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(DNA sequencing/dideoxynucleotides/T7 DNA polymerase/*Taq* DNA polymerase/fidelity)

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Contributed by Charles C. Richardson, March 28, 1995

ABSTRACT **Bacteriophage T7 DNA polymerase efficiently incorporates a chain-terminating dideoxynucleotide into DNA, in contrast to the DNA polymerases from *Escherichia coli* and *Thermus aquaticus*. The molecular basis for this difference has been determined by constructing active site hybrids of these polymerases. A single hydroxyl group on the polypeptide chain is critical for selectivity. Replacing tyrosine-526 of T7 DNA polymerase with phenylalanine increases discrimination against the four dideoxynucleotides by >2000-fold, while replacing the phenylalanine at the homologous position in *E. coli* DNA polymerase I (position 762) or *T. aquaticus* DNA polymerase (position 667) with tyrosine decreases discrimination against the four dideoxynucleotides 250- to 8000-fold. These mutations allow the engineering of new DNA polymerases with enhanced properties for use in DNA sequence analysis.**

All known DNA polymerases can be placed in one of four families based on sequence homologies (1, 2). Although *Escherichia coli* DNA polymerase I, *Thermus aquaticus* DNA polymerase (*Taq* DNA polymerase), and bacteriophage T7 DNA polymerase are members of the DNA polymerase I (Pol I) family, a number of their properties differ significantly (3). First, T7 DNA polymerase is responsible for the replication of a genome and, as such, interacts with other replication proteins, whereas *E. coli* DNA polymerase I and *Taq* DNA polymerase are mainly responsible for repair and recombination. Second, the three polymerases have different exonuclease activities: T7 has only a 3′ → 5′ activity, *Taq* has only a 5′ → 3′ activity, and *E. coli* DNA polymerase I has both 3′ → 5′ and 5′ → 3′ activities. The exonuclease activities reside in separate domains at the amino termini and can be inactivated selectively by genetic modification. Third, the thermostability of *Taq* DNA polymerase distinguishes it from the other two.

A fourth difference is their relative abilities to distinguish between a deoxy- and a dideoxyribose in the nucleoside triphosphate. *E. coli* DNA polymerase I (4–6) and *Taq* DNA polymerase (7) incorporate deoxynucleotides at a rate that is several hundred to several thousand times that of dideoxynucleotides, while T7 DNA polymerase incorporates dideoxynucleotides much more efficiently, preferring deoxynucleotides by only a factor of three (5, 6). To determine the molecular basis for this difference we constructed active site hybrids of the DNA polymerases and examined their ability to use ddNTPs relative to dNTPs. Inasmuch as chain termination by dideoxynucleotides is the basis of all commonly used enzymatic methods of DNA sequencing (5, 8), an understanding of this difference has utility for improving the DNA polymerases used for this purpose.

METHODS

Construction of Hybrid Genes. Hybrid genes were constructed by using synthetic oligonucleotides and the polymerase chain reaction. Hybrids of the T7 DNA polymerase gene were expressed under the control of the *lac* promoter in the vector pUC18; the parent vector (pGP5-12) contains the gene for T7 DNA polymerase with a deletion that encodes amino acid residues 118–145, inactivating the exonuclease (9). Hybrids of the *E. coli* DNA polymerase I gene were constructed in pKLEN-1, which encodes the large fragment of *E. coli* DNA polymerase I (beginning at residue 324) under the control of a T7 RNA polymerase promoter. Hybrids of *Taq* DNA polymerase were constructed in pTQΔ-1, which encodes a truncated fragment of *Taq* DNA polymerase (beginning at residue 289) under the control of a T7 RNA polymerase promoter. For characterization of the purified *Taq* hybrid polymerase C-Q5 (see Table 1), a fragment containing the mutation in pTQΔ-1 was transferred into the full-length *Taq* DNA polymerase gene.

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Perkin–Elmer (AmpliTaq) and the other five enzymes were purified using standard procedures. Polymerase activities in extracts and with purified proteins were determined by measuring the incorporation of [^{3}H]dTMP into a primed M13 template (9).

Incorporation Rate Ratios of dNMPs to ddNMPs. Relative incorporation rates were determined by gel analysis of a ^{32}P-end-labeled primer annealed to single-stranded M13 DNA extended using a fixed ratio of dNTPs to each ddNTP. Reaction mixtures (20 μl) contained 0.5 pmol of [5′-^{32}P]GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCA (500,000 cpm/pmol) annealed to single-stranded M13 mGP1-2 DNA (5)/25 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$/5 mM dithiothreitol/all four dNTPs (each at 120 μM)/one of the four ddNTPs at 20 μM/10 ng of yeast inorganic pyrophosphatase (12)/10 ng of the indicated DNA polymerase. Reactions were carried out for 15 min at 37°C (*E. coli* and T7) or 70°C (*Taq*). The reactions were terminated by the addition of 20 μl of 80% formamide/10 mM EDTA/0.02% bromphenol blue, and the samples were heated at 90°C for 2 min immediately prior to loading onto an 8% denaturing polyacrylamide gel.

The relative rate of incorporation of dNMPs to each ddNMP for the three DNA polymerases containing tyrosine at the ribose selectivity site was determined by quantitative analysis of the gel shown in Fig. 3. The radioactive bands in each lane were analyzed with a PhosphorImager, and the data were fit to an exponential decay curve to obtain the apparent ratio of dNTPs to ddNTPs. The incorporation rate ratio is the ratio of the apparent to the actual ratio of each dNTP to ddNTP. For each of the three DNA polymerases with phenylalanine, the relative rate of incorporation was determined by comparing the average length of fragments generated using varying dNTP to ddNTP ratios to those generated using the homologous polymerase with tyrosine; the increase in discrimination against ddNTPs with phenylalanine is the ratio of the dNTP/ddNTP ratios required to produce fragments of the same average length for the two polymerases.

RESULTS

Analysis of T7 DNA Polymerase/*E. coli* DNA Polymerase I Hybrid Genes. The three-dimensional structure of *E. coli* DNA polymerase I is known (13–15). The strongest homology with T7 DNA polymerase is in the crevice responsible for binding DNA and dNTPs (16). For the five most conserved regions, ranging in size from 14 to 29 amino acid residues, we constructed hybrid genes in which the DNA encoding each segment in *E. coli* DNA polymerase I was substituted for the homologous segment in the gene for T7 DNA polymerase (Fig. 1). Activities of extracts ranged from <1% that of WT T7 DNA polymerase for hybrids containing regions A, D, and E, 2% for region C, and 80% for region B.

To screen all the hybrids and avoid interference by the host DNA polymerases, we used an SDS–DNA activity gel assay for initial characterization (10, 11). SDS was added to cells containing the overproduced hybrid polymerases and the proteins were separated by electrophoresis in a polyacrylamide gel containing single-stranded DNA. After electrophoresis, the SDS was removed, allowing the proteins to renature, and polymerase activity was determined directly in the gel by incubation with [α-^{32}P]dNTPs. By comparing reactions carried out on duplicate gels in the presence and absence of ddTTP, we determined the ability of ddTTP to inhibit DNA synthesis by each of the hybrid polymerases (Fig. 2).

In the absence of ddTTP, DNA synthesis was relatively efficient with the hybrid polymerases containing regions A, B, and C of *E. coli* polymerase when compared to WT T7 DNA

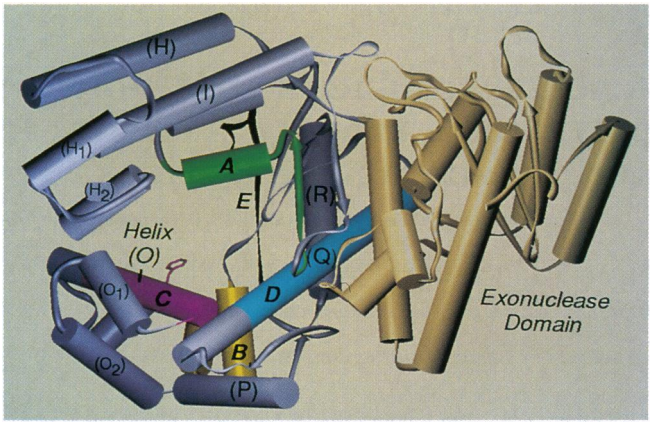

FIG. 1. Structure of the large fragment of *E. coli* DNA polymerase I showing the regions substituted into T7 DNA polymerase. The regions A–E (shown in different colors) were removed from *E. coli* DNA polymerase I and placed into T7 DNA polymerase. The specific residues substituted were as follows: A, 666–682 (*E. coli*) → 427–444 (T7); B, 710–734 (*E. coli*) → 480–506 (T7); C, 754–767 (*E. coli*) → 518–531 (T7): D, 843–867 (*E. coli*) → 609–633 (T7); E, 914–928 (*E. coli*) → 690–704 (T7). The structure shown was derived from the coordinates determined by Beese *et al.* (15). The letters within parentheses define specific helices (13, 15). The residue shown in helix O (region C) is phenylalanine-762.

hibited several 100-fold by a 20-fold excess of ddTTP over dTTP, DNA synthesis by the hybrid containing region C was resistant to this level of ddTTP. To define more precisely the residues responsible for this difference, we analyzed additional

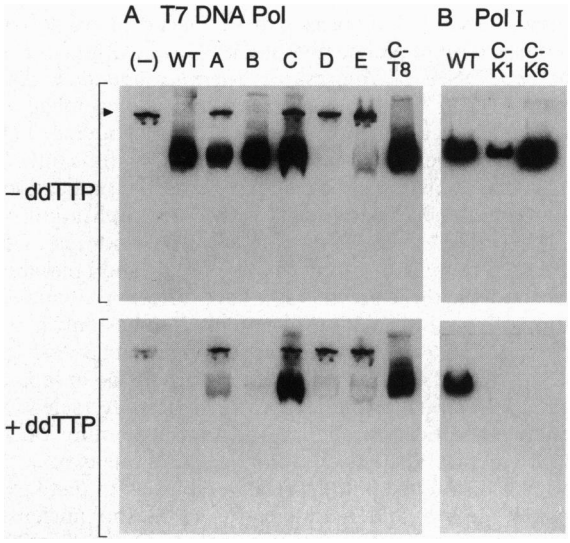

FIG. 2. SDS–DNA activity gel analysis of hybrid DNA polymerases. Cell aliquots were loaded on duplicate gels, and after electrophoresis, the SDS was removed and the polymerases were assayed for their ability to incorporate [α-^{32}P]dAMP into single-stranded calf thymus DNA in the absence (−) or presence (+) of ddTTP. (*A*) Analysis of WT T7 DNA polymerase I (WT) and hybrids in which regions A–E of T7 DNA polymerase were replaced with the homologous regions from *E. coli* DNA polymerase I (Fig. 1). C-T8 corresponds to cells containing the T7 DNA polymerase hybrid with the single Y526F mutation (Table 1). In the leftmost lane (−), the cells contain the control vector pUC18. The leftmost lane and those containing hybrids A, C, D, and E contain 10 times the amount of induced cells as in the other lanes to compensate for the low activity of these polymerases; the band visible at the top of each of these lanes is the result of DNA synthesis by the host *E. coli* DNA polymerase I (arrow-

hybrids containing smaller segments of region C (Table 1 and Fig. 2*A*). All of the hybrids that contained the replacement of tyrosine-526 in T7 DNA polymerase with phenylalanine, the residue at the homologous position in *E. coli* DNA polymerase I (position 762), were resistant to ddTTP. Thus modification of this single site is critical for increasing the ability of T7 DNA polymerase to distinguish between deoxy- and dideoxynucleotides.

A reciprocal series of hybrids in *E. coli* DNA polymerase I were constructed to determine whether substitution of region C from T7 DNA polymerase would decrease their ability to distinguish between deoxy- and dideoxynucleotides (Table 1 and Fig. 2*B*). SDS–DNA activity gel analysis of these hybrids defined the same single position as the sole determinant: when phenylalanine-762 in *E. coli* DNA polymerase I was replaced by tyrosine, DNA synthesis was strongly inhibited by ddTTP, reflecting a diminished ability of the hybrid polymerase to distinguish between deoxy- and dideoxynucleotides.

Analysis of *Taq* DNA Polymerase/T7 DNA Polymerase Hybrid Genes. Of the 16 known members of the Pol I family (1, 2, 17), one other besides *E. coli* DNA polymerase I, *Taq* DNA polymerase, is known to strongly prefer deoxy- over dideoxynucleotides (7). *Taq* DNA polymerase has a phenylalanine at residue 667, the site that corresponds to phenylalanine-762 in *E. coli* DNA polymerase I. We have therefore determined the effect of substituting region C of T7 DNA polymerase for the homologous region in *Taq* DNA polymerase (Table 1). Substitutions that include tyrosine for phenylalanine at residue 667 of *Taq* DNA polymerase increased dramatically the ability of ddTTP to inhibit DNA synthesis.

Table 1. Localization of the domain responsible for distinguishing between dNTPs and ddNTPs

Polymerase		Sequence	Inhibition of DNA synthesis by ddTTP
Pol I	754	RRSAKAINFGLIYG	−
Taq	659	RRAAKTINFGVLYG	−
T7	518	RDNAKTFIYGFLYG	+
Consensus		R AK G YG	
T7 WT		RDNAKTFIYGFLYG	+
T7 C-T2		RRSAKAINFGLIYG	−
T7 C-T3		RRSAKTFIYGFLYG	+
T7 C-T4		RDNAKAINFGFLYG	−
T7 C-T5		RDNAKAIIYGFLYG	+
T7 C-T6		RDNAKTFNFGFLYG	−
T7 C-T7		RDNAKTFNYGFLYG	+
T7 C-T8		RDNAKTFIFGFLYG	−
Pol I WT		RRSAKAINFGLIYG	−
Pol I C-K1		RDNAKTFIYGFLYG	+
Pol I C-K2		RRSAKTFIYGLIYG	+
Pol I C-K3		RRSAKTFNFGLIYG	−
Pol I C-K4		RRSAKAIIYGLIYG	+
Pol I C-K5		RRSAKAIIFGLIYG	−
Pol I C-K6		RRSAKAINYGLIYG	+
Taq WT		RRAAKTINFGVLYG	−
Taq C-Q1		RDNAKTINFGVLYG	−
Taq C-Q2		RRAAKTFIYGFLYG	+
Taq C-Q3		RRAAKTIIYGVLYG	+
Taq C-Q4		RRAAKTIIFGVLYG	−
Taq C-Q5		RRAAKTINYGVLYG	+
Specificity residue		↑	

The three aligned sequences at the top correspond to region C in Fig. 1; the number of the first residue shown for each polymerase is indicated at the left. Three sets of hybrids are presented, corresponding to substitutions of *E. coli* DNA polymerase I sequences into T7 DNA polymerase (T7), T7 DNA polymerase sequences in *E. coli* DNA polymerase I (Pol I), and T7 DNA polymerase sequences into *Taq* DNA polymerase (*Taq*). Substituted residues are underlined. Each hybrid protein was tested qualitatively for inhibition of DNA synthesis by ddTTP using the SDS–DNA activity gel assay described in the legend to Fig. 2; inhibition was categorized as >20-fold (+), reflecting efficient incorporation of a chain-terminating ddTMP, or <2-fold (−).

Analysis of Purified Proteins with Tyrosine Versus Phenylalanine at Ribose Selectivity Position. To analyze quantitatively the effect of tyrosine versus phenylalanine at the ribose selectivity site, we purified the three hybrid polymerases, T7 Y526F, *E. coli* F762Y, and Taq F667Y, and compared their properties to the WT DNA polymerases. The specific polymerase activities of each of the three hybrid proteins were within 25% of the respective WT enzymes. The autoradiograph in Fig. 3 shows the relative ability of the three WT and hybrid polymerases to incorporate each of the four ddNMPs. A 5′-^{32}P-labeled primer annealed to a single-stranded M13 template was extended by each of the DNA polymerases in the presence of a fixed (6:1) ratio of dNTP to each of the four ddNTPs. For all three DNA polymerases, the presence of phenylalanine resulted in strong discrimination against each of the four ddNTPs, while the presence of tyrosine resulted in efficient incorporation of all four ddNMPs.

We determined quantitatively the relative rate of incorporation of each ddNMP compared with the corresponding dNMP for each of the DNA polymerases shown in Fig. 3 (Table 2). Whereas with WT T7 DNA polymerase the average rate of incorporation of deoxynucleotides is three times that of dideoxynucleotides, with the hybrid T7 DNA polymerase Y526F the average rate of incorporation of deoxynucleotides is 8000 times that of dideoxynucleotides. On the other hand, whereas with WT *E. coli* DNA polymerase I and *Taq* DNA polymerase the average rate of incorporation of deoxynucleotides is 600 and 3000 times that of dideoxynucleotides, respec-

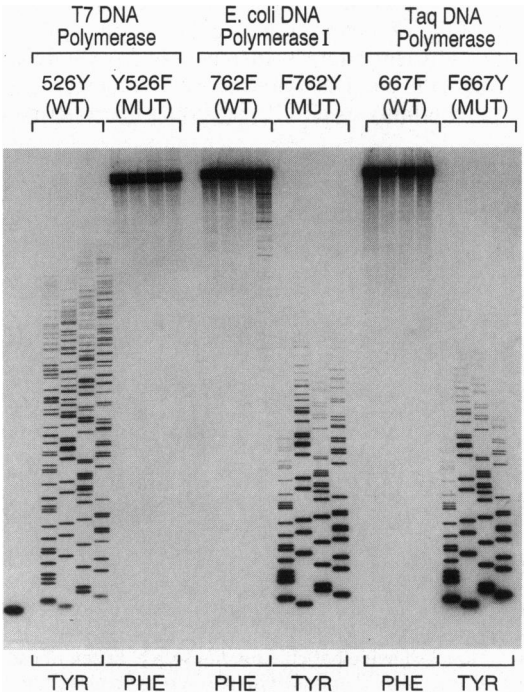

FIG. 3. Effect of phenylalanine versus tyrosine on discrimination against dideoxynucleotides. Purified T7 DNA polymerase, *E. coli* DNA polymerase I, and *Taq* DNA polymerase with either phenylalanine (F) or tyrosine (Y) at the ribose selectivity site were compared for their ability to extend a radioactive primer in the presence of a 6:1

Table 2. Effect of phenylalanine versus tyrosine at the ribose selectivity site on discrimination against ddNTPs

Polymerase	Incorporation rate ratio dG/ddG	dA/ddA	dT/ddT	dC/ddC	Average dNMP/ddNMP
WT T7 DNA polymerase (526Y)	3.2	3.3	2.8	3.7	3.0
T7 DNA polymerase Y526F	6400	7300	8400	11,000	8000
WT *E. coli* DNA polymerase I (762F)	140	720	1100	250	600
E. coli DNA polymerase I F762Y	0.56	0.72	0.54	0.75	0.6
WT *Taq* DNA polymerase (667F)	1400	4700	4500	2,600	3000
Taq DNA polymerase F667Y	0.45	0.59	0.56	0.32	0.5

T7 DNA polymerase, *E. coli* DNA polymerase I, and *Taq* DNA polymerase were compared with either phenylalanine (F) or tyrosine (Y) at the ribose selectivity site. In each case the unmodified or WT DNA polymerase (WT) is presented first. The relative rate of incorporation of each dNMP to ddNMP was determined by gel analysis of the average extension lengths at different ratios of dNTP to ddNTP (see text).

tively, the hybrid DNA polymerases *E. coli* F762Y and *Taq* F667Y actually prefer ddNTPs over dNTPs ≈2-fold.

DISCUSSION

Mechanism of Discrimination. The critical phenylalanine/tyrosine residue defining the ability of Pol I-type DNA polymerases to distinguish between deoxy- and dideoxyribose is located on the "O" helix facing into the crevice responsible for binding dNTPs and DNA (Figs. 1 and 4). In a crystal structure of the binary complex of *E. coli* DNA polymerase I with dCTP, the closest residue to the 3′-hydroxyl group of the dCTP is this phenylalanine (residue 762), separated by a distance of about 4.5 Å (14). While this binary complex must be viewed with caution since a primer-template is required for specific binding of the correct dNTP, genetic and structural data support the juxtaposition of these two moieties in the catalytically competent complex (17–20).

What is the mechanism that accounts for efficient incorporation of a nucleotide when there is a hydroxyl moiety on either the 3′ position of the ribose or on the aromatic residue of the polymerase but inefficient catalysis when both hydroxyls are absent? ddTTP binds to *E. coli* DNA polymerase I in a binary complex with the same affinity as dTTP, suggesting that discrimination

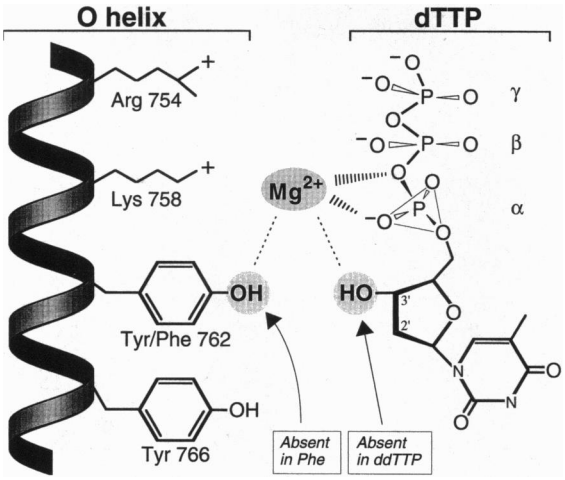

FIG. 4. Model showing possible relationship between ribose selectivity residue and a dNTP in Pol I-type DNA polymerases. On the left is helix O of *E. coli* DNA polymerase I (13); the four residues shown all face into the crevice responsible for binding DNA and dNTPs (Fig. 1). On the right is dTTP; the position of the ribose moiety is approximately that observed in a binary complex of dCTP with *E. coli* DNA polymerase I (14). The two critical hydroxyl moieties on residue 762 (tyrosine) and the 3′ position of the dNTP are shaded. A hypothetical

between a deoxy- and dideoxyribose occurs in a step subsequent to the initial binding (21). One model is that a hydroxyl moiety is required to restrict the space occupied by the nucleotide and thus stabilize a catalytically productive orientation. However, it is hard to reconcile this model with the fact that two hydroxyls in the same space, one on the enzyme and the other on the nucleotide, does not affect polymerization. Another model is that at least one of the hydroxyl moieties is necessary for binding an essential ligand. While this could be a water molecule, a more attractive possibility is a divalent cation. The number and location of metal ions required for polymerization in Pol I-type DNA polymerases are not known, although several conserved acidic residues have been implicated genetically as important in binding the divalent cation(s) (17–20). It is thought that one divalent cation acts as a Lewis acid to promote deprotonation of the 3′ hydroxyl of the primer while a second promotes the formation of the pentacovalent transition state at the α-phosphate of the dNTP (18). Perhaps the hydroxyl ion either on the nucleotide or on the enzyme is required to stabilize the binding of the metal ion that interacts with the α-phosphate of the dNTP (Fig. 4).

What is the effect of residues other than phenylalanine and tyrosine at the ribose selectivity site? The substitution of the polar uncharged residues cysteine, serine, and asparagine at residue 526 of T7 DNA polymerase reduces its activity by 10- to 50-fold, as measured by SDS–DNA activity gel analysis; the residual activity of these mutant enzymes is unable to distinguish between deoxynucleotides and dideoxynucleotides (data not shown). The substitution of the nonpolar residue leucine reduces T7 DNA polymerase activity 1000-fold; the residual activity incorporates deoxynucleotides preferentially over dideoxynucleotides. The only substitution that retains the high level of polymerase activity observed with phenylalanine and tyrosine is tryptophan, which results in a strong preference for deoxynucleotides over dideoxynucleotides. While these results show that a polar group at this site reduces the ability of a polymerase to distinguish between deoxy- and dideoxynucleotides, they also suggest a critical role of an aromatic moiety for efficient incorporation of all nucleotides. Consistent with these results, Astatke *et al.* (17) have shown that replacement of phenylalanine-762 of *E. coli* DNA polymerase I with alanine increases the K_m for dTTP by >100-fold, without affecting significantly the k_{cat}.

Relationship to Other DNA Polymerases. Mutations that increase the ability of a DNA polymerase to incorporate dideoxynucleotides into DNA have not, to our knowledge, been previously described. On the other hand, mutations that decrease the ability of DNA polymerases to incorporate dideoxynucleotides relative to deoxynucleotides have been described in T7 DNA polymerase (22), herpes DNA polymerase (23), and human immunodeficiency virus (HIV) DNA polymerase (24, 25). The nature of the changes observed in

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API

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Build custom dashboards for your attorneys and clients with live data direct from the court.

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Sync your system to PACER to automate legal marketing.