2), and (ii) the relative binding of polymerase to these mismatched and correctly matched base pairs (Table I). *Taq* polymerse appears to bind with about equal affinities to matched and mismatched primer-template 3′-termini, in agreement with earlier observations using AMV RT (16), HIV-1 RT (19), and T7 polymerase (25). However, the reverse transcriptases, which also lack proofreading exonuclease activity, appear to extend most mismatched termini more efficiently than *Taq* polymerase by about 2 to 3 orders of magnitude (15,16,19). Therefore, *Taq* polymerase appears to be ideal for discriminating between DNA templates which differ by one or a few nucleotide substitutions using PCR ('allele-specific' amplification), which is an important application of the method.

Allele-specific PCR is enhanced by DNA polymerases devoid of 3′- 5′ exonuclease activity since mismatched primer 3′-termini are extended with much lower efficiencies than correctly matched termini (13,15−17,19). However some single mismatches at 3′-primer ends are clearly not refractory to extension in PCR reactions (4,26). Kwok *et al.* (26) studied the effect of mismatches at the 3′-end of primers using *Taq* polymerase to amplify DNA using an HIV model system. Their purpose was to find conditions that allowed amplification delibrately in the presence of mismatches so that PCR conditions for finding rare variant virus genomes could be optimized. Using primers 29 base pairs in length, a primer annealing temperature of 55°C and standard PCR reaction conditions including 200 μM of each dNTP, only 4 of the possible 12 mismatches were inefficiently extended, C·C, G·A, A·G and to a lesser extent A·A. PCR product was readily detectable after 30 cycles in the case of all other mismatches.

The measurements of 'standard' mismatch extension efficiencies, f^0_{ext}, Eq. (1), are relevant to the allele selective amplification data of Kwok et al. (26). We showed that although all 12 possible mismatched primer-template complexes have approximately the same binding affinity for *Taq* polymerase, large differences exist in the efficiency with which they are elongated. No detectible extension of C·C, G·A and A·G mismatches were observed, $f^0_{ext} < 10^{-6}$, while the A·A mismatch was extended with an efficiency of $f^0_{ext} \sim 2\times10^{-6}$ (Figure 2). A discrepancy between kinetic and PCR data is that G·G mismatches produced PCR product with the same efficiency as G·C or C·G matches while the kinetic analysis gave an extension efficiency of G·G mismatches $< 10^{-6}$ (Figure 2), similar to that found for C·C, G·A, A·G mismatches. To account for this discrepancy we note that in the PCR experiment the position of the mismatch was followed by a 'downstream' C in the template strand; in this configuration, DNA containing a mismatched primer terminus may undergo transient local misalignment resulting in the primer G being 'correctly paired' with the adjacent template C located next to the mispair. As shown by Kunkel and coworkers (e.g., see Ref. 17), local misalignment of primer-template DNA resulting in a transient conversion base mispairs to correct pairs, is an important cause of mutagenic hot spots.

Table II. Summary of the Prognosis for Allele-specific Amplification

ALLELES 1 2	MISMATCHES	PROGNOSIS FOR ALLELE-SPECIFIC AMPLIFICATION
A•T T•A	A•A AND T•T	EXCELLENT FOR A•A, GOOD FOR T•T
C•G G•C	C•C AND G•G	EXCELLENT FOR BOTH
C•G T•A	A•C AND G•T	GOOD
	OR	
	T•G AND C•A	GOOD
G•C T•A	A•G AND C•T	EXCELLENT FOR A•G, POOR FOR C•T
	OR	
	T•C AND G•A	EXCELLENT FOR G•A, GOOD FOR T•C
A•T G•C	C•A AND T•G	GOOD
	OR	
	G•T AND A•C	GOOD
A•T C•G	G•A AND T•C	EXCELLENT FOR G•A, GOOD FOR T•C
	OR	
	C•T AND A•G	EXCELLENT FOR A•G, POOR FOR C•T

All of the possible allelic differences are shown on the left side of the table. For some allelic differences, there are two different possible sets of primers depending upon which of the two strands of the DNA are used as template for extension of the allele specific primers. A·T represents A(primer) · T(template); T·A represents T(primer) · A(template). Excellent, Good and Poor refer to an f^0_{ext} of $<10^{-5}$, 10^{-5}-10^{-3}, $> 10^{-3}$ respectively.

A number of systems for allele-specific PCR have been developed and make use of several different strategies (see review by Ugozzoli and Wallace, Ref. 4). A mismatch at the 3′-end of a primer will lower DNA melting temperature; shortening the length of both the matched and mismatched primers can enhance the difference with which matched and mismatched primers are extended under PCR conditions. Melting temperature effects can be further enhanced by placing additional mismatches in the primer close to the 3′-end. We have shown that mismatch extension efficiencies decrease with decreasing dNTP concentrations and decreasing polymerase processivity (15). Thus, allele specific amplification should be most effective when carried out with a relatively nonprocessive polymerase at low 'next correct' dNTP concentration.

Two groups have made use of reduced dNTP concentrations to enhance mismatch extension for allele-specific PCR (8,9). In both PCR studies, all 4 nucleotides were lowered. However, lowering one or all of the dNTPs reduces PCR efficiency, and additional cycles are required to produce enough product. The discrepency noted above comparing kinetic and PCR data for extension of G·G mismatches exists only at high nucleotide concentration (200 μM dNTP). When the dGTP concentration was reduced to 50 μM, the G·G mismatch was amplified poorly (26). At 6 μM of each dNTP only correct matches were amplified, although some product was detected for the T·G mismatch (26). The kinetic data show that T(primer)·G has a high standard extension efficiency, $f^0_{ext} \sim 10^{-3}$ (Figure 2).

It would be desirable to choose mismatches with small f^0_{ext} values when designing allele-specific PCR experiments. As a general 'rule of thumb', Table II, showing all of the possible mismatches in both primer and template, can be used as an aid

in selecting primer-template pairs to optimize allele specific amplification. Generally, it is extremely difficult to extend A·A, G·G, C·C, G·A and A·G mismatches. Based on previous mismatch extension data using AMV RT (15), we expected to find inefficient extension for the C(primer)·T mismatch. However, as shown in Figure 3, the C(primer)·T mismatch is extended with **highest** efficiency by *Taq* polymerase.

Although it is possible that efficient extension of C·T mispairs may be an inherent property of *Taq* polymerase, it is perhaps more likely the elevated rate of C·T extension may result from undefined effects of surrounding DNA sequence. It is well documented that nucleotide misinsertion and mismatch extension efficiencies can vary significantly (~ 5 to 100-fold) in different sequence contexts (27). Another factor that might cause significant perturbations in mispair extension efficiencies is solution pH. Extension of A·C mispairs, which are known to exist in a protonated a wobble configuration (28), could be influenced directly by a change in pH. A loss of a proton with increasing pH should destabilize the mispair (29) resulting in reduced A(primer)·C and C(primer)·A extension efficiencies.

To reduce extension of the most stable mismatches, e.g. G·T, T·G, it is advisable to use the lowest possible dNTP concentrations compatible with the requirement to obtain PCR product; additional stratgies to inhibit mismatch extension are discussed in reference 4, including adding several mismatches proximal to the 3′-primer terminus or shortening primer length. Special consideration should also be given to the identity of the 'downstream' template base to avoid primer-template misalignment driven mismatch extension. Our results suggest that changes in temperature are unlikely to have a significant effect on mismatch extension efficiencies for polymerases devoid of proofreading activity. Extension efficiencies for both matched (G·C) and mismatched (A·C and T·C) base pairs appear to change in a similar manner as a function of temperature (Figure 4), resulting in values of f^0_{ext} that are essentially constant.

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