Mismatch Extension by *Escherichia coli* DNA Polymerase III Holoenzyme*

(Received for publication, July 27, 1998, and in revised form, October 21, 1998)

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The in vitro fidelity of *Escherichia coli* DNA polymerase III holoenzyme (HE) is characterized by an unusual propensity for generating (−1)-frameshift mutations. Here we have examined the capability of HE isolated from both a wild-type and a proofreading-impaired mutD5 strain to polymerize from M13mp2 DNA primer-templates containing a terminal T(templates)C mismatch. These substrates contained either an A or a G as the next (5′) template base. The assay allows distinction between: (i) direct extension of the terminal C (producing a base substitution), (ii) exonucleolytic removal of the C, or (iii), for the G-containing template, extension after misalignment of the C on the next template G (producing a (−1)-frameshift). On the A-containing substrate, both HEs did not extend the terminal C (<1%); instead, they exonucleolytically removed it (>99%). In contrast, on the G-containing substrate, the mutD5 HE yielded 61% (−1)-frameshfts and 6% base substitutions. The wild-type HE mostly excised the mispaired C from this substrate before extension (98%), but among the 2% mutants, (−1)-frameshifts exceeded base substitutions by 20 to 1. The preference of polymerase III HE for misalignment extension over direct mismatch extension provides a basis for explaining the in vitro (−1)-frame- shift specificity of polymerase III HE.

The error rate of a DNA polymerase depends not only on the efficiency with which it discriminates against incorrect nucleotides during the insertion step but also on its efficiency in continuing synthesis from the mismatched primer-terminus that it created by misinsertion. This is most obvious for enzymes containing an associated proofreading activity. Proofreading and mismatch extension compete, and a slow extension rate is likely to lead to very few mismatches being able to escape removal by the exonuclease. Thus, the ability to extend mismatches is an important factor in determining polymerase fidelity.

The accuracy of the duplication of the genetic material in the bacterium *Escherichia coli* depends largely on the fidelity of the DNA polymerase III holoenzyme (HE), which is responsible for its chromosome replication (1). The HE is a large dimeric complex composed of 10 distinct subunits that is capable of simultaneously synthesizing the leading and lagging strands of the replication forks (2–4). In vitro fidelity studies of purified HE using either single-stranded phage templates (5, 6) or oligonucleotide substrates (7) have revealed that HE is quite accurate for base substitution errors. Interestingly, gap-filling synthesis of M13 DNA (6) using a forward mutational assay capable of detecting various types of synthesis errors revealed HE to be relatively inaccurate for (−1)-frameshift mutations. In fact, (−1)-frameshifts were the major class of errors generated by HE in this assay (6).

Studies of (−1)-frameshift mutations have lead to the proposal of several general mechanisms for their formation. One model proposed by Streisinger et al. (8) postulates the occurrence of slippage of the nascent DNA strand in homopolymeric sequences (the direct slippage model). Within this model, the frameshift mutation frequency increases with the length of the run, because larger runs produce more (and more stable) misaligned intermediates. This model has been suggested to contribute to spontaneous frameshift mutations in vivo (9–12) as well as frameshifts produced in vitro by a variety of DNA polymerases (13).

A second mechanism, the “misincorporation model” proposed by Kunkel and Soni (14), states that frameshift mutations can be initiated by misinsertion of a nucleotide. If the misinserted nucleotide is complementary to the next template base, then its 1-base forward misalignment can form a frameshift intermediate containing a correctly base-paired terminus (and an unpaired extra base in the template strand). As in the direct slippage model, this frameshift intermediate would be fixed into a (−1)-frameshift mutation by further extension. The misincorporation mechanism has been shown to operate during in vitro DNA polymerization by the Klenow polymerase (15) and human immunodeficiency virus type I reverse transcriptase (16).

A third possible model for (−1)-frameshifts generated by polymerase III HE in vitro results from recent work by Bloom et al. (7), who observed elevated misincorporation by polymerase III HE in cases where the misincorporated base was complementary to the next template base. The authors proposed a transient misalignment before incorporation, in which the incoming (incorrect) dNTP is aligned on the next template base (dNTP-mediated misalignment). Continued DNA synthesis from the misaligned intermediate (after incorporation of the misaligned dNTP) would also generate a (−1)-frameshift.

Based on a detailed analysis of in vitro mutation spectra produced by *E. coli* DNA polymerase III holoenzyme (both wild-type and MutD5), we had suggested that most of the observed (−1)-frameshift mutations produced by this enzyme occur via the misincorporation plus slippage mechanism (6). This conclusion was based on the following observations: (i) most of the (−1)-frameshifts occurred at nonreiterated tem-
plate positions, (ii) the (−1)-framen shift mutations were proofread with the same efficiency as the base substitution mutations, and (iii) (−1)-framen shift errors significantly increased under conditions of biased dNTP pools, specifically at template positions where the 5' neighbor is complementary to the dNTP provided in excess (6). We further suggested that the high level of (−1)-framen shift mutations created by this enzyme reflects a general inability to directly extend terminal mismatches and a greatly enhanced extension efficiency if the terminal mismatch can be misaligned on the next template base.

In this study, we have further investigated the mismatch extension capability of the wild-type and MutD5 holoenzyme by using a fidelity assay specifically designed to measure the relative probabilities by which a polymerase chooses each of the three possible pathways upon encountering a mismatched primer-terminus: (i) direct extension (yielding a base substitution), (ii) misalignment extension (yielding a 1-frameshift), or (iii) exonucleolytic proofreading (yielding no mutation). We show that for a mispaired primer-template in which misalignment is not possible, HE preferentially proofreads the mispaired base (>99%). However, for a primer-template in which the terminal primer base is complementary to the next template base, the polymerase extends from the slipped intermediate to yield a (−1)-framen shift mutation in a significant fraction of the cases. These data support models in which the in vitro framen shift specificity of HE results from its relatively low efficiency in direct mispair extension compared with its efficiency in misalignment and extension of the misaligned intermediate.

EXPERIMENTAL PROCEDURES

Materials and Chemicals—Bacteriophage M13mp2 and its mutant derivatives containing a T–G substitution at position 103 (mp2G103) or a G–A substitution at position 102 (mp2A102) in the lacZa gene were obtained from Dr. Katarzyna Bebene (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Strains MC1061, which was used to prepare competent cells, and CSH50, which was used as an α-complementation host to score plaque color, were stocks of our laboratory. The holoenzymes from a wild-type and a mutant strain were purified as described previously (6). Klenow polymerase (exo−), (Amicon Inc., Beverly, MA). Ultrapure dNTP, ATP, and E. coli single-stranded binding protein were purchased from Pharmacia Biotech, Inc.

Construction of M13mp2 Substrates with a 3'-Terminal Mismatch— M13mp2G103 RF DNA containing unique sites for restriction endonucleases Kpnl (15) was doubly digested with Kpnl and was blunt filled with calf spleen adenyl kinase to yield a 10-bp-long fragment containing a 3'-end mismatch. The fragment was purified on a 5% polyacrylamide gel and subcloned into the BamHI site of the M13mp2 replication vector. The resulting 30,000-bp-long fragment was sequenced completely and was used to transform competent MC1061 by electroporation with a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Inc.) set at 2.01 kV, 400 W, 25 μF. Immediately after electroporation, 1 ml of SOC medium was added. Plating was performed by adding the transfected cells to 3 ml of melted soft agar (42°C) containing 2.5 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.24 mg of isopropyl-1-thio-β-D-galactoside, and 0.25 ml of a mid-log culture of indicator strain CSH50. This mixture was poured onto minimal agar plates. The plates were inverted and incubated for 18–24 h at 37°C, followed by an additional 24-h incubation at room temperature. Plaques were isolated and classified according to their density, size, color, or colorless phenotype. The frequencies of mispair extension and misalignment extension were calculated by dividing the frequency of light blue plaques or colorless plaques, respectively, by 0.55. The latter represents the average 55% expression probability of a mutation contained in the (−)-strand of a full-length RF II molecule (17, 18). The use of a mismatch repair-proficient strain, such as MC1061, is preferred in this assay because it minimizes the occurrence of mixed (blue/colorless) plaques that make it difficult to score the light blue phenotype (17, 18).

RESULTS

To examine how E. coli DNA polymerase III HE processes terminal mismatches in vitro, we used a fidelity assay that permits distinction between the different pathways available to the polymerase upon encountering a primer-template containing a 3'-terminal mismatch. The mismatched substrates used in this study are described in Fig. 1. They are based on the M13mp2 lacZ α-complementation system and use differential plaque colors to distinguish the various reaction products. They represent a modification from those originally described by Kunkel and co-workers (17, 19–21), which have been used to test for the analysis of mismatch resolvase activity by the M13 polymerase. The mismatch resolvase assay was performed using plasmids containing a 3135-bp duplex region and a 4061-bp single-stranded region. The 3135-bp primer contains the terminal mismatch. Upon synthesis by polymerase III HE, the full-length RF BP II products can be readily separated from the starting molecules using a 0.8% agarose gel (see Fig. 2), thus allowing us to recover and separately analyze the full-length extension products. This feature is important for assaying rapid and highly processive polymerases such as HE, because it permits the analysis of extension products at very short reaction times, even when not all substrates are bound or extended.

Two substrates were prepared, both of which contained a T-C mismatch at the 3'-end of the primer strand (position 103 of the lacZa sequence) (Fig. 1). In one substrate, the next template nucleotide to be copied is a G, in the other substrate, it is an A. In both cases, the template strand is phenotypically wild-type (dark blue plaque), because the G→A change at position 102 is silent (17). However, the mismatched C in the primer strand is observed when a T→G base substitution mutant at position 103, which produces a light blue plaque. There are three possible outcomes for these two substrates when processed by HE. First, HE may simply extend the mispaired terminus C, creating a RF II heteroduplex molecule containing a base substitution mutation in the (−)-strand. Upon transfection, this molecule will yield a light blue plaque. (Actually, due to the action
of mismatch repair on the internal mismatch, upon transfer into the competent cells, the probability of producing a light blue plaque is about 55%; the remaining 45% carry the \( \text{(1)} \)-strand genotype and will be dark blue. The 55% \( \text{(2)} \)-strand expression efficiency has been determined experimentally for a large series of base base and frameshift mismatches (17, 18).

Second, in the case of the G-containing substrate, the terminal mismatched C may first misalign on the next template base G, followed by the extension of this misaligned structure. Upon transfection, this will yield (again at 55% efficiency) a mp2 frameshift mutant with a colorless plaque phenotype. Third, instead of extending the terminal mismatch, HE may remove the mispaired C by exonucleaseolytic proofreading. Upon extension, this would produce a wild-type dark blue plaque. Thus, a simple enumeration of light blue, colorless, and dark blue plaques reveals the fate of the terminal mismatch upon polymerization.

Extension reactions were performed for each DNA substrate with wild-type or MutD5 HE at two different dNTP concentrations: 50 and 1000 \( \mu \)M. The reaction products were separated by agarose gel electrophoresis (see Fig. 2), and full-length RF II molecules were purified and analyzed by transfection. Time-course experiments showed that the RF II product had already appeared after 15 s of reaction time, as expected for a rapid and highly processive enzyme such as HE (data not shown). As a control, we also performed extension reactions with the Klenow fragment (exo\(^-\); see “Experimental Procedures”).

The data in Fig. 3 show that with the substrate containing the T\( \_\)C mispair followed by a template A (right panel), most of plaques scored (>99%) in reactions with either the wild-type or MutD5 HE were dark blue, indicating that proofreading of the terminal base was the predominant outcome. The failure to detect light blue or colorless plaques even for the MutD5 HE, which possesses only limited (~5%) exonuclease activity (5), indicates that HE is very inefficient in extending directly from the mispaired terminus.

Extension by MutD5 HE of the substrate containing the T\( \_\)C mispair with the 5′ neighboring template G (Fig. 3, left panel) yielded high numbers of colorless and dark blue plaques and significantly fewer light blue plaques. Sequencing of randomly

**Fig. 1. Construction of terminally mismatched substrates.** M13mp2G103 RF DNA was digested with restriction endonucleases BamHI and KpnI to yield fragments of 3135 and 4061 bp. The 3135-bp fragment was purified and used as a primer. The primer was annealed to either wild-type single-stranded DNA (G at position 102) or modified single-stranded DNA (A at position 102) to produce heteroduplex molecules containing a T-C mispair at position 103 of lacZ with either G or A as the next template base (see “Experimental Procedures” for details).

**Fig. 2. DNA synthesis by polymerase III holoenzyme from a mispaired primer-terminus.** Lane 1, the starting substrate with terminal T-C mispair (top band). The lower two bands represent the single-stranded template and the 3135-bp primer fragment from which the substrate was constructed. Lane 2, the product of the extension reaction with 135 units of MutD5 HE (a 5-min reaction in the presence of 1000 \( \mu \)M of each of the four dNTPs) yielding a full-length RF II product. Reactions and electrophoresis were carried out as described under “Experimental Procedures.”
selected colorless and light blue plaques (10 each) showed that all colorless mutants had lost template T at the site of mispair, and that all light blue mutants carried the expected T to G base change at position 103. The proportion of colorless and light blue plaques was lower when the dNTP concentration was decreased from 1000 to 50 μM, reflecting the stimulatory effect of high dNTP concentrations on the two forward reactions as they compete with the proofreading step (6). The data with the G-containing substrate demonstrate that MutD5 HE is highly proficient in forming and extending a (−1)-framenshift intermediate (when permitted by the sequence context) as compared with direct extension of the mismatch, which is inefficient. The wild-type enzyme at either dNTP concentration produces a majority of dark blue plaques, indicating that proofreading is the predominating pathway. Nevertheless, among the mutants, colorless plaques outnumber the light blue plaques, indicating that misalignment extension is strongly preferred over direct extension in this case also. Note that the Klenow fragment greatly prefers direct extension over misalignment extension, as seen from the excess of light blue plaques versus colorless plaques (Fig. 3).

In Table I, we present the calculated probabilities for the G-containing substrate for each of the three pathways. At 1000 μM dNTP, the MutD5 HE chooses the misalignment extension pathway in 61% of the cases. The direct extension pathway is chosen in only 6% of the cases, while 33% of the mismatches are proofread. At 50 μM dNTP, the proportion of proofread molecules increases to 80.8%, whereas 17.8% are processed by misalignment extension, and only 1.5% are directly extended. Thus, the ratio between direct extension and extension from a misaligned intermediate at either dNTP concentration is about 1:10. The wild-type HE, which has a strong exonuclease proofreading activity (6), prefers to excise the mispaired C before polymerization. However, at 1000 μM dNTP, about 2% of the primer-templates are processed by HE to yield the (−1)-framenshift mutation, whereas only 0.1% is directly extended. Reactions sampled at different time points between 15 s and 5 min yielded the same ratios between direct extension, misalignment extension, or proofreading (data not shown). Because smaller HE subassemblies (core, polymerase III*, polymerase III⁺) cannot synthesize the required long stretch (−4000 bp) of DNA within a short time (and in the presence of single-stranded binding protein), there can be little doubt that the observed products are made by HE. The results obtained with HE are in contrast to those obtained with the Klenow polymerase.

The data obtained in this study reveal properties of E. coli DNA polymerase III HE that appear to be unique among the polymerases tested to date. First, when presented with a preformed terminal mismatch, direct extension of the mismatch by the enzyme to yield the expected base substitution is extremely infrequent. For example, for the A-containing substrate (Fig. 1), only about 0.2% is extended to yield the expected base substitution (Fig. 3), with the vast majority (>99.8%) being removed by proofreading. This is true even for the proofreading-impaired HE purified from the mutD5 mutator strain, in which only 5% or less of the exonuclease activity remains (6). Secondly, when presented with a terminal mismatch in a sequence context in which the terminal base in the primer strand is complementary to the next template base (the G-containing substrate; see Fig. 1), extension is greatly facilitated but now takes place from the misaligned intermediate, yielding a (−1)-framenshift instead of the base substitution mutation. In the case of the proofreading-impaired enzyme, this secondary pathway significantly exceeds the exonuclease pathway (61 versus 33%; see Table I). For the wild-type enzyme, proofreading still predominates; however, most significantly, misalignment extension exceeds direct extension by 20:1 (Table I). This strong bias favoring misalignment extension over direct extension is unique to polymerase III, because other enzymes for which misalignment extension has been documented, such as Klenow fragment (15, 21), avian myeloblastosis virus and Moloney

![Diagram](https://via.placeholder.com/150)

**Fig. 3. Terminal mismatch utilization by polymerase III holoenzyme.**

The two terminally mismatched substrates used in this study are shown. The next template base to be copied after the terminal mismatch is underlined (G and A in the left and right panel, respectively). The numbers represent the observed numbers of dark blue, light blue, or colorless plaques seen after transcription of the purified RF II product from the indicated extension reactions (see the text for a more detailed explanation).
murine leukemia virus reverse transcriptases (19), mamman-
lian polymerase β (19), and yeast polymerase I (20), generally
produce the frameshift mutation less efficiently than the base
substitution. To illustrate the extreme tendency of polymerase
III HE in this respect, we compared the enzyme side by side
with the Klenow fragment. This enzyme also performed mis-
alignment extension, but at only 5% of the rate of the direct
extension (Table 1).

The present data on mismatch extension by HE are relevant
for explaining the unusual spectrum of in vitro errors produced
by HE in a forward mutagenesis assay using the lacI gene as a
mutational target (6). In these spectra, wild-type and MutD5
HE produced a majority of (−1)-frameshift mutations at non-
reiterated sequences. We proposed (6) that these frameshifts
originated as base misinsertion errors that failed to be ex-
tended by the enzyme but were processed efficiently by exten-
sion from the slipped intermediate in sequence contexts where
the next template base is complementary to the misinserted
base. The present data demonstrating the clear preference for
misalignment extension over direct extension when HE is faced
with a (preformed) 3′ terminal mismatch are fully consistent
with this model. Although a preformed terminal mismatch may
not resemble a mismatch created during ongoing DNA synthe-
sis in all respects, the behavior displayed by HE is striking and
must reflect some property of HE that is likely relevant during
ongoing DNA synthesis as well.

An important alternative context in which to consider the
tendency of HE to create (−1)-frameshifts is provided by the
“dNTP-stabilized misalignment” model proposed by Bloom et al.
(7). These investigators noted increased misincorporation by
HE on an oligonucleotide template under conditions in which
the misincorporating nucleotide was complementary to the
next template base. The findings were interpreted to indicate
that the polymerase is able to read ahead and use the informa-
tion provided by the next template position to direct (misin-
corporation. First of all, this model provides an alternative
mode of misincorporation by HE compared with direct
base-base misinsertion. If correct, both base-base mismatching
and misincorporation by dNTP-stabilized misalignment may
provide the terminally mismatched substrates that HE may
extend from either the aligned or misaligned state. Secondly,
provided that the primer-terminus remains in the misaligned
state after phosphodiester bond formation, the reaction product
of a misincorporation via dNTP-stabilized misalignment is the
same as the intermediate that we suggested to arise from a
standard misincorporation and subsequent forward misalign-
ment. This feature of the dNTP-stabilized misalignment model
for misincorporation makes it a potentially attractive pathway
for the generation of the (−1)-frameshifts observed in vitro (6).
However, if the forward extension rate is slow relative to the
interconversion of the aligned and misaligned states, then any
distinction with regard to the initial mode of misincorporation
would disappear. Finally, it is likely that the preferred usage of
misaligned intermediates by HE and misincorporation by
dNTP-stabilized misalignment are manifestations of the same,
unusual enzymological property of HE that allows the enzyme
to generate and/or use misaligned intermediates with rela-
tively high efficiency. This property, which is obviously rel-
vant for the fidelity of the enzyme, is most intriguing and
deserves further investigation.

In addition to providing insight into the mechanism of frame-
shift mutagenesis by HE, our present study also provides infor-
mation about the contribution of proofreading to in vitro
fidelity. The data from Table I indicate that for the MutD5
enzyme, 33 and 81% of the terminal mismatches are proofread
at 1000 and 50 μM dNTP, respectively; for the wild-type en-
zyme, these numbers are 97.8 and 99.6%, respectively. Con-
verted into fidelity factors (the fold reduction in mutant frac-
tion due to proofreading), the proofreading activity of MutD5
HE contributes 1.5-fold [100/100−33] and 5.3-fold [100/100−81]
to the accuracy of extension synthesis at the two dNTP
concentrations, respectively, whereas for the wild-type enzyme,
these factors are 45- and 250-fold. Thus, the efficiency of the
MutD5 proofreading activity is reduced between 30- and 47-
fold compared with the wild-type enzyme. These data are con-
sistent with direct measurements of the exonuclease deficiency
associated with the MutD5 enzyme taken in the absence of
dNTPs that indicated that the exonuclease activity was de-
creased 26- to 47-fold (6). Thus, it appears that in the present
experiments, which use a preformed terminal mismatch, proof-
reading correlates directly with the strength of the exonucle-
ase. Interestingly, this correlation does not appear to hold for
the proofreading contribution during ongoing in vitro DNA
synthesis, in which we measured only a 4- to 6-fold difference in
the error rates (for both base substitutions and frameshifts)
between the wild-type and the MutD5 enzyme. This may be due
to a different binding mode of the enzyme in the two assays.
Possibly, upon initial binding to a primer, HE preferentially
binds with the primer in the exonuclease subunit, as has been
shown to be the case for T7 DNA polymerase (22). In contrast,
during ongoing DNA synthesis, the terminal nucleotide is in
the polymerase active site, and a kinetic barrier may exist for
transfer to the exonuclease site.

Whereas this study provides new insights into the fidelity
behavior of the replicative complex of E. coli, numerous ques-
tions still remain. Poor extension from terminal mismatches
can be readily seen as a valuable attribute for a high fidelity
enzyme because it will greatly increase the potential for exo-
nucleolytic removal, but it is not clear why a high tendency
to transform a terminal mismatch into a frameshift intermediate
is particularly useful. This tendency seems to be a property
intrinsic to the α subunit, because a similar frameshift predom-
nance was observed in fidelity experiments performed with the
isolated α subunit (23). Apparently, the numerous other sub-
units present in the HE do not play a significant role in pre-
venting these frameshifts. However, because high levels of
frameshift mutagenesis are not observed in vivo (even before
the action of mismatch repair) (24), the question of how they
are prevented inside the cell is interesting. We suggest that
this additional mode of error prevention is somehow related to
the functioning of HE at the in vitro replication fork. Further
experiments will be needed to address this important question.

Acknowledgments—We thank Drs. T. Kunkel and K. Bebenek
of National Institute of Environmental Health Sciences for kindly
providing the mutant M13mp2 phages used in this study. We thank
Drs. K. Bebenek and W. Osheroff for critically reviewing the manuscript.

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