The Mutational Specificity of the Dbh Lesion Bypass Polymerase and Its Implications*

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The Dbh polymerase of *Sulfolobus solfataricus* is a member of the recently described family of low fidelity DNA polymerases involved in bypass of DNA lesions. To investigate the enzymatic properties of Dbh, we characterized the errors made by this polymerase *in vitro*. Not only is Dbh much less accurate than the “classical” polymerases, but it showed a remarkable tendency to skip over a template pyrimidine positioned immediately 3’ to a G residue, generating a single-base deletion. Single-turnover kinetic measurements suggest possible mechanisms. First, Dbh shows a bias in favor of dCTP, such that the rate of incorporation of dCTP opposite a template G is about 10-fold faster than for the other three dNTPs opposite their complementary partners. On a DNA substrate corresponding to a frameshift hotspot, the rate of frameshift insertion of dCTP opposite a template G that is one residue 5’ to the expected templating position is approximately equal to the rate of the non-frameshifted C-dGTP insertion. We suspect that the unusual mutational specificity of Dbh (which is shared with other polymerases from the DinB branch of the bypass polymerase family) may be related to the type of DNA lesion(s) that it serves to bypass *in vivo*.

In the past few years it has become apparent that the DNA repair proteins of the UmuC/DinB/Rev1/Rad30 family are in fact DNA polymerases, now called the Y family (1–3). Studies *in vitro* on members of this family have shown two distinctive properties: an ability to synthesize past template lesions that would cause stalling of the “classical” replicative and repair polymerases, and a remarkably low fidelity when copying an undamaged DNA template (see e.g. Refs. 4–6). It is thought that these DNA polymerases function *in vivo* to allow replication forks to bypass blocking lesions, and that each bypass polymerase may have specificity for one or a subset of lesions. Low fidelity may be merely a consequence of the active site features that allow lesion bypass, or it may be an additional survival strategy, allowing increased mutation rate as a response to environmental stress (7).

At a molecular level, an obvious question is what modifications to the classical polymerase active site are responsible for the novel properties of the lesion bypass polymerases. Several recently published crystallographic studies have begun to address these issues (8–11). They show a polymerase domain that is clearly related to the classical polymerases, in that it can be described as having the familiar right-handed arrangement of palm, fingers, and thumb subdomains (12). Moreover, the palm subdomain shows the classical “polymerase fold,” the well-conserved secondary structure elements that provide the scaffold for the polymerase active site. Both homology-modeled and directly observed ternary complexes provide plausible explanations for the characteristic biochemical properties of the bypass polymerases. First, the bypass polymerases appear to make fewer contacts with their substrates when compared with other DNA polymerase ternary complex structures (reviewed in Ref. 13). Second, the relative positions of the fingers and palm subdomains suggest that the bypass polymerases may undergo very little shift in conformation in the transition from apo-enzyme to ternary complex. By contrast, other DNA polymerases show a substantial movement of the fingers subdomain on formation of the ternary complex (14–17), and it has been suggested that this conformational change may be important in ensuring polymerase accuracy.

Three of the four bypass polymerase crystal structures are of closely related polymerases, Dbh (two structures) and Dpo4, from subspecies of the thermophilic archaeon, *Sulfolobus solfataricus*. The availability of structural data makes these proteins very attractive as model systems for mechanistic studies. The Dbh (DinB homolog) and Dpo4 proteins are both from the DinB branch of the bypass polymerase family. Both have substantial sequence homology to the *Escherichia coli* DinB protein (18, 19), which has been shown to have DNA polymerase activity and a tendency to make single-base deletion errors *both in vitro* and *in vivo* (20–22). Expression of dinB is induced by DNA damage, implicating the gene product in a response to stress. However, inactivation of the gene does not result in a noticeable decrease either in viability or in the ability to withstand UV or oxidative damage (23). To date, the processes shown to require dinB function are untargeted mutagenesis of phage λ, stationary-phase adaptive point mutation, and replication of DNA containing benzo[a]pyrene-G adducts (23–27).

Here we report some initial biochemical studies on the Dbh polymerase. We have generated a mutational spectrum of the polymerase. We have also carried out kinetic studies that explain the prevalence of some types of errors made by this polymerase.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides were synthesized by the Keck Biotechnology Resource Laboratory at Yale Medical School and were purified as described previously (28). Primer strands were 5’-labeled with 32P and were annealed with suitable template strands to produce the substrates used in polymerase kinetics measurements. Full-length Dbh polymerase with an N-terminal His6 tag, purified as described previously (8), was kindly provided by Bo-Lu Zhou. Purification of the 3’-5’

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exonuclease-deficient (D424A) Klenow fragment has been described (29, 30).

**HSV-tk Forward Mutational Assay**—The method developed by Eckert and coworkers (31) was used to determine the frequency and type of mutations made during DNA synthesis in vitro by Dbh polymerase. A limited survey of mutations made by Klenow fragment in the same assay was carried out for comparison purposes. DNA synthesis reactions (50 μl) contained 2 pmol of primed single-stranded M13tk3.5, which carries the HSV-tk gene. Reaction conditions for Klenow fragment were 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM DTT, 1 mM each dNTP, and 1.2 pmol of exonuclease-deficient (D424A) Klenow fragment. Incubation was at 37°C for 1 h. For Dbh polymerase, the reaction contained 10 mM HEPES-NaOH, pH 8.5, 10 mM MgCl₂, 5 mM DTT, 1 mM each dNTP, and 2 μM polymerase, and was incubated for 2.5 h at 65°C under mineral oil. The 203-bp target fragment from the tk gene was isolated by restriction enzyme digestion and assembled into a full-length tk gene by hybridization to a gapped duplex plasmid bearing a functional chloramphenicol (Cm) resistance gene on the product-containing strand. The resulting plasmids were introduced by electroporation into strain FT334 (recA13, tk), plating on chloramphenicol to select for progeny of the DNA strand synthesized in vitro, and plating on chloramphenicol plus 5-fluoro-2′-deoxyuridine (FUDR) to select for inactivating tk mutants. To increase the probability that the plasmids sequenced would contain independent mutations, the FT334 cells after selection were plated onto chloramphenicol plus trimethoprim plates. For Klenow fragment, the error rate was determined by dividing the number of colonies on Cm alone by the number of detectable sites for that class of errors (information provided by K. Eckert). Because the tk mutants generated by Dbh polymerase contained multiple mutations, frequently frameshifts, we did not need to take account of whether individual mutations were detectable. Instead, the error rate for a particular class of errors was determined by calculating the fraction of the total errors contributed by that class, multiplying by the mutant frequency, and dividing by the number of detectable sites for that class of errors (see Tables II and III).

**Calculation of Error Rates**—The overall mutant frequency was calculated by dividing the number of colonies on Cm/FUDR by the number of colonies on Cm alone. For the purpose of calculating error rates, we used the portion of the tk target sequence from nucleotides 89 to 255, because the region from 256 to the EcoRV site at 286 did not consistently give reliable sequence data. For Klenow fragment, the error rate for a particular class of errors was determined by calculating the fraction of the total errors contributed by that class, multiplying by the mutant frequency, and dividing by the number of detectable sites for that class of errors (information provided by K. Eckert). Because the tk mutants generated by Dbh polymerase contained multiple mutations, frequently frameshifts, we did not need to take account of whether individual mutations were detectable. Instead, the error rate for a particular class of errors was calculated by dividing the number of times that error was observed by the number of opportunities for committing that error within the target sequence of the 46 sequenced clones (see Tables II and III).

**Kinetiic Measurements**—Single-turnover kinetic measurements of the polymerase reaction catalyzed by Dbh were carried out in a mixture containing 0.1 μM duplex DNA oligonucleotide, 5′-32P-labeled on the primer strand, and variable concentrations of one or more dNTPs in 10 mM HEPES-NaOH, pH 8.5, 10 mM MgCl₂, and 5 mM DTT. The reaction was initiated by addition of Dbh polymerase to 5 μM and was incubated at 22°C unless otherwise indicated. Under these conditions, the reaction rate was sufficiently slow to allow manual sampling. Samples were fractionated on a denaturing polyacrylamide gel, and the amounts of substrate and products were quantitated using a phosphorimaging device, as described previously (28). By showing that the reaction rate was unaffected by an increase in Dbh concentration to 20 μM, we established that all the DNA substrate was enzyme-bound under our reaction conditions.

**RESULTS**

**Reaction Conditions for Dbh Polymerase**—We carried out a series of reactions to enable us to choose appropriate reaction conditions for the Dbh polymerase. The enzyme was maximally active at pH values between 7.5 and 9.5, with lower activity below pH 7 (pH values above 9.5 were not tested). The polymerase was inhibited about 3-fold by the addition of 100 mM NaCl and much more severely by NaCl concentrations of 200 mM and above. Based on these observations, we chose standard buffer conditions of pH 8.5, with no added salt. The reaction rate increased steadily with increasing temperature, such that the rate at 65°C was 40-fold higher than the rate at 22°C (Fig. 1); Sulfolobus strains usually grow in environments at temperatures between 75 and 95°C (32). Most of the kinetic studies were carried out at 22°C, for convenience of sampling. For the mutational specificity determination, where more extensive DNA synthesis was required, the reactions were incubated at 65°C.

**Fidelity of Dbh Polymerase**—We used the HSV-tk forward mutation assay developed by Eckert and coworkers (31) to investigate the fidelity of the Dbh polymerase. In this assay the DNA polymerase being studied is used to copy a part of the HSV-tk gene in vitro. The newly synthesized DNA strand is

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7 The abbreviations used are: HSV-tk, herpes simplex virus thymidine kinase; DTT, dithiothreitol; Cm, chloramphenicol; FUDR, 5-fluoro-2′-deoxyuridine; pol, polymerase.
then hybridized to a tk-containing plasmid, which has a single-stranded gap in the relevant part of the tk gene. After transformation into a tk strain of E. coli, plasmid-containing cells are selected by resistance to chloramphenicol; those containing an inactivating mutation in the tk gene are, in addition, resistant to the nucleoside analog 5'-fluoro-2'-deoxyuridine (FUdR). Preliminary experiments established that the background mutation rate (background mutations per plasmid per generation) was 1.6 \times 10^{-8} (average of three determinations), in agreement with published data (31). Also in agreement were our measurements of mutation frequency after transformation of the gapped plasmid (4.7 \times 10^{-8}, average of three determinations), and the frequency of tk mutants generated by exonuclease-deficient Klenow fragment (3.8 \times 10^{-8}, average of three determinations). Transformation with a plasmid carrying an inactivating mutation in the tk gene gave very similar numbers of colonies on Cm and Cm/FUdR, indicating that there was no significant difference in plating efficiency on the two media.

When Dbh was used for primed synthesis of the tk target, the apparent mutant frequency (number of colonies on Cm/FUdR divided by number of colonies on Cm) was 0.17 (average of two determinations). On sequencing tk^− clones, almost all contained multiple mutations, an average of about six per clone (Table I). The high frequency of mutations per sequenced clone (98% had two or more mutations) indicates clearly that essentially all of the DNA molecules synthesized by Dbh contained at least one mutation, and therefore that the true mutant frequency was close to 1.0; this value was used in calculating the frequency of particular errors.

Sequencing of tk^− clones revealed that 80% of the Dbh-generated mutations were one- or two-base deletions (Table I and Fig. 2). By contrast, the mutant collection resulting from primed synthesis catalyzed by Klenow fragment was primarily made up of base substitutions (~60% of the total), consistent with published data for this and other classical DNA polymerases (33). To investigate the possibility that the unusual error distribution in the Dbh spectrum might have resulted from DNA damage caused by the high temperature (65°C) used in the enzymatic primed synthesis reaction, we carried out primed synthesis by Klenow fragment with an additional heating step, heating either the DNA template before the polymerase reaction, the primed synthesis products before fractionation, or the gapped plasmid with hybridized small fragment before transformation. These modifications resulted in at most a 3-fold increase in mutant frequency; moreover, there was no noticeable increase in the frequency of deletion mutations recovered from heat-treated DNAs.

Analysis of the one- and two-base deletions recovered in the Dbh spectrum revealed a pronounced sequence dependence to the mutation frequency (Table II). Frameshift hotspots involved deletions of a template pyrimidine, with a slight preference for C over T; compare frequencies of deletion of the repeated base within 5′-GCC, 5′-GTT, and 5′-GAA. Deletions occurred overwhelmingly adjacent to a 5′-G on the template strand; compare 5′-GCC with 5′-ACC and 5′-TCC. Additionally, there was a preference for deleting a base situated within a run of two or more identical bases (compare 5′-GC, 5′-GCC, and 5′-GCCC, or 5′-GT, 5′-GTT, and 5′-GTTT), although, unlike classical polymerases, Dbh showed little or no increase in deletion frequency as the run length increased beyond two bases. Examination of Fig. 2 suggests that the above three criteria may be all that is required to define a deletion hotspot in the Dbh spectrum, because similar sequence motifs in different surrounding sequences gave very similar numbers of errors. For example, three separate 5′-GCC sequences gave 26, 28, and 23 deletions, and three separate 5′-GCC sequences gave 18, 20, and 19 deletions. The high mutation frequencies at these hotspots corresponded to the polymerase making an error about half of the time when it copied these particular sequences.

Because base substitution mutations were a minor component of the Dbh mutational spectrum, the number recovered
been heated at 65 °C obtained. The standard reaction carried out at 22 °C synthesis reaction. In our Klenow fragment mutational analysis, one C-to-T transition was obtained out of a total of 15 mutants analyzed from the 28160 reported for classical polymerases in the absence of proofreading, the error rate was about 10- to 100-fold higher than that interesting trends were apparent (Table III and Fig. 2). First, the extension of mispaired primer termini. The large number of tagenesis, namely dNTP selection and discrimination against both of the processes that contribute to base substitution mutagenesis, namely dNTP selection and discrimination against context of base substitution error frequencies of Dbh polymerase.

### Table II

| Deleted base | Sequence context | Number of sequences | Number of deletions | Error frequency$^d$ |
|-------------|------------------|---------------------|--------------------|---------------------|
| A           | (G)AAAA          | 2                   | 5                  | $5.4 \times 10^{-2}$ |
|             | (C)AA            | 0                   | $1.1 \times 10^{-2}$ |
|             | (G)AA            | 4                   | $4.3 \times 10^{-3}$ |
|             | (C)A             | 0                   | $2.2 \times 10^{-3}$ |
|             | (G)A             | 2                   | $8.7 \times 10^{-3}$ |
|             | (T)A             | 1                   | $3.6 \times 10^{-3}$ |
| C           | (T)CCCC          | 1                   | 7                  | 0.15 |
|             | (G)CCC           | 77                  | $3.9 \times 10^{-2}$ |
|             | (A)CC            | 9                   | 0.41 |
|             | (G)CC            | 57                  | 6.5 $\times 10^{-2}$ |
|             | (T)CC            | 3                   | 5.4 $\times 10^{-3}$ |
|             | (A)C             | 2                   | $1.7 \times 10^{-3}$ |
|             | (G)C             | 13                  | $4.3 \times 10^{-2}$ |
|             | (T)C             | 5                   | 2.3 $\times 10^{-2}$ |
| G           | (T)GGGG          | 1                   | 0                  | $2.2 \times 10^{-2}$ |
|             | (C)GGG           | 2                   | 1                  | $1.1 \times 10^{-2}$ |
|             | (A)GG            | 0                   | $2.2 \times 10^{-2}$ |
|             | (C)GG            | 0                   | $3.6 \times 10^{-3}$ |
|             | (A)G             | 8                   | $2.7 \times 10^{-3}$ |
|             | (C)G             | 13                  | $1.7 \times 10^{-3}$ |
|             | (T)G             | 5                   | $4.3 \times 10^{-3}$ |
| T           | (G)TTT           | 1                   | 6                  | 0.13 |
|             | (G)TT            | 37                  | 0.27 |
|             | (A)T             | 5                   | 0                  | $4.3 \times 10^{-2}$ |
|             | (C)T             | 6                   | 7.2 $\times 10^{-2}$ |
|             | (G)T             | 3                   | 2.2 $\times 10^{-2}$ |

$^a$ Refers to the template strand as shown in Fig. 2.

$^b$ The base 5’ to the deleted base (or run containing the deleted base) is shown in parentheses.

$^c$ The number of occurrences of each type of sequence within the 167-nucleotide region of the tk target for which reliable sequence data were obtained.

$^d$ Calculated by dividing the number of deletions obtained by the number of times the corresponding type of sequence was encountered in 46 sequenced tk mutants; e.g. for the (G)AAA sequence, error frequency = 5 ÷ (2 × 46).

### Table III

| Template base | Number$^a$ | Mutation | Mispair | Number of mutations$^b$ | Error rate (Dbh) $(\times 10^{-3})$ | Error rate (KF) $(\times 10^{-3})$ |
|---------------|------------|----------|---------|-------------------------|------------------------------------|----------------------------------|
| A             | 37         | A → G    | A · dCTP| 5 (4)                   | 2.9                                | 0.0065                           |
|               |            | A → C    | A · dGTP| 1                       | 0.59                               | $0.0059$                        |
|               |            | A → T    | A · dATP| 1                       | 0.59                               | 0.0084                          |
| C             | 57         | C → T    | C · dATP| 16$^c$                  | 6.1                                | 0.053                           |
|               |            | C → A    | C · dTTP| 3 (2)                   | 1.1                                | $0.011$                         |
|               |            | C → G    | C · dCTP| 5 (5)                   | 1.9                                | $0.0063$                        |
| G             | 50         | G → A    | G · dTTP| 2                       | 0.87                               | 0.0092                          |
|               |            | G → C    | G · dGTP| 1                       | 0.43                               | 0.053                           |
|               |            | G → T    | G · dATP| 2                       | 0.87                               | 0.040                           |
| T             | 23         | T → C    | T · dGTP| 4                       | 3.8                                | 0.071                           |
|               |            | T → A    | T · dTTP| 0                       | $0.95$                             | 0.0063                          |
|               |            | T → G    | T · dCTP| 2 (2)                   | 1.9                                | 0.026                           |

$^a$ The number of occurrences of each template base within the 167-nucleotide region of the tk target for which reliable sequence data were obtained.

$^b$ The number of mutations that could have resulted from misalignment-mediated misinsertion is shown in parentheses.

$^c$ The calculation is analogous to that described in Table II.

$^d$ Error rates for exonuclease-deficient Klenow fragment (KF) are taken from Ref. 33.

The large number of C-to-T transition mutations may have resulted from deamination of C at the temperature of 65 °C.

### Analysis of one- and two-base deletions formed by Dbh polymerase

Analysis of 228 single-base deletions and 4 two-base deletions, grouped with respect to the identity of the deleted base.

### Table III

| Template base | Number$^a$ | Mutation | Mispair | Number of mutations$^b$ | Error rate (Dbh) $(\times 10^{-3})$ | Error rate (KF) $(\times 10^{-3})$ |
|---------------|------------|----------|---------|-------------------------|------------------------------------|----------------------------------|
| A             | 37         | A → G    | A · dCTP| 5 (4)                   | 2.9                                | 0.0065                           |
|               |            | A → C    | A · dGTP| 1                       | 0.59                               | $0.0059$                        |
|               |            | A → T    | A · dATP| 1                       | 0.59                               | 0.0084                          |
| C             | 57         | C → T    | C · dATP| 16$^c$                  | 6.1                                | 0.053                           |
|               |            | C → A    | C · dTTP| 3 (2)                   | 1.1                                | $0.011$                         |
|               |            | C → G    | C · dCTP| 5 (5)                   | 1.9                                | $0.0063$                        |
| G             | 50         | G → A    | G · dTTP| 2                       | 0.87                               | 0.0092                          |
|               |            | G → C    | G · dGTP| 1                       | 0.43                               | 0.053                           |
|               |            | G → T    | G · dATP| 2                       | 0.87                               | 0.040                           |
| T             | 23         | T → C    | T · dGTP| 4                       | 3.8                                | 0.071                           |
|               |            | T → A    | T · dTTP| 0                       | $0.95$                             | 0.0063                          |
|               |            | T → G    | T · dCTP| 2 (2)                   | 1.9                                | 0.026                           |

$^a$ The number of occurrences of each template base within the 167-nucleotide region of the tk target for which reliable sequence data were obtained.

$^b$ The number of mutations that could have resulted from misalignment-mediated misinsertion is shown in parentheses.

$^c$ The calculation is analogous to that described in Table II.

$^d$ Error rates for exonuclease-deficient Klenow fragment (KF) are taken from Ref. 33.

The large number of C-to-T transition mutations may have resulted from deamination of C at the temperature of 65 °C.

In our Klenow fragment mutational analysis, one C-to-T transition was obtained out of a total of 15 mutants analyzed from the standard reaction carried out at 22 °C, whereas three were obtained, out of a total of 21, from a reaction in which the M13 template had initially been heated at 65 °C for 2 h.

C-to-T transitions in the Dbh spectrum should probably be viewed with some caution, because they may have resulted from deamination of template C residues (see Table III). In both the Dbh and Klenow fragment spectra, about one-third of the base substitutions could potentially have taken place through a misalignment mechanism. In the Dbh spectrum, the majority of these (11 of 13) involved misinsertion of a C, resulting in a mutation of 5'→XC to 5'→CC on the synthesized strand (5'-GX to 5'-GG on the template strand). By contrast, none of did not constitute a good statistical sample. Nevertheless, some interesting trends were apparent (Table III and Fig. 2). First, the error rate was about 10- to 100-fold higher than that reported for classical polymerases in the absence of proofreading, exemplified by Klenow fragment (exo-). This suggests that Dbh is less stringent than the classical polymerases in one or both of the processes that contribute to base substitution mutagenesis, namely dNTP selection and discrimination against extension of mispaired primer termini. The large number of
the eight possible misalignment-mediated substitutions in the Klenow fragment spectrum involved misinsertion of a C (data not shown), although this comparison is somewhat suspect because none of the putative misalignment-mediated base substitutions in the Dbh spectrum would have been detected in the Klenow fragment experiment. The Dbh-generated substitutions were present in clones that also contained frameshifts, and therefore there was no requirement that the base substitutions cause tk deficiency. By contrast, all but one of the tk/H11002 clones generated by Klenow fragment were singly mutant, and therefore the mutations must have caused tk deficiency to be detectable; this allows only five sites at which a template 5'-GG change could occur (31).

Kinetic Measurements — We measured the kinetics of nucleotide incorporation by Dbh to better understand the error specificity of this DNA polymerase. Reactions were carried out in the presence of a large excess of enzyme to ensure that all the DNA substrate was enzyme-bound at the start of the reaction. Under these conditions the maximal reaction rate reflects \( k_{pol} \), the rate-limiting step up to the completion of phosphoryl transfer, and the dNTP dependence of the reaction rate gives \( K_{D}(dNTP) \). The kinetic parameters for insertion of a correct dNTP opposite its complementary template base show that the reaction is much less efficient than that catalyzed by a polymerase such as Klenow fragment (Table IV). The \( K_{f}(dNTP) \) values were in the range of 300 to 1000 \( \mu \text{M} \), compared with 1 to 10 \( \mu \text{M} \) for Klenow fragment (34). Additionally, the reaction rate (at 22 °C) was 10\(^{-3}\) to 10\(^{-4}\) fold slower than that catalyzed by Klenow fragment. Even allowing for the fact that, at 22 °C, Dbh is further from its optimum temperature than is Klenow fragment, this would still amount to \( -100\)-fold difference in reaction rate. Incorporation of the \( \alpha\)-thio analog dCTP\(\alpha\)-S opposite G in the first DNA substrate shown in Table IV was 4-fold slower than incorporation of the normal oxy substrate (data not shown). A similar elemental effect (4-fold) was seen for incorporation of the three incorrect dNTPs opposite the same template G.

When inserting correctly paired dNTPs, the Dbh polymerase showed a preference for dCTP, with \( k_{pol} \) for correct incorporation of this nucleotide being about 10-fold higher than for the other three nucleotides opposite their complementary partners. The reaction rate was also influenced by the nature of the sequence on the template strand following the templating base. The reaction was 3- to 4-fold slower when the templating base was followed by a run of G residues than when it was followed by runs of Ts (Table IV), As or Cs, or a non-run sequence (data not shown). The strong influence of sequence context on reaction rate is also demonstrated by the data in Table V. The limited comparison shown in Table IV suggests that the preference for dCTP insertion is maintained regardless of sequence context.

Frameshifting in Vitro — Using an oligonucleotide substrate corresponding to one of the frameshift hotspots within the tk target sequence (last entry of Table IV), we obtained a product consistent with a single-base deletion event. Incorporation of dCTP (the putative frameshift reaction) occurred at a rate very similar to the rate of the non-frameshifted correct dGTP incorporation (Fig. 3a and Table IV), implying that Dbh should make a frameshift within the hotspot sequence about 50% of the time, consistent with the results of the mutational assay. The alternative interpretation, that the dCTP incorporation resulted from C-dCTP misinsertion, is extremely unlikely, based on the very slow rate of dCTP incorporation observed with a control substrate that differed only in replacement of the downstream template G by a T residue (Fig. 3b). Because our data showed the rate of frameshift dCTP insertion to be approximately equal to the rate of correct C-dGTP incorporation on the same DNA sequence, and because, in other sequence contexts, correct (non-frameshifted) dCTP insertion was -10-fold faster than correct insertion of the other three dNTPs
(Table IV), we estimate the kinetic cost of frameshifting to be a modest ~10-fold decrease in rate.

To investigate factors that appeared to influence the frequency of frameshifhst mutagenesis, as deduced from Table II, we compared the rates of addition of correct and frameshifted single dNTPs on several different DNA substrates, at a single dNTP concentration (Table V). In every case that was tested, the frameshifting reaction was faster than the alternative re-action of misincorporation of the added dNTP opposite the first unpaired template position, which was estimated using an otherwise identical DNA sequence that did not present any opportunity for frameshifting. The DNA sequences used in this experiment were derived from the frameshift hotspot sequence described in Table IV. Other sequences were derived from F1 to test factors that might influence frameshifting.

Column sequences were compared in Table V, it appears that the incorporation rate of correct dNTP incorporation (Table V). From the sequence was, and indicated the likelihood that a frameshifted product would be obtained in a primed synthesis reaction in the presence of all four dNTPs. As already deduced from the kinetic parameters (Table IV), the rates of frameshift and non-frameshift incorporation on the F1 substrate were approximately equal, consistent with this sequence being a mutational hotspot. The probability of frameshifting on the other sequences was 7- to 35-fold lower, although this decrease was as likely to result from a faster rate of correct insertion (e.g. F4) as from a slower rate of frameshift incorporation.

Although the rate data indicate that the dNTP insertion re-actions that initiate single-base deletion events are relatively facile, examination of the reaction products suggests that subsequent realignment of the primer terminus also takes place. In Fig. 3a, addition of a second C residue can be seen at long incubation times after the initial frameshift dCTP insertion. Because of the extremely slow rate of C-dCTP misinsertion (Fig. 3b), we attribute the second C addition to insertion opposite dGTP after realignment of the primer terminus (forming a mispair) rather than to C-dCTP misinsertion. The formation of a product extended by four G residues suggests realignment of some fraction of the primer termini after frameshifting.

**Bypass of Abasic Sites**—As previously reported (8), Dbh can bypass an abasic site. To determine which dNTP is chosen by Dbh for insertion in response to an abasic site, we examined synthesis in the presence of only a single dNTP on templates containing abasic residues (Fig. 4). When a single abasic resi-due is followed by unmodified nucleotides, frameshifting appears to play a role in the bypass, because the preferred dNTP for insertion opposite the abasic site is the dNTP complementary to the abasic spacer. We tested two DNA substrates in which template information was absent from the positions immediately beyond the primer ter-minus: these were a blunt-ended duplex and one having five

### Table V

| Sequence | Correct dNTP (corr) | Frameshift (fs) | Ratio fs/corr | Mispaired dNTP |
|----------|-------------------|---------------|---------------|---------------|
| 5'-CCG   | dGTP 0.081        | dCTP 0.055    | 0.7           | C-dCTP 0.00032 (F2 CTCC) |
| 3'-AGCGGCCC | dGTP 0.054   | dCTP 0.0046  | 0.09          | C-dATP 0.00063 (F1 CGCC) |
| 5'-CGG   | dGTP 0.12        | dCTP 0.0023   | 0.02          | C-dCTP 0.00032 (F2 CTCC) |
| 3'-GCTGCCCC | dGTP 1.06     | dCTP 0.11    | 0.1           | T-dCTP 0.0079 (F5 TCCC) |
| 5'-CCA   | dATP 1.44        | dGTP 0.050    | 0.03          | T-dGTP 0.011 (F4 TGCC)  |
| 3'-GCTCCCC | dGTP 0.90      | dCTP 0.018   | 0.02          | A-dCTP 0.0015 (ACCC)    |
| 5'-CTT   | dCTP 0.86        | dCTP 0.033    | 0.04          | A-dCTP 0.0015 (ACCC)    |

* Rates were measured as described in “Experimental Procedures” at a single dNTP concentration of 200 μM.

* For clarity, only the sequence close to the primer terminus is shown. The F1 substrate corresponds to the frameshift substrate listed at the end of Table IV. Other sequences were derived from F1 to test factors that might influence frameshifting.

* Incorporation of the dNTP complementary to the first unpaired template base.

* The rate of formation of the indicated mispair was measured on a closely related substrate which differed in the sequence of unpaired template bases so that it did not present any opportunity for a competing frameshift incorporation. The template sequence, from 3’ to 5’, is shown; in most cases the substrate corresponds to one used elsewhere in this experiment, as indicated by the sequence number.


**DISCUSSION**

*Error Specificity of the Dbh Polymerase—*Our data show that Dbh has a remarkable tendency to make single-base deletions and, to a lesser extent, base substitutions immediately 3' to the deleted base. Because the DinB study analyzed mutations recovered in phage λ after growth on a strain overexpressing DinB, it was unclear which DNA strand served as the template during the error-producing synthesis event, and therefore the precise nature of the mutational pathway could not be determined with certainty. An advantage of mutational analyses of the type we have used, in which a single-stranded template is copied in *vitro*, is that the DNA strand ambiguity is removed, allowing us to conclude that the 5'-G residues that appear to be important in determining the mutational outcome are located on the template strand when the polymerase error takes place.

**Fig. 4.** Dbh-catalyzed nucleotide addition on templates containing abasic sites. *a,* sequence of the DNA substrates. The primer strand was 5'-labeled with 32P. The four template bases immediately 5' to the primer terminus (shown as NNNN) were varied as indicated in the schematic representations in *b* and *c.* Exceptions were the blunt-ended substrate, which had no DNA beyond the primer terminus, and the substrate whose single-stranded template consisted of five abasic spacers with no additional DNA. The "x" represents a stable abasic site. *b,* gel electrophoresis of the products of reactions testing the addition of single dNTPs to the indicated DNA substrates. The reactions contained 40 nM duplex DNA substrate, 0.5 μM Dbh, and 0.5 mM of a single dNTP and were incubated at 37°C for 35 min. Each group of four lanes corresponds to the testing of one template sequence with the four separate dNTPs. *c,* bar graph representation of the yield of extended product obtained after 15-min reaction of each of the DNA substrates with each dNTP. Throughout, the asterisks represent the 32P label at the 5'-end of the primer strand of each DNA substrate.

Single-base deletions in sequences with a G residue 5' to the deleted base. Because the DinB study analyzed mutations recovered in phage λ after growth on a strain overexpressing DinB, it was unclear which DNA strand served as the template during the error-producing synthesis event, and therefore the precise nature of the mutational pathway could not be determined with certainty. An advantage of mutational analyses of the type we have used, in which a single-stranded template is copied in *vitro*, is that the DNA strand ambiguity is removed, allowing us to conclude that the 5'-G residues that appear to be important in determining the mutational outcome are located on the template strand when the polymerase error takes place.

**Mammalian DNA polymerase k (pol k),** another member of the DinB branch of the bypass polymerase family, has also been reported to make a high frequency of single-base deletion errors (35-37). The error specificity of human pol k described by Ohashi *et al.* (37) resembles that of Dbh in that more than 50% of the one- and two-base deletion errors were deletions of a template pyrimidine in a 5'-Gpy sequence (see Fig. 2 of Ref. 37). However, this error was not as dominant as in the Dbh error spectrum, where it accounted for more than 80% of the frameshifts, and, moreover, human pol k did not produce a particularly high frequency of 5'-G to 5'-G mutations.

Wagner and Nohmi (22) presented a model for DinB errors in which they postulated that the initiating event was misinsertion of a C residue at the primer terminus by DNA polymerase III holoenzyme, which subsequently dissociates. Extension of this mutagenic intermediate by DinB would then be favored...
either if realignment of the primer terminus could take place, pairing the terminal C with a neighboring G and generating a single-base deletion, or if the mispair could be extended by addition of a further dCTP residue, resulting in mutation of 5'-GX to 5'-GG on the complementary strand. Our data for Dbh imply that the error specificity characteristic of DinB-like polymerases can be generated by the bypass polymerase alone, without the intervention of other cellular polymerases. Moreover, our kinetic data indicate that Dbh prefers to insert dCTP, at least in some sequence contexts, which could account for the tendency of the Dbh polymerase to cause frameshifts adjacent to template G residues. The kinetic parameters for frameshift insertion of dCTP opposite G on a model substrate (Table IV) suggest that the energetic cost of accommodating a slipped template base is relatively modest, so that incorporation of dCTP opposite G in the frameshift position might compete effectively with incorporation of A, G, or T at the correct (non-frameshifted) position. Further synthesis without rearrangement would give a single-base deletion, whereas realignment of the primer terminus followed by extension of the resulting mismatch would result in a 5'-GX to 5'-GG base substitution on the template strand. The preference for deletion of template pyrimidines and for a run of two or more identical bases may suggest that the extra base on the template strand can be more easily accommodated if it is a pyrimidine and if it can be rearranged to an alternative position just within the primer-template duplex. Because the deletion frequency in homopolymeric runs longer than two bases is not substantially increased over that in runs of two identical bases, there appears to be no advantage in moving the extra base further into the duplex region. This contrasts with the effect of homopolymeric run length on deletion frequency in classical polymerases (38).

Frameshift Reactions—Our studies on model oligonucleotide substrates in the presence of single dNTPs (Table V) have allowed us to measure the rates of the individual competing steps and thus deduce plausible pathways for frameshift-related processes. Dbh polymerase generated probable frameshift products from all of the sequences tested, not merely from those (F1 and F4) that represented the most likely frameshift hotspots. In principle, the observed products might have resulted from misincorporation opposite the first (non-frameshifting) template position. To address this possibility, we measured the rate of the corresponding misincorporation reaction on a DNA duplex that differed only at the second template position such that it did not present any opportunity for frameshifting with the incoming dNTP under investigation. In every case that we tested, frameshift incorporation opposite the complementary base was faster than the alternative misincorporation reaction; often there was a substantial difference in rate, so that the frameshift pathway would be expected to predominate over misincorporation.

From the single-nucleotide reactions, we can identify factors that determine whether a frameshift is likely to result when Dbh copies a template in the presence of all four dNTPs. First, frameshift incorporation must compete effectively with incorporation of the complementary nucleotide at the non-frameshift template position. We found that the rates of frameshift incorporation on different sequences did not always agree with our expectations from the mutational analysis. Because the rates of correct dNTP insertions by Dbh are exquisitely sensitive to the DNA sequence around the primer terminus, the probability of obtaining a frameshift mutation is as likely to be influenced by the rate of correct (non-frameshifted) dNTP incorporation as by the rate of the frameshift reaction. In primed synthesis reactions on the tk template sequence in the presence of all four dNTPs, we have noted that the Dbh polymerase reaction is slower when traversing the rather GC-rich sequences around the frameshift hotspots. Although the correlation is not perfect, it raises the possibility that sequence context may influence frameshifting through an effect on the rate of ongoing (non-frameshift) polymerase synthesis. In the situation where frameshift insertion has taken place to initiate the mutagenic pathway, then the eventual outcome (deletion or base substitution) will depend on whether the primer terminus is realigned before further extension takes place. The abundance of one- and two-base deletions in the mutational spectrum of Dbh suggests that, for the hotspots at least, extension of the frameshift intermediate is preferred over realignment and mismatch extension. The balance between these two competing processes in other sequence contexts will probably be influenced by the ease with which each particular frameshift intermediate is accommodated within the active site versus the preference for extending particular mispairs.

Frameshift incorporation also appears to play a role in synthesis by Dbh on templates containing abasic sites. We consistently observed incorporation of the dNTP complementary to the template base 5' to the abasic site, implying that the addition was templated by this base. Similar behavior has been reported for eukaryotic DNA polymerase β (39). When the template is entirely devoid of coding information, either by having a blunt end or by having a run of four abasic spacers, Dbh polymerase prefers to add dATP, suggesting that, like many DNA polymerases, it uses A as the default nucleotide opposite a non-informational lesion.

Dbh Polymerase Kinetics—Single-turnover kinetics of Dbh gave a rate (k_{pol}) that was around 100-fold lower than that of a classical polymerase (Klenow fragment), even allowing for the different temperature optima of the two enzymes. A similarly low k_{pol} value was reported in single-turnover studies of DNA polymerase η (40). In vivo the catalytic rate of Dbh, like that of E. coli DinB, is probably enhanced by interaction with the corresponding sliding clamp processivity factor (5, 41, 42). Perhaps the dependence of these bypass polymerases on replisome accessory subunits targets their activity to the required sites on the chromosome and avoids undesirable low fidelity synthesis elsewhere. Unlike pol η, Dbh showed very weak affinity for dNTP substrates; as noted above, this is consistent with the small number of contacts seen in the polymerase active site.

As noted in the discussion of error specificity, our kinetic data indicated a more rapid rate (k_{pol}) for correct insertions involving dCTP. Steady-state kinetic data for other DinB-like polymerases also show a slight bias toward dCTP insertion, when compared with other correct dNTP insertions (5, 19, 36). Moreover, a steady-state analysis of misincorporation by Dbh showed a bias toward mispairs involving incoming dCTP (11). The ability of Dbh to form misaligned primer termini means that misinsertion kinetics should be interpreted with caution, because an apparent misinsertion reaction may in fact have been templated by the 5’ neighbor of the expected templating base. (See, for example, Ref. 19, where the DNA substrate had a T 5’ to the first template base, and dATP was the preferred misinsertion regardless of the identity of the first template base.) A similar mechanism (called “dNTP-stabilized misalignment”) has been proposed to account for anomalously high misinsertion efficiencies by E. coli DNA polymerase III holoenzyme in particular sequence contexts (43).

The small sulfur elemental effect (~4-fold) in both correct and incorrect dNTP insertions by Dbh resembles the data for pol η and is subject to the same caveats (40). Most noteworthy
is that this behavior is entirely different from that seen with classical polymerases, where a small elemental effect for incorporation of a correct dNTP contrasts with a much larger elemental effect for misinsertion (44). In the classical polymerases, the widely accepted interpretation is that the chemical (phosphoryl transfer) step of the polymerase reaction is rate-limiting for misinsertion, but that a preceding non-covalent (“conformational change”) step is rate-limiting for insertion of a correct dNTP. Our data do not distinguish between two plausible explanations for the Dbh elemental effects: either the chemical step is not rate-limiting even with an incorrect nucleotide or the chemical step is not associated with as large an elemental effect as in the classical polymerases. The latter scenario is not unreasonable, given that the elemental effect in classical polymerases may have a substantial steric component (45) and the active site in the bypass polymerases is much less enclosed than in the classical polymerases. The possibility that the bypass polymerases do not have a conformational change step preceding chemistry is unlikely in the face of convincing evidence for a two-step mechanism in the pol η system (40).

**Bypass Polymerase Structures**—Our data can be examined within the framework of the recently solved crystal structures of bypass polymerases. Particularly relevant to our work are the two structures of uncomplexed Dbh (8, 11) and the ternary complex (polymerase-DNA-dNTP) of the Dpo4 polymerase from another strain of S. solfataricus (10). The Dbh and Dpo4 polymerases are 63% identical over the N-terminal 200 amino acids, which encompass the five conserved motifs common to all polymerases are 63% identical over the same region. Given the congruence we see in the two structures of uncomplexed Dbh (8, 11) and the ternary complex (polymerase-DNA-dNTP) of the Dpo4 polymerase from another strain of S. solfataricus (10). The Dbh and Dpo4 polymerases are 63% identical over the N-terminal 200 amino acids, which encompass the five conserved motifs common to all bypass polymerases. By contrast, Dbh and E. coli DinB are only 40% identical over the same region. Given the congruence we have described between the mutational specificities of Dbh and DinB, it is reasonable to assume that the properties of Dpo4 will be essentially identical. Therefore, our results with Dbh should be interpretable in terms of the polymerase active site seen in the Dpo4 ternary complex.

As had been surmised from the structures of the uncomplexed bypass polymerases, the Dpo4 ternary complex (10) showed a binding site for the incoming nucleotide that had fewer protein side-chain contacts than are seen in the classical polymerase families, consistent with the unusually high dissociation constants for dNTP binding in our kinetic experiments. In the Dpo4 cocrystal with DNA and a correctly paired incoming nucleotid, the template base on the 5’ side of the nascent base pair is unstacked from its neighbor and rotated out of the active site, similar to the conformation of the analogous template base in ternary complex cocrystals of DNA polymerases from the bacterial pol I family (14, 15). It has been suggested that the dislocation of base stacking immediately 5’ to the templating base may serve to discourage frameshifting (46). By contrast, the second cocrystal ternary complex described for Dpo4 has some of the characteristics expected for a −1 frameshift intermediate (10). There is no unstacking of the two template bases immediately beyond the primer terminus, so that both remain within the active site region. An incoming ddGTP, rather than forming a mispair with the first templating base (a G), pairs with a complementary C that is the second base beyond the duplex terminus. However, this ternary complex clearly cannot be a catalytically competent conformation for, otherwise, reaction would have taken place because the primer terminus has a 3’-hydroxyl. A possible explanation is revealed on close inspection of the structure: the space between the primer-terminal base and the base of the incoming ddGTP is occupied by a molecule of ethylene glycol. Perhaps, if this molecule were absent, the primer terminus and incoming nucleotide could adopt conformations that bring the primer 3’-OH and the dNTP α-phosphate into a position where phosphoryl transfer can occur. It is also possible that the relative orientation of the reactive groups in a frameshift ternary complex is influenced by the immediate sequence context, particularly the identity of the skipped template base, and this might explain the profound sequence dependence of frameshift frequency that we have observed.

Is Frameshifting Related to DinB Function?—The mutational hallmark of the DinB branch of the bypass polymerase family is a high frequency of misalignment errors at template G positions, resulting in either frameshifts or base substitutions. To what extent does this shed light on the role of the DinB family in vivo? In E. coli, DinB has been shown to be required for adaptive point mutation in stationary phase cells (23, 25), and it has been hypothesized that DinB functions to accelerate genetic change in response to stress (7). In the experiments demonstrating the requirement for DinB, adaptive mutation was measured by the ability to revert a single-base insertion mutation; therefore, it is not particularly surprising to observe a dependence on a gene product with a demonstrated ability to create single-base deletions at high frequency. However, we feel it is unlikely that evolution would select a DNA polymerase that makes frequent frameshift errors (3 per 100 bases synthesized) as the means for promoting genetic change under conditions of environmental stress. Arguably, a base substitution mutator could provide greater genetic diversity with less risk of lethality.

An alternative explanation, which we prefer, is that the frameshifting properties we have observed for Dbh provide a clue as to the lesion bypass abilities of this and related polymerases. We hypothesize that DinB and its homologs are present in cells to bypass a particular type of DNA damage and that the sequence dependence of frameshifting mirrors the type of structure that is encountered during lesion bypass. Because Dbh appears best suited to form a frameshift intermediate in which a template pyrimidine is skipped to insert dCTP opposite a neighboring template G, we suggest that DinB might be able to bypass, in an error-free manner, a template G having a bulky covalent adduct located to its 3’ side and occupying approximately the same space as would a skipped pyrimidine base. If this reasoning is correct, a survey of published data suggests the most likely candidate for the lesion bypassed by DinB and its homologs to be a benzo[α]pyrene-modified G, or a similar structure. In E. coli, DinB is required for error-free bypass of benzo[α]pyrene-N2-dG lesions in vivo, and bypass of benzo[α]pyrene-G adducts has been observed in vitro, both with E. coli DinB and human DNA polymerase κ (27, 47). By contrast, the data for bacterial, archaeal, and mammalian DinB enzymes do not show a consistent pattern of bypass of any other types of DNA lesions (5, 19, 26, 41, 47–50).

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