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ABSTRACT

Mutant DNA polymerases have become an increasingly important tool in biotechnology. The ability to examine the activity and specific properties of enzymes has a crucial role in the characterization of the enzyme. We have developed several systems for characterizing DNA polymerases that combine random mutagenesis with in vivo selection systems. However, in vivo screening systems for specific properties are sometimes unavailable. The ability to quickly screen for polymerase activity has many applications, including the identification of compounds that can inhibit polymerase activity, identifying the properties of newly discovered polymerases, and engineering new biological properties into existing polymerases. These applications can both expand the knowledge of the basic science of polymerases and can further industrial efforts to identify new drugs. We have previously published a paper that focused on creating an hPolη mutant library by using a yeast-based complementation system and analyzing a single mutant for its ability to bypass several site-specific lesions. The current paper describes a high-throughput method to quickly screen for polymerase activity in vitro and to use this assay to analyze the properties of mutant polymerases. We illustrate this procedure by screening 90 human DNA polymerase η mutants for activity and damage bypass and by analyzing fluorescent nucleotide analog incorporation. The screen permitted several inferences concerning structure-function relationships of polymerase η. Furthermore, our screening assay identified an enhanced lesion bypass ability by one mutant and revealed enhanced fluorescent nucleotide incorporation by another mutant. This procedure and modifications thereof should enable multiple applications, including analyzing structure-function relationships, engineering novel properties into polymerases, and screening combinatorial libraries for polymerase inhibitors.

INTRODUCTION

DNA polymerases with altered catalytic activities have been extensively utilized in biotechnology for DNA sequencing, detection of polymorphisms, and PCR (12,14,23). Site-directed mutagenesis together with structural information has facilitated the rational design of a limited number of polymerase variants with specific desired properties (5,18). Nevertheless, rational strategies for engineering polymerases with specific properties has been limited by our inadequacy at predicting changes in catalytic properties caused by multiple and even single amino acid substitutions. Alternatively, evolution strategies that couple large libraries containing random nucleotide substitutions with genetic selection make it possible to identify rare mutant proteins with specific phenotypes or altered properties (10,24). Positive selection by functional complementation of genetically deficient host strains is a particularly efficacious method of isolating desired proteins from large numbers of variants. However, for DNA polymerases, such complementation has been difficult to achieve with the notable exceptions of a mutant E. coli DNA polymerase I host strain (28) and a mutant yeast rad30 host strain (11). Even in these cases, complementation has been limited to Taq DNA polymerase I (Promega, Madison, WI, USA), E. coli DNA polymerase I, DNA polymerase β, HIV reverse transcriptase, and human polymerase η (hPolη) (11,16,24, 27,29). In the absence of genetic complementation, rapid in vitro screening methods are required to survey large numbers of mutant enzymes, such as those generated by random sequence mutagenesis or gene shuffling (2,6). Screening methods can also be utilized to characterize large numbers of mutant DNA polymerases identified by genetic complementation.

We have previously published a paper that focused on creating an hPolη mutant library by using a yeast-based complementation system and analyzing a single mutant for its ability to bypass several site-specific lesions. The current paper describes a high-throughput method to quickly screen for polymerase activity in vitro and to use this assay to analyze the properties of mutant polymerases. We illustrate this procedure by screening 90 human DNA polymerase η mutants for activity and damage bypass and by analyzing fluorescent nucleotide analog incorporation. The screen permitted several inferences concerning structure-function relationships of polymerase η. Furthermore, our screening assay identified an enhanced lesion bypass ability by one mutant and revealed enhanced fluorescent nucleotide incorporation by another mutant. This procedure and modifications thereof should enable multiple applications, including analyzing structure-function relationships, engineering novel properties into polymerases, and screening combinatorial libraries for polymerase inhibitors.

DRUG DISCOVERY
AND GENOMIC TECHNOLOGIES

Research Report
In Vitro Production and Screening of DNA Polymerase η Mutants for Catalytic Diversity

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MATERIALS AND METHODS

In Vitro Transcription-Translation of hPolη Mutant Proteins

DNA plasmids encoding hPolη (11) were used as templates for PCR amplification in a 96-well format, with an upstream primer that contained the T7 phage promoter sequence (5′-TTATC-GAAATTAATACGACTCACTATAG-GGAGACCCAAGCTTGGTACCGAGCTCGGATCCAAAATGGCTACTG-GACAGGATC-3′) and a downstream primer encoding a hexahistidine tag (5′-CGGAATTCCTAATGGTGATGGTGATGAGCGGCCGCATGTGT-TAATGGCTTAAAAAATGATTCC-3′).

Five microliters of the amplified product (2240 bp) containing the hPolη cDNA under control of the T7 promoter served as a template for in vitro transcription and translation using 20 µL/well of TNT® T7 Quick for PCR DNA kit (Promega) in a 96-well plate. The plates were incubated at 30°C for 90 min, and the product was used immediately or frozen in small aliquots at -80°C for further use.

High-Throughput Quantitation of DNA Polymerase Activity

DNA polymerase activity was measured at 37°C for 30 min in 10 µL reaction mixtures containing 1 µg of activated calf thymus DNA, 25 µM each dNTP, 1 µCi α[32P] dGTP (3000 Ci/mmol) (NEN® Life Science Products, Boston, MA, USA), and 1 µL of in vitro-synthesized polymerase in 40 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 60 mM KCl, 10 mM DTT, 250 µg BSA, and 2.5% glycerol. The reaction was terminated by the addition of 2.5 µL of formamide solution; the products were analyzed by electrophoresis through 14% denaturing polyacrylamide gels and quantified by phosphorimager analysis using ImageQuant software. The templates used for bypass of DNA lesions (8-oxo-G, O6-MeG, O4-MeT, an abasic site, ethenoA, or no lesion) were synthesized by the Midland Certified Reagent company and contained one of the above adducts in the N position: 5′-TTGGC(N)GCAGAATATTGCTAGCGGGAATTCGGCGCG-3′. The primer was 32P-5′-CGCGCCGAATTCGGCGCG-3′.

Screening for the Ability to Incorporate a Fluorescent Analog

Assay mixtures (20 µL) contained 1 µg activated calf thymus DNA template-primer, 25 µM each dATP and dCTP, and either dTTP or the rhodamine green-labeled dUTP, 5 µM dGTP, 250 nCi α[32P] dGTP (3000 Ci/mmol), Taq PCR buffer, 5 mM MgSO4, and 0.01-1 U commercially available polymerase or 1 µL in vitro-synthesized polymerase. The reactions were incubated at 72°C for thermostable polymerases or 37°C for hPolη and stopped after 30 min by the addition of EDTA to a final concentration of 50 mM. Before direct-
ly measuring fluorescence incorporation on the microtiter filter plates, the unincorporated dye-labeled nucleotides were removed from the samples by using a QIAquick™ PCR purification kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. Rhodamine green or 32P-dGTP associated with the filter was quantified by phosphorimeter analysis as described earlier.

RESULTS

High-Throughput In Vitro Synthesis and Polymerase Assay

To elucidate structure-function relationships in the Y family of DNA polymerases, we established a system to produce and screen libraries for mutant DNA polymerases that exhibit specific properties. DNA polymerase η is one of the recently identified error-prone DNA polymerases that have the ability to copy DNA containing alterations that block synthesis by other DNA polymerases. We previously established a library of mutant hPolη genes by substituting random nucleotides for segments of motif II within the polymerase active site (11). Genes encoding active mutant polymerases were selected based on their ability to complement the UV sensitivity of a yeast strain lacking both Polη encoded by RAD30 and the RAD52 gene product (22). The in vivo complementation of UV sensitivity by hPolη harboring random nucleotide substitutions within motif II yielded more than 2000 independent colonies, each expressing a different mutant Polη. To investigate the properties of a large number of these mutants, we developed a fast and simple in vitro procedure to produce and screen DNA polymerases. We illustrate this new high-throughput in vitro synthesis and polymerase assay (HTPA) by screening hPolη mutants for novel properties.

The HTPA consists of high-throughput, coupled, in vitro transcription-translation of DNA polymerase genes followed by a filter-binding activity assay, all in a 96-well format. Plasmids or amplified cDNAs encoding hPolη mutants linked to an upstream primer containing the bacteriophage T7 promoter sequence served as templates for the production of mRNAs and for translation into active polymerases by a reticulocyte lysate. Translation of mutant polymerases in the presence of 35S-labeled methionine revealed approximately 10% variation in the amounts of a single radioactive band migrating at the size expected for hPolη (78 kDa) (data not shown). The in vitro-synthesized polymerases were then assayed for their ability to incorporate 32P-labeled nucleotides using activated calf thymus DNA as a template-primer. Reaction mixtures containing the newly synthesized proteins were loaded onto Biodyne B microtiter filter plates. The filter was washed to remove residual unincorporated nucleotides, dried, and analyzed to quantitate polymerase activity. These filter plates possess both a high nucleic acid binding capacity and a non-brittle filter that can be removed to enhance detection. To validate the procedure, we compared the results obtained in the traditional test tube acid precipitation assay (17) to those obtained in the 96-well HTPA, using a set of hPolη mutants with varying amounts of activity. Figure 1 shows that the two protocols yielded nearly identical results, even though the quantitation of 96 reactions using the HTPA is at least 50 times faster than the traditional filter assay. The throughput of the HTPA can be further increased by performing the assay on pools of up to 50 different enzymes in each well. Thus, pooling strategies (21) can increase screening efficiency to thousands of polymerases per plate.

Activity and Damage Bypass by In Vitro-Synthesized hPolη Mutants

We utilized the HTPA to quantitate the activity of 90 different mutant hPolη. The amount of 32P-labeled nucleotide incorporated by the mutants varied from 2% to 190% of that incorporated by the wild-type (sample A3) enzyme (Figure 2A). Because comparable amounts of the hPolη protein were synthesized in the in vitro transcription-translation reactions, based on multiple 35S methionine labeling analyses, the HTPA results reflect differences in the specific activities of the mutant polymerases. If variable amounts of mutant protein were produced, then the data had to be normalized to the amount of enzyme contained in each reaction.

Wild-type hPolη has been shown to bypass chemically diverse DNA le-
sions. To measure damage bypass by the selected hPolη mutants, we used the in vitro-synthesized proteins in primer extension assays that monitor the copying of oligonucleotide templates containing site-specific DNA lesions. We tested the 90 hPolη mutants for relative efficiencies to bypass different DNA modifications introduced into oligonucleotide template at a specific site, as exemplified in Figure 2B. The pattern of lesion bypass observed for the wild-type and mutant polymerases was similar. The likelihood of bypass was greatest for small adducts generated by oxygen damage (8-oxo-G) and alkylating agents (O4-MeT) (Figure 2B). Opposite an abasic site, the wild-type and mutants incorporated a single nucleotide but failed to significantly extend the incorporated nucleotide (data not shown). No significant incorporation was observed opposite 1,N6-ethenoadenine (ethenoA) (data not shown), a lesion associated with a metabolite(s) of the carcinogen, vinyl chloride DNA.

**Incorporation of Nucleoside Triphosphate Fluorescent Analogs**

Nonradioactive labeling and detection methods are becoming a viable alternative to the use of radionucleotides. Methods such as nick translation, random primer extension, end-labeling,

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**Figure 2. Screening hPolη mutants for polymerase activity and lesion bypass by the HTPA and gel assays, respectively.** (A) hPolη mutant proteins were synthesized in vitro from PCR-amplified templates and tested for polymerase activity on an activated calf thymus DNA template-primer. The phosphorimager results for 90 hPolη mutants are shown with the percent activity of each mutant relative to the wild-type hPolη indicated. Wild-type hPolη is labeled (WT) along with control wells that contain no sample (Blk), or no polymerase (-pol). (B) Damage bypass by in vitro-synthesized hPolη mutants on an unmodified template and templates containing site-specific lesions. Bypass of the alterations in the same sequence context was compared in primer extension assays. The template contains either no lesion, 8-oxo-G, or O4-MeT. The position of the lesion (*) is three nucleotides downstream of the 3′ primer terminus (→). Assay mixtures, containing 2 μL of a 1:20 dilution of in vitro-synthesized wild-type or mutant hPolη were as follows: wild-type (A3), A56E (A6), F57L (A9), R61K (B2), S62G (B4), and W64C (B8). The reactions were terminated after 15 min by the addition of formamide stop solution. The reactions were analyzed by electrophoresis through 14% denaturing polyacrylamide gels and quantified by phosphorimager analysis. Ctr, control reactions containing polymerase with very low damage bypass ability.
Southern blots, Northern blots, dot-blots, and in situ hybridizations are increasingly being carried out using nonradioactive techniques (1,13,20). The ability to incorporate fluorescent analogs into a DNA strand is integral to developing technologies, including DNA microarray and single molecule sequencing efforts (8,9,26,31). We used the HTPA to measure the ability of polymerases to incorporate nucleotides from a mixture of dNTPs containing a rhodamine green-labeled dUTP analog in place of dTTP. To assess the utility of this procedure for screening for polymerases that efficiently utilize fluorescent nucleoside triphosphates analogs, we tested three commercial DNA polymerases: Taq, Vent® exo- (New England Biolabs, Beverly, MA, USA), and Deep Vent® exo- (New England Biolabs). Measurements of fluorescence indicated that all three polymerases were able to incorporate the fluorescently labeled dUTP analog into the activated calf thymus DNA template-primer (Figure 3A). However, quantitation of the fluorescent signal revealed both a high background and quenching of the signal because of high-density incorporation of the fluorescent analog (25) (data not shown). Therefore, we used α[32P] dGTP as a secondary reporter to quantify incorporation (Figure 3B). Incorporation in the reaction lacking dATP represents the background of the reaction, resulting from incorporation of α[32P] dGTP before termination opposite a template dT. The efficiency of the incorporation of the fluorescent nucleotide was ascertained by comparing the incorporation in reactions containing dTTP versus fluorescent dUTP. The results show that the radioactive signal increased with increasing polymerase concentration for mixtures containing four normal dNTPs or for mixtures containing the fluorescent analog. The average relative incorporation in assays containing the fluorescent analog as compared to normal dNTPs was determined over the range of 0.01–0.1 U enzyme (Figure 3C).

Fluorescent Nucleotide Incorporation by Mutant hPolη

Although the 3-D structure of the Y family DNA polymerases is similar to the structures found in other DNA polymerase families and resembles a human right hand (19,30), certain modifications appear in the Y family that allow synthesis past bulky lesions, including T-T dimers and lesions generated by oxygen damage and alkylating agents. As shown in Figure 4, we used the HTPA to assess the ability of hPolη mutants to incorporate a fluorescent nucleoside triphosphate analog. Similar to the case of lesion bypass (Figure 2), the ability of the mutant polymerases to replicate the DNA template in the presence of the fluorescent analog (Figure 4) is correlated, with catalytic activity measured in the HTPA. In this initial screen, sample B2, which contains a single amino acid substitution at position 61 that changes arginine to lysine (R61K), showed an increased ability to incorporate the fluorescent analog relative to its ability to incorporate natural dNTPs (Figure 4).
DISCUSSION

We have developed a simple and easily automatable polymerase activity assay that can be combined with in vitro transcription and translation to quickly identify DNA polymerases with altered properties. This high-throughput screening method can also be used to analyze polymerases for differences in their functionality. The method can be used to screen for compounds that produce DNA adducts or strand breaks, inhibiting primer extension by the polymerase. It can also be used to screen polymerase libraries for mutant polymerases based on the ability to bypass DNA lesions, to incorporate modified nucleotides, or to operate under altered conditions. These types of analyses will help to both expand the basic knowledge of polymerases and advance the use of polymerases in performing new functions that can benefit the biotechnology field.

We utilized the HTPA to measure the activity of 90 hPol η mutants (Figure 2A). Because comparable amounts of hPol η protein were synthesized in the in vitro transcription-translation reactions, based on multiple 35S methionine labeling analyses, the HTPA results reflect from 2% to 190% differences in the specific activities of the mutant polymerases. These specific activities can be correlated with the amino acid substitutions in the variant proteins, revealing the importance of particular amino acids for the catalytic activity of the enzyme. Sample B4 contains a single amino acid substitution at position 62 that changes serine to glycine (S62G) and exhibits enhanced activity relative to wild-type hPol η. Multiply mutated samples that harbored the S62G substitution (samples E10, E12, H3, and H8) retained at least two thirds of the wild-type activity, which suggests that a positive effect on activity was retained in the presence of a variety of other mutations. Similar observations were made for sample B8, containing a tryptophan to cysteine substitution at position 64 (W64C) that was also present in multiply mutated samples (E7, G3, G11, H1, H2, and H7).

Structures for the binary and ternary complexes of hPol η have not yet been solved. The tertiary structure of another member of the Y family DNA polymerases, Dpo4, reveals that the residues in positions homologous to serine 62 and tryptophan 64 (i.e., glycine 58 and proline 60) interact with the DNA template (19), providing a rationale for the importance of these residues for the activity of hPol η. Unlike the substitution at serine 62 and tryptophan 64, substitution at alanine 68 was associated with reduced activity in both the single mutant (sample C6) and multiply mutated variants (C7, D6, E4, and F10), which suggests that the alanine 68 side chain may contribute to wild-type levels of activity. Further study of defined single and double mutants is required to address the predictions of this initial analysis.

In most of the mutant polymerases, the ability to bypass DNA lesions was tightly correlated with activity in the HTPA on an activated DNA template-primer (Figure 2A). The mutant also catalyzed less lesion bypass than wild-type hPol η (Figure 2B). These observations could reflect reduced processivity conferred by the W64C substitution, which is consistent with the interaction of the homologous wild-type residue with the DNA template in the related Dpo4 polymerase (19).

The ability to efficiently incorporate nucleotide analogs has many uses in biotechnology. Fluorescent analogs and affinity-based groups such as biotin or digoxygenin are being used in place of radioactive detection techniques (15). The incorporation of these bulky hydrophobic groups into a growing strand of DNA can be challenging (3). However, hPol η, which is able to bypass various DNA lesions, may be more efficacious in incorporating these nucleotide analogs. For each of the mutants, the relative ability to incorporate the dye analog was determined, eliminating biases that could take place by analyzing different amounts of enzyme.
We show by using the HTPA that the wild-type hPolη and several of the variant proteins are able to incorporate a fluorescent nucleoside triphosphate analog (Figure 4). Mutant B2 (R61K) exhibits an increased ability to incorporate this nucleotide analog. Studies of Dpo4 show that the amino acid corresponding to arginine 61 in hPolη is located in the catalytic pocket and may interact with the incoming nucleotide (19). Alterations of this amino acid may influence the ability of the polymerase to incorporate various nucleotide analogs.

Mutant libraries can be generated by several different methods, including random genetic damage, PCR mutagenesis, DNA shuffling, and random oligonucleotide mutagenesis. An overview of these methods has been previously described (7). With each of these techniques, it is useful to first screen the mutant library in vivo to identify the subset of active mutants. However, an in vivo selection system often does not exist for the polymerase of interest or for the desired properties; therefore, the library must be screened by in vitro methods. The HTPA can be used to both analyze the subset of active mutants identified by in vivo selection and to screen an unselected library of mutants. For example, in our previous publication, we described an in vivo selection strategy to isolate 2000 hPolη mutants that were capable of bypassing DNA damage by UV irradiation. Here we describe a method to quickly screen for polymerase activity in vitro and to use this assay to analyze unique properties of the mutant polymerases. For the Polη library, the preselection strategy created a pool of mutants that were able to bypass UV damage in vivo. Within these preselected Polη mutants that can accommodate thymine dimers, there is a high probability of isolating mutants that accommodate other bulky groups. From an unselected library, approximately 100 clones can be expressed, combined, and assayed in a single well of the 96-well filter plate. This will allow for the screening of nearly 10,000 clones per plate. Any wells that are found to contain an active polymerase can then be further analyzed to identify the specific clone that contains the active mutant. The HTPA can also be easily automated since it requires no centrifugation steps and little manipulation.

The ability to replicate, manipulate, and sequence DNA easily has underpinned the growth of the biotechnology industry. Methods such as PCR and fluorescent DNA sequencing have made the human genome project feasible and have led to the discovery of thousands of new genes. These methods have continually improved, in part by specifically manipulating the polymerases. Today, the production and alteration of DNA polymerases for research purposes is an industry in itself. Nucleosides corresponding to nucleoside triphosphates that inhibit DNA polymerases have proven to be valuable agents in chemotherapy of cancer and viral infection.

Using human DNA polymerase η as a representative polymerase, we present a high-throughput, automatable procedure for the production and analysis of mutant DNA polymerases. We analyzed the catalytic activity and lesion bypass capability of hPolη mutants and identified amino acid substitutions that affect activity, bypass ability, and processivity. The presence of a flexible active site in the Y family DNA polymerases, taken together with results on bulky analog incorporation suggests that this family of DNA polymerases should be an appropriate candidate for a large-scale screening of mutants that efficiently incorporate bulky fluorescent nucleotide analogs. In both screens (bypass ability and nucleotide analog incorporation), we identified mutants (B4 and B2, respectively) that showed increased activity relative to the wild-type enzyme. Our procedure, and modifications thereof, should have multiple applications, including the screening of combinatorial

![Figure 4. Incorporation of a fluorescent nucleotide analog by in vitro-synthesized hPolη mutants.](image-url)

(A) Quantification and (B) filter plate image showing the amount of the secondary reporter (d[32P]dGTP) incorporated into activated calf thymus DNA by each mutant. The HTPA was performed with the four normal dNTPs or a nucleotide mixture containing a fluorescent dUTP analog in place of dTTP. The assays, containing 2 µL of a 1:20 dilution of in vitro-synthesized wild-type or mutant hPolη, were: wild-type (A3), A56E (A6), F57L (A9), R61K (B2), S62G (B4), W64C (B8), K69M (C10), and K70E (C11).
libraries for effective inhibitors of DNA and RNA polymerases and reverse transcriptases, identifying new polymerases, and engineering novel properties into existing polymerases.

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