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DNA Polymerase Insertion Fidelity

GEL ASSAY FOR SITE-SPECIFIC KINETICS*

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A quantitative assay based on gel electrophoresis is described to measure nucleotide insertion kinetics at an arbitrary DNA template site. The assay is used to investigate kinetic mechanisms governing the fidelity of DNA synthesis using highly purified *Drosophila* DNA polymerase α holoenzyme complex and M13 primer-template DNA. K_m and V_{max} values are reported for correct insertion of A and misinsertion of G, C, and T opposite a single template T site. The misinsertion frequencies are 2×10^{-4} for G•T and 5×10^{-5} for both C•T and T•T relative to normal A•T base pairs. The dissociation constant of the polymerase-DNA-dNTP complex, as measured by K_m, plays a dominant role in determining the rates of forming right and wrong base pairs. Compared with K_m for insertion of A opposite T (3.7 ± 0.7 μM), the K_m value is 1100-fold greater for misinsertion of G opposite T (4.2 ± 0.4 mM), and 2600-fold greater for misinsertion of either C or T opposite T (9.8 ± 4.2 mM). These K_m differences indicate that in the enzyme binding site the stability of A•T base pairs is 4.3 kcal/mol greater than G•T pairs and 4.9 kcal/mol greater than C•T or T•T pairs. In contrast to the large differences in K_m, differences in V_{max} are relatively small. There is only a 4-fold reduction in V_{max} for insertion of G opposite T and an 8-fold reduction for C or T opposite T, compared with the correct insertion of A. For the specific template T site investigated, the nucleotide insertion fidelity for *Drosophila* polymerase α seems to be governed primarily by a K_m discrimination mechanism.

Gel electrophoresis in conjunction with DNA sequencing strategies makes it possible to resolve each base in an arbitrary DNA sequence and permits the analysis of a wide variety of localized phenomena including site-specific mutations. Sequencing gels have recently been used to detect base substitution errors by DNA polymerase *in vitro* (1-3). In the present study of polymerase fidelity, we develop a quantitative gel assay to measure kinetic parameters (V_{max} and K_m) for nucleotide insertion at arbitrary single sites along a DNA template strand. We illustrate the method with *Drosophila* DNA polymerase α (4) for the misinsertion of G, T, and C opposite a template T site relative to normal insertion of A, on primed M13 DNA. The misinsertion frequency is evaluated in terms of relative V_{max} and K_m values found with each nucleotide substrate. Recently, we have used this assay to measure nucleotide misinsertion kinetics of *Drosophila* polymerase α opposite template abasic (apurinic/apyrimidinic) sites (5).

The kinetic measurements are compared with the predictions of a model for DNA synthesis fidelity based on K_m discrimination (6-9). In this model, the relative rates of inserting matched *versus* mismatched nucleotides are governed by the relative residence times of dNTP substrates in the enzyme-DNA-dNTP complex, and the fidelity of DNA synthesis is attributed primarily to a much higher dissociation constant for wrong *versus* right dNTP substrates in the enzyme complex. Differences in catalytic rate constants for insertion resulting in V_{max} discrimination are assumed to play a much less significant role in fidelity. In K_m discrimination, the degree of fidelity is dependent on free energy differences between right and wrong base pairs at the binding site of polymerase (10) as opposed to mechanisms involving nucleotide selection or rejection at the catalytic site.

EXPERIMENTAL PROCEDURES

Materials—Purified *Drosophila* DNA polymerase α, consisting of at least three polypeptide subunits including primase (4), was a generous gift of Dr. I. R. Lehman, Stanford University. The primer, a 23-base deoxynucleotide (5′-GGCCTTGATATTCACAAACGAAT-3′) complementary in sequence to bases 2248-2225 in wild-type M13 DNA (11), was synthesized by conventional solid-phase methods in an automatic Microsyn 1460 synthesizer (Systec, Inc.) and was provided by Dr. R. E. Eritja, City of Hope. The template was single-stranded DNA isolated from wild-type M13 phage grown in *Escherichia coli* strain JM103. HPLC[1]-purified dNTP substrates were purchased from Pharmacia Biotechnology, Inc.

There seemed to be no detectible cross-contamination of any of the dNTPs. The absolute level of cross-contamination of a given dNTP with another was less than 1 part in 10^5. This upper bound was established by HPLC chromatography of each dNTP substrate, first alone and then in the presence of added dNTP "contaminant" at a concentration just sufficient for detection. The ratio of detectable contaminant to substrate concentration was used to define the upper bound. AmPur deoxynucleotide (dAmPurTP) was prepared as described (7). Radioactive nucleotides, [γ-^{32}P]ATP and [α-^{32}P]dATP, were purchased from ICN Radiochemicals; T_4 polynucleotide kinase, from U. S. Biochemicals.

Primer End-labeling—Primer 5′ termini were labeled with ^{32}P in a reaction buffer (100 μl) of 56 mM Tris·HCl (pH 7.7), 7 mM $MgCl_2$ and 13 mM dithiothreitol, containing 170 nM primer oligonucleotide, 0.4 μM [γ-^{32}P]ATP (4500 Ci/mmol), and 5 units of T_4 polynucleotide kinase. The solution was incubated at 37 °C for 1.5 h and the reaction was terminated by heating at 100 °C for 5 min.

Primer-Template Annealing—The ^{32}P-labeled primer was annealed to M13 DNA template in an annealing buffer (230 μl) of 50 mM Tris·HCl (pH 8.0), 2 mM β-mercaptoethanol, and 50 μg/ml acetylated bovine serum albumin, containing 74 nM primer and 81 nM template. The solution was incubated at 100 °C for 6 min and then cooled slowly to room temperature.

Based on a computer analysis of the base sequence in M13 DNA,

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[1] The abbreviations used are: AmPur, 2-aminopurine; dAmPurTP, 2-aminopurine deoxyribonucleoside triphosphate; HPLC, high performance liquid chromatography.

the primer was expected to form productive hybrids only with the site (bases 2225–2248) to which it was perfectly homologous. No other 23-base sequence showed greater than 60% homology with the primer. Furthermore, no other sequence showed perfect homology to the last six bases at the 3′-end of primer. In our experiments, therefore, primer extension by DNA polymerase was unlikely to occur at any other site in M13 DNA.

DNA Polymerase Reactions—Equal volumes of solution A containing enzyme-primer-template complex and solution B containing dNTP substrates were mixed to start polymerization reactions for kinetic studies. Solution A was made by adding 2 μl of *Drosophila* polymerase α stock solution (2,000 units/ml) and 9 μl of concentrated bovine serum albumin (25 mg/ml) to 80 μl of the original primer-template annealing solution. Solution B contained various concentrations of dNTPs in 52 mM Tris·HCl (pH 8.0), 16 mM $MgCl_2$, and 5 mM dithiothreitol. A 10-μl reaction mixture of solutions A and B contained approximately 0.2 unit of polymerase activity (specific activity, 50,000 units/mg, a unit being capable of incorporating 1 nmol of dNTP into acid-soluble material in 60 min at 37 °C (4)). The estimated ratio of primer-template to enzyme is 30:1. The reaction mixture was incubated at 37 °C for time periods of 1 or 4 min. Reaction was terminated by adding 20 μl of 20 mM EDTA in 95% formamide.

Reaction conditions for time course experiments to determine a suitable reaction time for kinetic studies were similar except that reaction times varied between 1 and 16 min.

Gel Electrophoresis and Autoradiography—Samples (5 μl) of DNA polymerase reaction mixture were denatured at 100 °C for 5 min, cooled on ice, loaded on 16% polyacrylamide gel (30 × 40 cm × 0.4 mm) containing 8 M urea, and electrophoresed for 4 h at 2000 V (50 V/cm) to obtain good resolution of extended primers. For autoradiography of the ^{32}P-labeled primer bands, gels were vacuum-dried on Whatman No. 3MM filter paper and overlayed with blue medical x-ray film (Kodak GPB-1) for 1–12-h exposures.

Densitometry—The bands in each lane of a gel autoradiograph were scanned on a Hoeffer GS300 densitometer at 6.5 cm/min to obtain maximum resolution of absorbance changes. Band intensities were evaluated with a Hoefer GS350 data system by integrating the area under the absorbance curve for each band, above a base line drawn by linearly connecting points of minimum absorbance on each side of the band.

Calibration against a photographic step tablet purchased from Kodak showed the densitometer readings to be proportional to absorbance up to $A = 2.0$. Varying quantities of ^{32}P-labeled primer were run on a gel and exposed over 3-h to 2-day time periods to determine the range over which film response (absorbance after development) was linearly related to counts of radiation. Exposed films were selected so that peak absorbance for each labeled primer and product band lay within the linear response range of the film. Plots of film exposure time *versus* integrated band intensity in arbitrary units showed (*a*) linearity up to 12,000 units, representing a peak absorbance around 2.0 and band width around 6 mm, and (*b*) a threshold value of 150 units. Accordingly, a band intensity measured as n units was assigned a corrected value of $n + 150$ units. In kinetic experiments, film exposure time was selected so that the highest band intensity measured on film did not exceed 10,000 units.

Competition Assay—The same primer-template and DNA polymerase conditions were used except that primer was not labeled and added substrates included 0.75 μM [α-^{32}P]dATP (3000 Ci/mmol). The intensity (I) of the [α-^{32}P]dAMP-labeled primer band(s) after 16 min reaction time was determined by gel electrophoresis, autoradiography, and densitometry as above. The initial intensity (I^0) was determined with 50 mM dGTP as the only other substrate. This substrate allowed primer to be extended to the site at which [α-^{32}P]dAMP was inserted opposite template T. The decrease in primer band intensity ($I^0 - I$) with added dNTP was used to determine the misinsertion frequency, f, for substrate dNTP relative to dATP at the template T site.

The ranges of unlabeled dNTP substrates used to compete out [α-^{32}P]dATP were: 0.7–5.0 μM dATP, 0.7–2.2 mM dGTP, 1.5–7.5 mM dCTP, and 4 μM–6 mM dTTP. Low concentrations of dTTP were used to observe the kinetics of extending the primer template to the template A site next to the target T (Fig. 1, *site 3*).

RESULTS

The experimental design allows one to compare the kinetics of inserting right *versus* wrong nucleotides at a single target site along a DNA template strand. Here we investigate the insertion of A, G, C, and T opposite a template T site (Fig. 1) using highly purified *Drosophila* DNA polymerase α, which is devoid of any detectible 3′ → 5′ proofreading exonuclease activity when present as an intact polymerase-primase holoenzyme complex (4, 12). The target T site, designated as *site 3*, is located three bases downstream from the original primer. Before reaching the target site, polymerase must first insert two properly paired Gs at sites 1 and 2. The primer is labeled with ^{32}P at its 5′-end so that primers extended by one, two, or three nucleotides can be observed as discrete bands on an autoradiograph of the polyacrylamide gel.

The experimental objective is to measure v_{23}, the velocity of primer extension from site 2 to site 3, for correctly paired and mispaired nucleotide substrates. The expression relating v_{23} to band intensities on the gel (see "Theoretical Analysis," below) is

$$v_{23} = \frac{I_2 + I_3}{t}\left(\frac{I_3}{I_2}\right) \quad (1)$$

where I_2 and I_3 are the integrated intensities at sites 2 and 3, expressed as percentage of total primer, and t is the reaction time (1–16 min). With 50 μM dGTP in the reaction mixture, $(I_2 + I_3)$ is close to its maximum value at a given t and does not change significantly with added dNTP (including additional much larger amounts of dGTP). When t is held constant and $(I_2 + I_3)$ stays constant over the range of dNTP substrate concentrations used (Table I), the I_3/I_2 term in Equation 1 measures the *relative* value of v_{23} at each substrate concentration.

An advantage in using $v = I_3/I_2$ in place of v_{23} (Equation 1) is that there is no need to correct for loading error (inadvertant loading of different sample volumes on the gel). Proper evaluation of v_{23} requires normalization by dividing by the total integrated intensity of all bands on the gel $(I_0 + I_1 + I_2 + I_3)$. Usually, two gel exposures are needed to photograph the very dark primer band (I_0) and much lighter extension bands within the linear region of the film. However, I_3/I_2 can be evaluated without normalization or dual exposure. As shown below, I_3/I_2 at $t = 4$ min provides a suitable relative measure of v_{23} for kinetic studies.

Time Course Studies—The evolution of each band intensity as a function of t up to 16 min is illustrated in Figs. 2 and 3. When 3 μM dATP is used for insertion of A opposite T (A·T) in site 3, along with 50 μM dGTP for insertion of G opposite C (G·C) in sites 1 and 2, there is a rapid increase in I_3 (Figs. 2*a* and 3*a*). Note that band 3 accumulates continuously because flow occurs into but not out of this site. At sites 1 and 2, flow occurs both into and out of each site. At site 1, very little net accumulation of band intensity is observed (Fig. 3), because in and out flows are similar and both involve G·C.

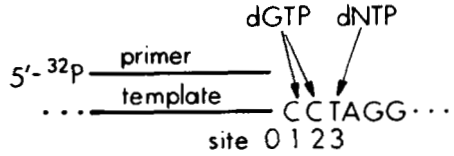

FIG. 1. **Diagram of system used to measure enzymatic rates of insertion for matched and mismatched dNTP substrates opposite base T in template.** The synthetic primer strand is 23 nucleotides long, labeled at the 5′-end with ^{32}P and annealed to a complementary section (bases 2248–2225) of circular M13 DNA template. *Site 0* is the 3′-end of primer opposite base 2225 in M13 DNA. Substrate dGTP (50 μM) is added to allow primer extension from *site 0* to *site 2*, by insertion of G opposite C in *sites 1* and *2*. Substrate dNTP (various concentrations) is added to allow insertion of N opposite T in *site 3*.

TABLE I

Examples of gel assay results showing the behavior of the insertion velocity (v_{23}) as a function of time and substrate concentration

The value of v_{23} is the product of $(I_2 + I_3)/t$ and I_3/I_2, as measured on gels. Experimental data are shown for substrate dCTP at concentrations of (*a*) 1 mM, (*b*) 3 mM, and (*c*) 6 mM, in the presence of 50 μM dGTP. At each dCTP concentration, as t is increased from 2 to 16 min, one observes that I_3/I_2 increases while $(I_2 + I_3)/t$ decreases, so that their product v_{23} is nearly constant. On the other hand, if t is held constant as [dCTP] is increased from (*a*) to (*c*), $(I_2 + I_3)/t$ stays nearly constant so I_3/I_2 itself becomes a relative measure of v_{23}. The value of I_3/I_2 at $t = 4$ min is used to measure relative velocity (v) for V_{max} and K_m determinations (Table II).

Concentration	t	$(I_2 + I_3)/t$	I_3/I_2	v_{23}
	min	*% min*$^{-1}$		*% min*$^{-1}$
(*a*)	2	14.2	0.079	1.12
	4	11.2	0.093	1.04
	8	7.5	0.132	0.99
	16	5.1	0.197	1.00
(*b*)	2	14.1	0.137	1.92
	4	10.5	0.179	1.87
	8	7.7	0.265	2.04
	16	5.1	0.429	2.20
(*c*)	2	15.4	0.221	3.40
	4	10.8	0.315	3.40
	8	7.7	0.481	3.70
	16	5.3	0.726	3.85

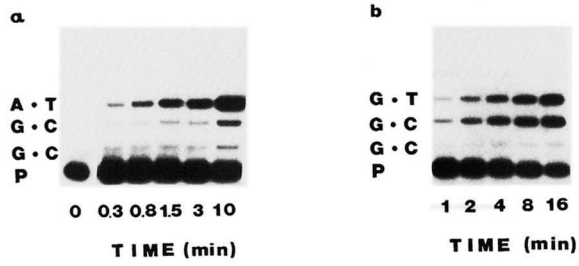

FIG. 2. **Gel autoradiograms showing bands arising by primer extension with *Drosophila* DNA polymerase α as reaction time increases.** The substrate added for insertion opposite T in the template (Fig. 1) is *a*, dATP (3 μM), and *b*, dGTP (3 mM). Shown *above* the unextended primer band (*P*) in each lane are two extended primer bands (labeled *G·C*) arising by addition of G opposite C in sites 1 and 2, followed by a third band labeled *A·T* (*a*), arising by insertion of A opposite T in site 3, or *G·T* (*b*), arising by misinsertion of G opposite T in this site.

If instead of adding 3 μM dATP one adds much more dGTP (3 mM), then G is misincorporated in place of A in site 3 (Figs. 2*b* and 3*b*), but at a slower rate. Comparing Fig. 3*b* with Fig. 3*a*, one sees that at each time point I_3 is now lower but I_2 is higher, such that their sum $(I_2 + I_3)$ stays approximately the same. In other words, when either dATP or much more dGTP is added to 50 μM dGTP, $(I_2 + I_3)$ remains constant while I_3/I_2 changes. A similar observation is made when either a high concentration of dCTP or dTTP is added to 50 μM dGTP (Figs. 3*c* and 3*d*).

Kinetic Studies: v versus [dNTP]—The behavior of v_{23} is examined as a function of the concentration of dNTP substrate used for incorporation opposite T in site 3 of the primer-template system (Fig. 1). With 50 μM dGTP in the reaction mixture, DNA polymerase α incorporates G opposite C in sites 1 and 2 at near maximum velocity, without causing detectible incorporation of G opposite T in site 3. At each concentration of added dNTP, v_{23} as found by Equation 1 remains essentially constant for t up to 8 min in the case of dATP and up to 16 min in the case of other substrates, *e.g.* dCTP (Table I). This result indicates that the enzyme-DNA-substrate complex at site 3 is in steady state for such time

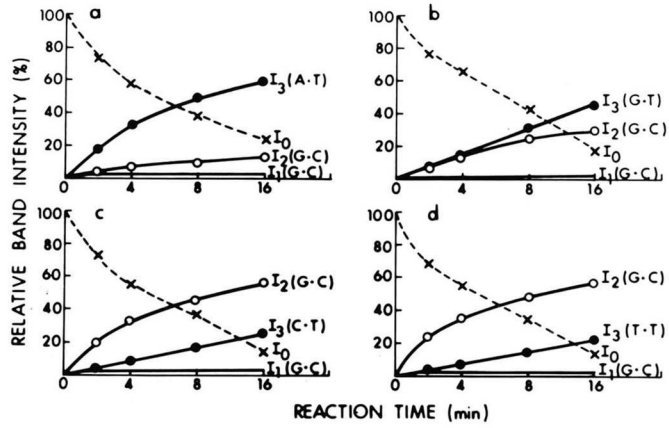

FIG. 3. **Gel band intensity changes as a function of reaction time.** Relative intensity values for each band (I_0, I_1, I_2, I_3), shown as percentage of integrated intensity of all bands, are plotted *versus* reaction time. The dNTP substrate added in each case, for insertion opposite T, is *a*, dATP, 2 μM; *b*, dGTP, 2 mM; *c*, dCTP, 2 mM; *d*, dTTP, 2 mM. Extension of the ^{32}P-labeled primer molecules by *Drosophila* DNA polymerase α occurs by the addition of G opposite C in site 1 to give I_1 (G·C) (*lowest solid line*), and in site 2 to give I_2 (G·C) (*open circles*), followed by addition of N opposite T in site 3 to give I_3 (N·T) (*filled circles*). The *dashed curve* I_0 shows original primer band disappearing with increasing time. In each case, except *b*, there is 50 μM dGTP present in the reaction mixture to obtain close to maximum rates of G insertion opposite C in sites 1 and 2. This amount of dGTP is insufficient to contribute measurable insertion of G opposite T in site 3.

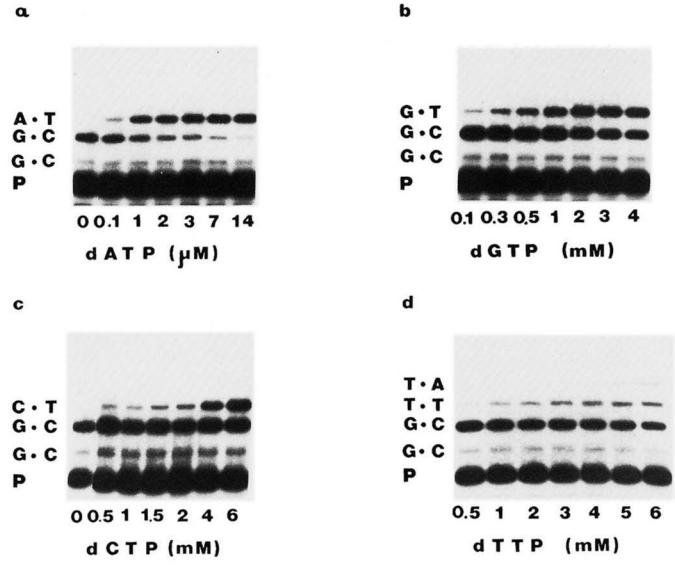

FIG. 4. **Gel autoradiogram showing band intensities as a function of added dNTP substrate.** DNA polymerase reactions are run for 4 min at varying concentrations of *a*, dATP; *b*, dGTP; *c*, dCTP; and *d*, dTTP. In each case except *b*, there is 50 μM dGTP present in the reaction mixture to obtain close to maximum rates of G insertion opposite C (G·C) in the first two sites without significant insertion opposite T in the third site. In case *d*, DNA polymerase α inserts a small amount of T opposite A following insertion of T opposite T, as seen by a faint T·A band following T·T.

periods. Accordingly, $t = 4$ min is a suitable reaction time for K_m and V_{max} determinations.

The relative value of v_{23}, as measured by $v = I_3/I_2$ at $t = 4$ min, varies with dNTP concentration as shown (Figs. 4 and 5). The plot of v *versus* [dNTP] shows typical saturation kinetics for each nucleotide N = A, G, and AmPur (Fig. 5). The relationship conforms to a Michaelis-Menten equation, as indicated by linearity in a Hanes-Woolf plot of [dNTP]/v

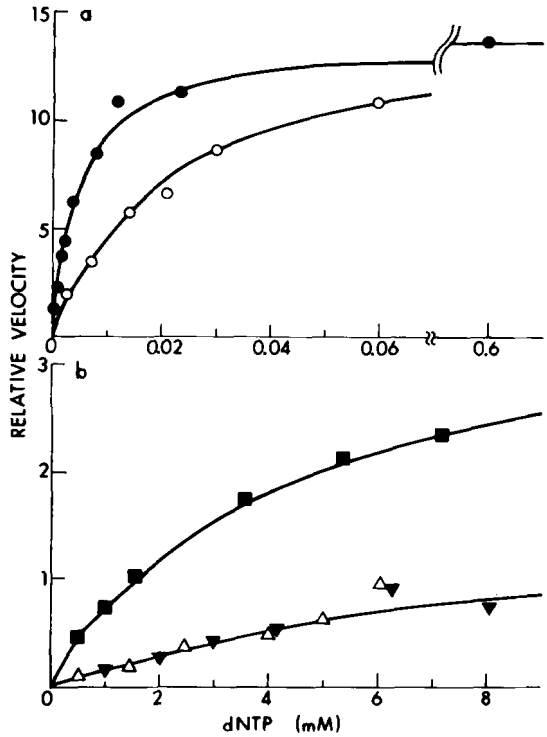

FIG. 5. **Plot showing relative velocity, $v = I_3/I_2$ measured on gels, as a function of dNTP concentration.** In addition to 50 μM dGTP, for insertion of G opposite C in sites 1 and 2, the following substrate is provided for insertion opposite T in site 3: *a*, dATP (●), dAmPurTP (○); *b*, dGTP (■), dCTP (▼), dTTP (△). Reaction time with *Drosophila* polymerase α is 4 min.

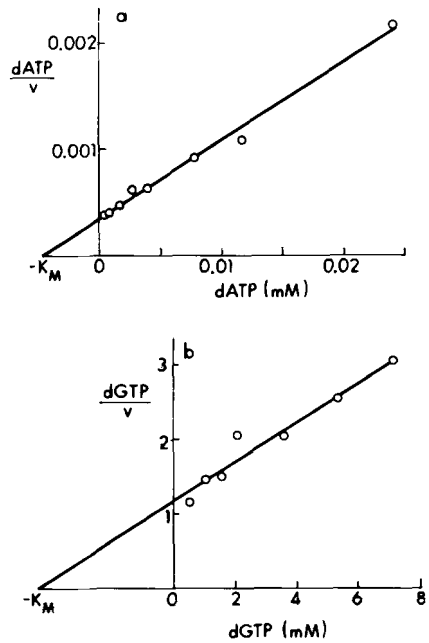

FIG. 6. **Hanes-Woolf plot to determine K_m and V_{max} for nucleotide insertion opposite T.** Variable substrates are: *a*, dATP; *b*, dGTP. The lines were obtained by a linear least squares fit to data obtained by evaluating $v = I_3/I_2$ for 4 min reactions with *Drosophila* polymerase α.

versus [dNTP] (Fig. 6) or Lineweaver-Burk plot of $1/v$ *versus* 1/[dNTP] (not shown). From a Hanes-Woolf plot, by linear least squares fit, we determine V_{max} corresponding to the maximum value of I_3/I_2 and K_m corresponding to the value of [dNTP] when I_3/I_2 is at half-maximum.

The K_m value found for misinsertion of G opposite T (4.2 mM) is 1100 times greater than the value (3.7 μM) for correct insertion of A opposite T (Table II). In contrast, the V_{max} value is less by only a factor of 4, being 3 for dGTP compared with 13 for dATP (Table II).

The ratio, V_{max}/K_m, which is the initial slope in a plot of v *versus* [dNTP] (Fig. 5), measures the efficiency of nucleotide insertion by polymerase (13). The ratio of the insertion efficiencies for wrong *versus* right base pairs indicates the *misinsertion frequency* (f) and the *fidelity* ($1/f$) of polymerase. Our results (Table II) show *Drosophila* DNA polymerase α has $f = 2.1 \times 10^{-4}$ for misinsertion of G opposite T in the site selected on M13 DNA (Fig. 1).

The f value for transversion mismatches C·T and T·T is about one-fourth that for the transition mismatch G·T (Table II). The observation of only a slight curvature in v with increasing [dCTP] or [dTTP] (Fig. 5) allows only rough estimates of K_m and V_{max} for these substrates. On the basis of Hanes-Woolf and Lineweaver-Burk plots, we estimate K_m ~10 mM and relative V_{max} ~1.7 in each case (Table II), *i.e.* K_m about 2-fold higher and V_{max} about 2-fold lower than those found for dGTP in the target T site.

It is important to point out that fidelity values deduced from V_{max}/K_m for correct *versus* incorrect insertions are based on measurements carried out by varying the concentration of a particular dNTP in the *absence* of its competitor, *e.g.* insertion of G in the absence of A. Thus, it is important to verify that a similar result would be obtained if G and A were present together in direct competition with each other for insertion opposite T.

To carry out a direct competition assay using the gel system, we start with the same primer template configuration (Fig. 1) and use 50 μM dGTP for primer extension to site 2, but use unlabeled primer and 0.75 μM [α-^{32}P]dATP as labeled substrate for insertion of A opposite T in site 3. Insertion of labeled A results in a band whose integrated intensity (I^0) can be measured as before. In the presence of added dNTP, the total band intensity (I) diminishes as N competes with A for insertion opposite T (data not shown).

The ratio of [α-^{32}P]dATP concentration to the dNTP concentration when $I = \frac{1}{2} I^0$ provides a measure of the misinsertion frequency (f) for dNTP relative to dATP. The following

TABLE II

Relative V_{max} and K_m values obtained by gel assay for dNTP substrates used for insertion opposite T

The relative velocity of insertion was measured as the ratio of band intensities, $v = I_3/I_2$, observed by gel electrophoresis after 4 min reaction time. A Hanes-Woolf plot of [dNTP]/v *versus* [dNTP] was fitted linearly by least-squares to determine the intercept (K_m/V_{max}) and slope ($1/V_{max}$). Shown are the mean K_m and V_{max} values ± S.D. found in repeated experiments with each substrate (number in parentheses). Also shown is the corresponding insertion efficiency, V_{max}/K_m, and resultant misinsertion frequency (f) relative to dATP. The f values obtained by competition assay are shown in parentheses for comparison.

Substrate		K_m	V_{max} $(I_3/I_2)_{max}$	V_{max}/K_m	f
		μM		M^{-1}	
dATP	(6)	3.7 ± 0.7	12.9 ± 1.7	3.5×10^6	1.0 (1.2)
dAmPurTP	(2)	35 ± 5	13.0 ± 2.0	3.7×10^5	1.1×10^{-1}
dGTP	(9)	4,200 ± 900	3.1 ± 0.2	7.4×10^2	2.1×10^{-4} (3.1×10^{-4})
dTTP	(3)	10,000 ± 4,000	1.7 ± 0.4	1.7×10^2	4.9×10^{-5} (6.7×10^{-5})
dCTP	(3)	10,000 ± 4,000	1.7 ± 0.4	1.7×10^2	4.9×10^{-5} (4.3×10^{-5})

equation is used to describe I as a function of dNTP concentration:

$$\frac{I^0 - I}{I} = f\frac{[\text{dNTP}]}{[[\alpha\text{-}^{32}\text{P}]\text{dATP}]} \quad (2)$$

where I^0 is the value of I when [dNTP] = 0. In each experiment, $[[\alpha\text{-}^{32}\text{P}]\text{dATP}]$ is held constant as [dNTP] is increased, so that a linear relationship is observed between $\frac{I^0 - I}{I}$ and [dNTP]. The slope of the line is used to determine f. Note that f is also the ratio of $[[\alpha\text{-}^{32}\text{P}]\text{dATP}]$ to the value of [dNTP] when $I = \frac{1}{2} I^0$. The f values shown in brackets in Table II are obtained by least squares fits to Equation 2 over [dNTP] ranges given for the competition assay under "Experimental Procedures." Within experimental error, these results agree with the corresponding evaluations from V_{max} and K_m measurements (Table II).

A significant amount of data has been published comparing the insertion of the mutagenic base analogue AmPur in competition with A in different sequences (14, 15), and K_m and V_{max} measurements comparing AmPur with A have also been made (7). We find, using the gel assay, that V_{max} values for AmPur and A are equal (Fig. 5 and Table II), consistent with the standard DNA polymerase assay (7). The gel assay also shows a 10-fold higher K_m for AmPur than for A, in agreement with expectations when G is present as the 5′-nearest neighbor base on the primer strand (16).

THEORETICAL ANALYSIS

Target Velocity—Consider the DNA primer-template system illustrated in Fig. 1. With dGTP present at 50 μM concentration, DNA polymerase adds G to primer rapidly in sites 1 and 2, but only very slowly in site 3. The objective is to derive an expression for the velocity of insertion at target site 3.

With [dGTP] = 50 μM, primers are extended from 0 to 2 at close to maximum velocity, because K_m for G insertion opposite C is only about 5 μM. As [dGTP] or any other [dNTP] is increased, the velocity for 0–2 extension (v_{02}) remains nearly constant, while that for 2–3 extension (v_{23}) increases. To derive a general expression for v_{23}, we proceed as follows. Note that this derivation makes no assumptions about the processivity of polymerase.

Consider the time required on average to extend a primer molecule from 0 to 3, namely τ_{03}. This is equal to the time from 0 to 2 (τ_{02}) plus the time from 2 to 3 (τ_{23}).

$$\tau_{03} = \tau_{02} + \tau_{23} \quad (3)$$

Because times for elongation are inversely related to velocities, one can also write the following.

$$1/v_{03} = 1/v_{02} + 1/v_{23} \quad (4)$$

Rearrangement of Equation 3 leads to the following expression for the velocity at the target site.

$$v_{23} = (v_{02})(v_{03})/(v_{02} - v_{03}) \quad (5)$$

Velocity Measurements—The reaction is started by adding a certain concentration of substrate, [S] = [dGTP] for example, to a fixed amount of enzyme and preformed primer-template complex with ^{32}P on the 5′-end of primer. After a short reaction time (t), EDTA is added to stop the reaction. The amount of primer extension in this time period is observed by gel electrophoresis, autoradiography, and densitometry. In addition to band 0, corresponding to unreacted primer, one observes bands 1, 2, and 3. We will now show that v_{23}, the velocity of primer extension from site 2 to 3, is proportional to I_3/I_2, where I_2 and I_3 are the integrated intensities of bands 2 and 3, respectively.

For primer extension from 0 to 3 the velocity corresponds to I_3 divided by reaction time.

$$v_{03} = I_3/t \quad (6)$$

On the other hand, v_{02} is given by

$$v_{02} = (I_2 + I_3)/t \quad (7)$$

since every primer that has reached position 3 has also reached position 2. Substituting Equations 6 and 7 in Equation 5, we find Equation 1 or its equivalent, Equation 8.

$$v_{23} = v_{02}(I_3/I_2) \quad (8)$$

Now at [dGTP] = 50 μM, v_{02} is already near maximum and changes very little as either [dGTP] or any other [S] is increased. As seen in Table I for the case of S = dCTP, the value of $[I_2 + I_3]/t$ at each t does not change significantly with increasing [S]. As long as this condition holds, the ratio I_3/I_2 is itself an appropriate relative measure of v_{23}. Furthermore, the relative velocity, $v = I_3/I_2$, has the advantage of being insensitive to loading error.

While I_2 and I_3 increase with time, I_1 remains low and nearly constant at all times in the 2–16 min range (Fig. 3). One expects I_1 to be low if the enzyme is processive, *i.e.* has low tendency to dissociate from primer template. The more processive the enzyme, the lower the value of I_1 should be. The near constancy of I_1 at 2–3% implies that polymerase α is moderately processive en route to the target site.

Kinetic Analysis—Initially, at $t = 0$, only the original primer band is present ($I_0 = 100\%$). As t increases, I_0 decreases while the extended primer bands (I_1, I_2, I_3) rise to different levels. A plateau is first reached by I_1, then by I_2, and finally by I_3 (Fig. 3). Because I_2 levels off before I_3, the ratio I_3/I_2 increases with t while $(I_2 + I_3)/t$ decreases. The product of I_3/I_2 and $(I_2 + I_3)/t$ is nearly constant for several minutes (up to t = 16 min as seen in Table I). This means that for several minutes the amount of substrate-enzyme-primer-template complex contributing to v_{23} remains in steady state (see "Appendix" for kinetic analysis). Under steady-state conditions, a Michaelis-Menten equation is expected to hold for v_{23} as [S] is increased, namely

$$v_{23} = (I_3/I_2)(I_2 + I_3)/t = V_{23}[S]/(K_m + [S]) \quad (9)$$

where V_{23} is the maximum value of v_{23} and K_m is the value of [S] when $v_{23} = V_{23}/2$. As long as $(I_2 + I_3)$ remains constant for a given t, we also expect

$$v = I_3/I_2 = V_{max}[S]/(K_m + [S]) \quad (10)$$

where $V_{max} = (I_3/I_2)_{max}$. The relationship between V_{max} and V_{23} is as follows.

$$V_{23} = V_{max}(I_2 + I_3)/t \quad (11)$$

As shown by a linear Hanes-Woolf plot of $[S]/v$ *versus* [S], Equation 10 seems to hold for [S] up to 6 mM (Fig. 6).

Comparison with Normal Base Insertion—By setting [dGTP] = 50 μM and varying [S] = [dATP], we can determine V_{max} and K_m for the insertion of A opposite T in the same site. Because V_{max} is now higher, it is desirable to use shorter reaction times. To compare V_{max} values obtained for two different time periods (a and b) we refer to Equation 11 and find

$$[V_{max}]_a/[V_{max}]_b = [(I_2 + I_3)/t]_b/[(I_2 + I_3)/t]_a \quad (12)$$

recognizing that V_{23} remains constant in the range t = 1–16 min (Table I).

Insertion of C or T opposite T—With [dGTP] set at 50 μM and [*S*] = [dCTP] or [dTTP] varied, it is theoretically possible to measure V_{max} and K_m for either C or T insertion opposite T. In practice, however, a problem arises if K_m is above 7 mM, because above this concentration substrate inhibition becomes significant. If we limit [*S*] to 7 mM, we also limit our ability to determine V_{max} and K_m. However, the ratio V_{max}/K_m can still be determined from the initial slope of v *versus* [*S*] (Fig. 5).

DISCUSSION

We have developed a quantitative assay to measure DNA polymerase insertion kinetics at any specific template site by using polyacrylamide gel electrophoresis to resolve primers extended by polymerase. The procedure makes it possible to evaluate nucleotide insertion velocity by simply measuring the intensity ratio of adjacent bands in a gel lane. Measurements at different substrate concentrations allow relative V_{max} and K_m values for correct and incorrect base pairs to be determined with natural or synthetic DNA templates and normal or analogue dNTP substrates. The present study focuses on enzymatic mechanisms underlying DNA polymerase insertion fidelity, using highly purified *Drosophila* DNA polymerase α. In future work, we intend to use the same approach to analyze the kinetic basis for base substitution mutational hot spots in DNA.

Before discussing experimental results, let us examine theoretical mechanisms by which a DNA polymerase (*E*) may discriminate between competing nucleotides for insertion opposite a given DNA template site. The enzymatic steps leading to the extension of primer DNA from *n to n* + 1 nucleotides by insertion of either a complementary (right) or noncomplementary (wrong) dNTP substrate may be described simply as follows (see "Appendix" for a more comprehensive treatment).

$$E\text{-DNA}(n) + \text{dNTP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-DNA}(n)\text{-dNTP} \xrightarrow{k_{cat}} E + \text{DNA}(n+1) + \text{PP}_i \quad (13)$$

The insertion velocity, $v = k_{cat}$ [*E*-DNA(*n*)-dNTP], obeys a classical Michaelis-Menten equation, with steady-state Michaelis constant $K_m = (k_{-1} + k_{cat})/k_1$ and maximum velocity $V_{max} = k_{cat}$ $[E\text{-DNA}(n)]_t$, where $[E\text{-DNA}(n)]_t$ is the total concentration of enzyme-DNA and enzyme-DNA-dNTP complex involved in the reaction. As a function of the concentration of enzyme-DNA and dNTP, the velocity is as follows (13).

$$v = \frac{k_{cat}}{K_m}[E\text{-DNA}(n)][\text{dNTP}] \quad (14)$$

Suppose two dNTP substrates, one right (*r*) and the other wrong (*w*) are competing for reaction under the same conditions. If $v(r)$ and $v(w)$ are their respective velocities of insertion, the misinsertion frequency (f) is given by the following.

$$f = \frac{v(w)}{v(r)} = \frac{K_m(r)}{K_m(w)} \times \frac{k_{cat}(w)}{k_{cat}(r)} = \frac{K_m(r)}{K_m(w)} \times \frac{V_{max}(w)}{V_{max}(r)} \quad (15)$$

In other words, discrimination between right and wrong dNTP substrates can occur theoretically because of K_m and/or V_{max} differences (8, 9). Base mispairs are expected to show a higher K_m because of their greater tendency to dissociate and lower V_{max} because of their less favorable geometry.

Normal base pairs are stabilized by hydrogen bonding between complementary bases and stacking interactions with neighboring bases in B DNA geometry. Base mispairs are less stable because of lack of complementary and unfavorable geometry for stacking. Hence, a mispair between a noncomplementary substrate and template target base should be relatively unstable, rapidly dissociating to release the mismatched dNTP from the enzyme-DNA complex. A higher tendency to dissociate means a higher dissociation rate constant k_{-1} and therefore a higher K_m value. An enzyme in which misinsertion frequencies are governed primarily by relative K_m values for wrong versus right dNTPs, *i.e.* K_m discrimination, achieves fidelities limited by the differences in dissociation energy for correct and incorrect base pairs in the environment of the active site (6, 9, 10).

Once dNTP is bound in the active site, a second discrimination step can occur based on geometrical constraints that affect the rate of phosphodiester bond catalysis and thereby alter the rate constant k_{cat}. Binding of a noncomplementary dNTP might occur in an unfavorable geometry. An enzyme that has very rigid geometrical constraints in the active site is capable, in principle, of attaining a higher level of fidelity than is possible by K_m discrimination alone (9). Such an enzyme should exhibit a marked decrease in V_{max} for wrong dNTPs.

Using the gel assay for the template T site on M13 DNA (Fig. 1), we compare the correct insertion of A and misinsertion of G, C, and T by highly purified *Drosophila* polymerase α. A misinsertion frequency of 2.1×10^{-4} is found for G and 5×10^{-5} for C and T (Table I). These low f values are principally the result of high K_m values for wrong base pairs. Compared with A·T (A insertion opposite T), G·T shows a 1100-fold higher K_m but only a 4-fold lower V_{max}. Similarly, C·T and T·T both show a 2600-fold higher K_m but only an 8-fold lower V_{max}. These results indicate that K_m discrimination is predominantly the mechanism determining the fidelity of DNA synthesis by *Drosophila* polymerase α.

The large K_m values for wrong nucleotides are attributed primarily to a large increase in the dissociation rate constant (k_{-1}) in Equation 13. A high k_{-1} means a low affinity between substrate base and template base. For wrong bases, k_{-1} is much larger than k_{cat}, so that K_m is very close to the dissociation equilibrium constant k_{-1}/k_1. Even for a right base, such as A opposite T, k_{-1} is likely greater than k_{cat} (6), so that K_m is not much different from the dissociation equilibrium constant.

The binding energy for a wrong base at the polymerase active site is less than that for a right base by the amount, $RT \ln[K_m(w)/K_m(r)]$. From the K_m values found experimentally (Table I), we find that the G·T mispair is 4.3 kcal mol^{-1} less stable than the normal A·T pair, whereas C·T and T·T mispairs are 4.9 kcal mol^{-1} less stable. These free energy differences are 2–4 times as large as those indicated by melting temperature differences between DNA oligomers containing matched *versus* mismatched base pairs in aqueous solution (17). We have proposed that amplifications of this magnitude may be explained by the partial exclusion of water at the polymerase active site (10).

The geometry of G·T mispairs has been shown by NMR (18) and x-ray crystallography (19) to be that of a wobble base pair, which differs slightly in dimensions from a normal A·T pair. The influence of this steric difference on catalysis (k_{cat}) could be responsible for the 4-fold reduction in V_{max} that we observe with polymerase α. Whether or not other enzymes are catalytically more sensitive to differences in base-pair geometry remains to be seen. In any event, the large difference in stability in the binding site seems to be the main reason why polymerases select A·T over the mismatches (G·T, C·T, and T·T) for incorporation into DNA. The latter two mispairs are expected to be less stable and more distorted than G·T, consistent with our finding of a further 2-fold increase in K_m and 2-fold decrease in V_{max} (Table II).

Data obtained by our gel assay seem consistent with results

obtained using a standard DNA polymerase assay in which incorporation of radioactive nucleotides into acid-insoluble DNA is measured by liquid scintillation counting. First, for *Drosophila* DNA polymerase α complex (consisting of at least three subunits including primase), K_m for the insertion of A opposite T was reported to be 3.7 μM (4), the same as obtained here (Table II). Second, we have previously shown with polymerase α from KB cells (8) and also with bacteriophage T_4 polymerase (7) that insertions of base mispairs involving the mutagenic base analogue AmPur are governed by K_m discrimination in agreement with gel assay results for *Drosophila* polymerase α (Table II and Fig. 5).

An estimate of the absolute velocity for inserting A opposite T can be deduced from the kinetic model ("Appendix"). Our estimate of k_{cat} = 2.2 nucleotides/s/enzyme is similar to a value of 2 nucleotides/s/enzyme previously determined on singly primed ϕX174 DNA for the same preparation of *Drosophila* polymerase α (20).

The misinsertion frequency of 2.1×10^{-4} found for G·T, relative to normal A·T pairs, implies that *Drosophila* α polymerase-primase complex exhibits an accuracy similar to α polymerases purified from other organisms. Loeb and Kunkel (21), using synthetic polynucleotide templates, report misinsertion frequencies (G·T) in the range $1–3 \times 10^{-4}$ for various α polymerases.

In another type of fidelity assay, a natural DNA template such as ϕX174 or M13 is copied by DNA polymerase *in vitro* and then transfected into *E. coli*, where single-site revertants (22) or different classes of forward mutants (23) are selected. *Drosophila* polymerase α has been observed to revert the *am3* site on ϕX174, by forming A·A or G·A mispairs, at a frequency of about 2×10^{-6}, similar to the reversion frequency by *E. coli* polymerase III holoenzyme complex (20). The polymerase III holoenzyme contains a $3' \rightarrow 5'$ proofreading exonuclease activity (24, 25), whereas there is no detectable exonucleolytic activity found in the intact polymerase-primase multisubunit complex of *Drosophila* polymerase α (4, 12). The fidelity of *Drosophila* polymerase inferred from the ϕX174 transfection assay seems to be about 25-fold greater than indicated in the gel assay. Although this difference in fidelities might be attributable to higher probabilities to form the mispairs C·T or T·T measured here as opposed to A·A or G·A (20), we suggest another reason for possible discrepancies in the two assays.

The reversion frequency in the ϕX174 transfection assay is determined by the product of two probabilities, the nucleotide misinsertion probability multiplied by the probability of primer extension beyond the misinsertion site to generate a nonrepaired, viable DNA molecule. Primers having a misinserted 3'-terminal nucleotide are less likely to be extended efficiently than primers having properly paired termini. Thus, error frequency measurements based on the transfection assay may be lower than "true" nucleotide misinsertion values, because DNA strands containing a mismatch may either be repaired or elongated inefficiently following transfection.

Finally, it is important to verify whether misinsertion ratios based on V_{max} and K_m values of individual dNTP substrates are similar to those obtained under conditions where competing wrong and right dNTPs are *simultaneously* present in the assay. In a competition experiment, incorporation of hot nucleotide ([α-^{32}P]dATP) opposite template T is measured by extending an unlabeled primer and evaluating the band intensity of the extended primer by gel electrophoresis and autoradiography. Increasing concentrations of cold dNTP are then added to compete with hot dATP for insertion at the target T site. The band intensity is reduced by a factor of 2 when a competing dNTP is misinserted opposite T at a rate equal to the correct insertion of A. Misinsertion frequencies obtained from the competition experiment are in agreement with those found from the V_{max} and K_m values (Table II).

APPENDIX

A kinetic model for the elongation of primer from site 0 to site 3 (Fig. 1) is desired to describe velocity v_{23} in terms of kinetic constants for individual reaction steps. The model is required to include each polymerization reaction and to allow for dissociation of polymerase from primer-template and yet be amenable to solution by the steady-state approximation. The following simple model is proposed for this purpose:

$$E + D_0 \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} ED_0$$

$$ED_0 + N_1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ED_0N_1 \xrightarrow{k_f} ED_1 + PP_i$$
$$\qquad\qquad\quad k_d \downarrow \qquad\qquad k_d' \downarrow \uparrow k_b'$$
$$\qquad\qquad\quad E + D_0 + N_1 \qquad\quad E + D_1$$

$$ED_1 + N_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ED_1N_2 \xrightarrow{k_g} ED_2 + PP_i$$
$$\qquad\qquad\quad k_c \downarrow \qquad\qquad k_c' \downarrow \uparrow k_a'$$
$$\qquad\qquad\quad E + D_1 + N_2 \qquad\quad E + D_2$$

$$ED_2 + N_3 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ED_2N_3 \xrightarrow{k_{cat}} D_3 + E + PP_i \qquad \text{(A1)}$$
$$\qquad\qquad\quad k_s \downarrow$$
$$\qquad\qquad\quad E + D_2 + N_3$$

where E represents DNA polymerase devoid of exonuclease proofreading activity. D_0 is the original primer bound to template DNA; D_1, D_2, and D_3 are primers extended by the addition of 1, 2, and 3 nucleotides, respectively. ED_0, ED_1, and ED_2 are the corresponding enzyme-DNA complexes. ED_0N_1, ED_1N_2, and ED_2N_3 are the enzyme-DNA-substrate complexes with substrates N_1, N_2, and N_3, respectively. In our experiments, N_1 and N_2 are dGTP, and N_3 is either dATP (right substrate) or dGTP, dCTP, or dTTP (wrong substrate).

The side pathways (*downward pointing arrows*) leading to dissociation of complexes ED_0N_1, ED_1N_2, and ED_2N_3 with rate constants k_d, k_c, and k_s, respectively, are assumed to be irreversible. The reverse association requires the simultaneous interaction of three components, an unlikely event. Reassociation of polymerase and extended primer (D_1 and D_2) can occur via k_b' and k_a' (*upward pointing arrows*).

The velocity of primer extension at the target site is $v_{23} = k_{cat}\,[ED_2N_3]/[D]_t$, expressed as a fraction of total primer $[D]_t$. A general expression for the steady-state concentration of ED_2N_3 is

$$[ED_2N_3] = [E]_t \left[1 + \frac{K_M}{[N_3]} + \frac{1}{C}\left(k_{cat} + k_s + \frac{k_c'\,K_M}{[N_3]}\right) \cdot \left\{\frac{1}{k_g}\left(1 + \frac{K_G}{[N_2]}\right) + \frac{(C-1)}{k_a'\,[D_2]}\left[1 + \frac{k_b'\,[D_1]}{k_f\,(B-1)}\left(1 + \frac{K_F}{[N_1]}\right)\right]\right\}^{-1}\right] \qquad \text{(A2)}$$

where

$$[E]_t = [E] + [ED_0] + [ED_0N_1] + [ED_1] + [ED_1N_2] + [ED_2] + [ED_2N_3] \qquad \text{(A3)}$$

$$K_M = \frac{k_{-3} + k_{cat} + k_s}{k_3} \qquad \text{(A4)}$$

$$K_G = \frac{k_{-2} + k_g + k_c}{k_2} \qquad \text{(A5)}$$

$$K_F = \frac{k_{-1} + k_f + k_d}{k_1} \qquad \text{(A6)}$$

$$B = 1 + \frac{k_b'\,[D_1]}{k_f k_0\,[D_0]}\left(\frac{k_{-0}\,K_F}{[N_1]} + k_f + k_d\right) \qquad \text{(A7)}$$

$$C = 1 + \frac{k_a'\,[D_2]\,(B-1)}{k_g\,k_b'\,[D_1]\,B}\left(\frac{k_d'\,K_G}{[N_2]} + k_g + k_c\right) \qquad \text{(A8)}$$

Some of the terms in Equation A2 are small enough to be neglected. Because the substrate for insertion in sites 1 and 2 is correct ($N_1 = N_2$ = dGTP), dissociation constants k_d and k_c are very small in relation to catalytic constants k_f and k_g for a processive enzyme. Furthermore, [dGTP] is set high enough (50 μM) to approach the maximum rate of incorporation in sites 1 and 2, so that $K_F/[N_1]$ and $K_G/[N_2]$ are each $\ll 1$. Also, for short time periods, the concentration of extended primers (D_1 and D_2) is very small in relation to original primer (D_0).

Other terms are sufficiently similar to be equated. Because association of polymerase with D_0, D_1, and D_2 occurs with similar rate constants, we can set $k_b' = k_a' = k_0$. Similarly, for dissociation, $k_d' = k_c' = k_{-0}$. Furthermore, because the same substrate is incorporated in positions 1 and 2, $k_g = k_f$.

A further simplification can be made by setting $k_{cat} + k_g = k_f$. This can be justified as follows. For correct insertion at site 3 (N_3 = dATP), k_{cat} is similar to k_f, whereas k_s is very small in relation to k_f. On the other hand, for incorrect insertion (N_3 = dGTP, dCTP, or dTTP), k_s should rise as k_{cat} falls. The rise in k_s, resulting from a less stable enzyme-DNA-substrate complex, may compensate the fall in k_{cat}, resulting from the less favorable geometry. Hence, $k_{cat} + k_s$ may be close to k_f even for wrong nucleotides in position 3.

The resultant simplified version of Equation A2 is given here.

$$[ED_2N_3] = [E]_t \left[3 + \frac{k_f}{k_0[D_0]} + \frac{K_M}{[N_3]} \left(1 + \frac{2k_{-0}}{k_f} + \frac{k_{-0}}{k_0[D_0]} \right) \right]^{-1} \quad \text{(A9)}$$

This leads to a Michaelis-Menten equation for v_{23} as follows:

$$v_{23} = k_{cat} \frac{[ED_2N_3]}{[D]_t} = \frac{V_{23}[N_3]}{K_{M\,app} + [N_3]} \quad \text{(A10)}$$

where V_{23} is the maximum velocity,

$$V_{23} = k_{cat} \frac{[E]_t}{[D]_t} \left(\frac{1}{3 + \frac{k_f}{k_0[D_0]}} \right) \quad \text{(A11)}$$

and $K_{M\,app}$ is the apparent Michaelis constant,

$$K_{M\,app} = K_M \left(\frac{1 + \frac{2k_{-0}}{k_f} + \frac{k_{-0}}{k_0[D_0]}}{3 + \frac{k_f}{k_0[D_0]}} \right) \quad \text{(A12)}$$

Note that $K_{M\,app}$ is identical to K_m when $k_{-0} = k_f$ (moderately processive enzyme) but is less than K_m when $k_{-0} \ll k_f$ (highly processive enzyme). In the case of DNA polymerase α, a moderately processive enzyme (26), we expect k_{-0} to be comparable to k_f, in which case the observed K_m should be close to the true K_m. For the insertion of A opposite T, the K_m observed by gel assay with *Drosophila* DNA polymerase α is the same as reported (3.7 μM) by conventional assay (4).

In Equation A11 for the maximum velocity (V_{23}), the term $k_{cat}\,[E]_t/[D]_t$ is *divided* by the factor $(3 + k_f/k_0[D_0])$. This factor has a minimum value of 3, when enzyme is saturated with primer, *i.e.* $[D_0] \gg k_f/k_0$. For lower primer concentrations, the factor will be larger.

If a factor of 3 is assumed, then a *minimum* value of k_{cat} can be estimated from the observed value of $V_{23} = V_{max}$ ($I_2 + I_3)/t$ at $t = 4$ min. For normal based insertion (N_3 = dATP), we find $V_{max} = 13$ (Table II) and $(I_2 + I_3)/t = 11\%$ min^{-1} = 0.11 min^{-1} (Table I). In the reaction mixture, $[D]_t = 33$ nM, assuming all primer is hybridized to template. The corresponding $[E]_t$ is 1.1 nM, assuming a molecular weight of 360,000 for polymerase α holoenzyme (4). Hence $[E]_t/3[D]_t = 0.011$ and $V_{23} = 1.43$ min^{-1} = 0.011 k_{cat}. The resultant k_{cat} = 2.2 s^{-1} agrees with the finding of 2 s^{-1} for normal base insertion on singly primed ϕX174 DNA (20).

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