Exonucleolytic editing is a major contributor to the fidelity of DNA replication by the multisubunit DNA polymerase (pol) III holoenzyme. To investigate the source of editing specificity, we have studied the isolated exonuclease subunit, ε, and the pol III core subassembly, which carries the ε, θ, and α (polymerase) subunits. Using oligonucleotides with specific terminal mismatches, we have found that both ε and pol III core preferentially excise a mispaired 3' terminus and therefore have intrinsic editing specificity. For both ε and pol III core, exonuclease activity is much more effective with single-strand DNA; with a double-strand DNA, the exonuclease is strongly temperature-dependent. We conclude that the ε subunit of pol III holoenzyme is itself a specific editing exonuclease and that the source of specificity is the greater melting capacity of a mispaired 3' terminus.

DNA replication is carried out with extremely high accuracy. Error frequencies during duplication of the Escherichia coli genome are 10⁻⁷-10⁻¹⁰ per base replicated (1). To achieve this fidelity, a DNA polymerase must have an exceptional ability to discriminate against incorrect base pairs, which may exhibit only slight structural and energetic differences from the correct base pairs. Fidelity is achieved by a polymerase in a two-step process: (1) base selection, correct selection of the complementary dNTP during 5' → 3' incorporation; (2) editing, 3' → 5' exonucleolytic excision of a noncomplementary deoxyribonucleotide misinserted at the 3' end of a growing DNA chain. With the additional contribution of postreplicative mismatch repair, the high fidelity of genome duplication is achieved (2–4).

DNA pol III holoenzyme is the enzyme primarily responsible for chromosomal replication in E. coli and therefore is probably the major determinant of the fidelity of genome duplication. The pol III holoenzyme contains 10 distinct polypeptide subunits: α, ε, θ, τ, γ, δ', ζ, χ, and δ (5, 6). The ε subunit, the dnaQ gene product, is the 3' → 5' proofreading exonuclease (7, 8). The α subunit, the dnaE gene product, is the 5' → 3' polymerase (9, 10). The α, ε, and θ subunits compose pol III core, the smallest subassembly of pol III prepared from the holoenzyme (11). By the use of isolated ε subunit and pol III core, we can study the mechanism of editing in the presence and absence of the polymerase subunit.

Earlier studies with a single mismatch indicated that both pol III core and isolated ε have a preference for a mispaired 3' terminus (8, 12). In this work we have examined in more detail the specificity and the mechanism of exonucleolytic proofreading.

First, we wanted to determine if editing specificity is a property of the exonuclease subunit alone. Based on the crystal structure, the editing specificity of DNA polymerase I appears not to be intrinsic to the exonuclease domain, but depends on the transfer of single-strand DNA from the polymerase domain to the exonuclease domain (13, 14). The exonuclease active site must function solely as a single-strand exonuclease, because there is no room for duplex DNA (13, 15); therefore the exonuclease site makes no direct selection for a mispaired base. Kinetic studies have suggested that editing by pol I is achieved mainly by a delay in elongation from a mismatched primer terminus (16). Thus editing does not result from the intrinsic specificity of the exonuclease, but rather depends on a kinetic delay in polymerization, which allows more time for transfer of single-strand DNA into the exonuclease site. If the editing mechanism suggested for pol I was also used by pol III, the isolated ε subunit would not distinguish a correctly paired from a mispaired 3' terminus. In this work, we have examined the editing specificity of the ε subunit and pol III core with a series of correctly paired or mispaired oligonucleotides annealed to bacteriophage M13 DNA. These DNA substrates provided all 16 possible combinations of correct and incorrect base pairs at the primer 3' terminus. We have found that both pol III core and ε specifically excise incorrectly paired 3' termini more rapidly than correctly paired termini.

We also wanted to determine the feature of DNA structure that allows the exonuclease to recognize mispairs. There are two plausible general mechanisms for selectivity in editing: recognition of departures from the equivalent geometry of the Watson-Crick base pairs, and melting capacity of the 3' terminus. Geometric recognition has been implicated as the critical determinant for the specificity of base selection in the polymerase reaction (17, 18). In the melting model, the exonuclease is intrinsically a single-strand nuclease that preferentially removes a misinserted base because the mismatch at the 3' end is more often in a single-strand configuration (19, 20). In the case of pol I, Brutlag and Kornberg (19) demonstrated that the exonuclease works best on single-strand DNA and that there is a marked increase in exonuclease activity with duplex DNA as temperature is increased. These data suggest that melting of the 3' terminus is the primary determinant for editing. As noted above, structural studies with pol I have also supported a melting model for editing by pol I. In this study, we have examined the effect of temperature and strand specificity on the exonuclease activity.

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The abbreviations used are: pol, polymerase; N, nucleoside.
of pol III core and $\epsilon$; our data support the melting model for editing by pol III.

Finally, we have carried out a steady-state analysis of the exonuclease activity of pol III core to determine the kinetic parameters that provide editing specificity. We have found that $V_{\text{max}}$ discrimination is the primary kinetic property that characterizes preferential excision of a misinserted base at the 3'-terminus.

**EXPERIMENTAL PROCEDURES**

Materials—Purified $\epsilon$ subunit was prepared as described previously (8). Purified core subassembly was prepared as described previously (11). T4 polynucleotide kinase was purchased from New England BioLabs. Oligonucleotide substrates and the 35-mer template were synthesized by conventional solid phase methods. Single-strand M13 DNA was purified from *E. coli* strain JM103 using published procedures (21). [\(\gamma^{-32}\text{P}\)]ATP (>5000 Ci/mmol, 10 mCi/ml) was purchased from Amer sham Corp.

Preparation of DNA Substrates—Sixteen oligonucleotide substrates were annealed to M13 DNA to generate the 16 possible correct and incorrect base pairs. Oligonucleotides (20-mers) were synthesized complementary to four unique M13 sites with a variable 3'-terminal single-strand M13 DNA. Exonuclease assays were performed with plate DNA either at a 1.5:1 molar ratio (M13 template in excess) or at a 1:1 molar ratio (oligonucleotide template). Annealing was carried out by adding NaCl to a final concentration of 50 mM, then heating the oligonucleotide template mixture to 85 °C for 10 min, followed by slow cooling to room temperature over 2-4 h.

Exonuclease Assays—For standard exonuclease assays, reaction mixtures (10 μl for epsilon and 15 μl for core) contained 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM dithiothreitol, 1 μM [\(\gamma^{-32}\text{P}\)]ATP, 20 units of T4 polynucleotide kinase, and varying amounts of oligonucleotide substrate. The reaction was incubated at 37 °C for 80 min and was terminated by heating to 100 °C for 5 min. Oligonucleotide substrates were annealed to template DNA either at a 1:5:1 molar ratio (M13 template in excess) or at a 1:1 molar ratio (oligonucleotide template). Annealing was carried out by adding NaCl to a final concentration of 50 mM, then heating the oligonucleotide template mixture to 85 °C for 10 min, followed by slow cooling to room temperature over 2-4 h.

**RESULTS**

**Exonuclease Specificity of $\epsilon$ and pol III Core**—To determine the specificity of pol III core and the isolated $\epsilon$ subunit, we have used a gel electrophoresis assay to measure the rate of exonuclease action. DNA substrates were oligonucleotides, labeled with \(^{32}\text{P}\) at the 5' end, with a 3' terminus that was either mispaired or correctly paired to the M13 template. The velocity of exonuclease action was measured by electrophoresis of the oligonucleotide on a sequencing gel that resolves as discrete bands single nucleotide differences in length. Assays were carried out with oligonucleotide substrates that provided all 16 possible combinations of correct and incorrect terminal pairs. The experiments were performed under conditions in which the relative velocity of the exonuclease reactions should be proportional to the $V_{\text{max}}/K_{\text{m}}$, specificity parameter (substrate concentration very much less than $K_{\text{m}}$).

In every case, exonuclease activity on an incorrectly paired base at the 3' terminus proceeded more rapidly than removal of a correctly paired base. Autoradiographic data are shown in Fig. 1 for the exonuclease activity of $\epsilon$ on correct and incorrect base pairs opposite template C. Removal of a 3'-terminal G from a G-C pair proceeded more slowly than for any of the other mispaired bases opposite C. After 8 min, less than 10% of the correctly paired G was removed, whereas more than 90% of the three incorrectly paired bases were excised. The autoradiographs were scanned by a densitometer, and the peaks corresponding to primer bands were integrated. The ratio of the intensity of the primer band to the sum of the intensities of all bands present was plotted against time using a semi-log scale. By fitting the data to a first order exponential curve, kinetic rate constants for the exonuclease reaction were determined. Examples of the graphed data are shown in Fig. 2 for correct and incorrect base pairs opposite template C (Fig. 2, A and B) and T (Fig. 2, C and D); data are presented for $\epsilon$ (Fig. 2, A and C) and for pol III core (Fig. 2, B and D). The rate constants are presented in Table I for all 16 combinations for $\epsilon$ and pol III core.

Both $\epsilon$ and core preferentially attack a mispaired 3' terminus, although the relative specificities vary. We observed the greatest specificity for mispairs opposite template C and G, presumably because the correctly paired 3' terminus is so resistant to exonucleolytic attack. Conversely, we found the least specificity for mispairs opposite template A and T, for which the correctly paired 3' terminus was relatively sensitive to degradation. The major conclusion of our data on exonuclease specificity is that $\epsilon$ has intrinsic discrimination for a mispaired 3' terminus. Moreover, the properties of the $\epsilon$ exonuclease are qualitatively very similar to those of the pol III core.

**Evidence for a Melting Mechanism for $\epsilon$ and pol III Core**—As noted in the introduction, there are two general mechanisms for exonucleolytic specificity toward a mispaired 3' terminus: geometric recognition and melting capacity. For the melting model, there are three clear predictions: (i) and A-T correct pair should be more sensitive to exonuclease than a G-C pair; (ii) the exonuclease activity on duplex DNA should be strongly temperature-dependent; (iii) the exonuclease activity should be most effective with a single-strand DNA template DNA used for the assay is shown at the top of the figure. The 20-mer oligonucleotides, each containing a different 3'-terminal base, were labeled with \(^{32}\text{P}\) at the 5' end and annealed to single-strand M13 DNA. Exonuclease assays were performed with isolated $\epsilon$ as described under "Experimental Procedures." Aliquots from the reaction were removed at the indicated times and subjected to gel electrophoresis. The top band in each lane represents the intact oligonucleotide. The lower bands represent oligonucleotide substrates that have been degraded by one and two nucleotides, respectively. Data are shown for exonuclease assays carried out using substrates containing 3'-terminal G-C, A-C, T-C, and C-C pairs.

![Fig. 1. Exonuclease activity of $\epsilon$ on mispairs opposite template C.](image-url)
FIG. 2. Specificity of ε and core exonuclease for mispaired terminal bases. A and B, activity of ε and pol III core, respectively, on substrates containing mispairs opposite template C. 3' terminal bases are: G·C (——, □), A·C (— — , ■), T·C (— – , ○), and C·C (— — , □). C and D, activity of ε and pol III core, respectively, on 3' terminal mispairs opposite template A. 3' terminal bases are: A·T (——, □), G·T (— — , ■), T·T (— – , ○), and C·T (— — , □).

TABLE 1
Rate constants for exonuclease of pol III core and isolated ε

<table>
<thead>
<tr>
<th>3' Terminus</th>
<th>N</th>
<th>kcat x 10^3</th>
<th>koff/kcat</th>
<th>kcat x 10^3</th>
<th>koff/kcat</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>N-G</td>
<td>5.3</td>
<td>1.0</td>
<td>3.4</td>
<td>1.0</td>
</tr>
<tr>
<td>N-A</td>
<td>T</td>
<td>34</td>
<td>1.0</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>N-T</td>
<td>G</td>
<td>53</td>
<td>1.5</td>
<td>31</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>49</td>
<td>1.4</td>
<td>130</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>53</td>
<td>1.6</td>
<td>130</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>20</td>
<td>1.0</td>
<td>4.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>57</td>
<td>2.9</td>
<td>14</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>140</td>
<td>7.4</td>
<td>16</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>65</td>
<td>3.3</td>
<td>20</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>2.3</td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>49</td>
<td>21</td>
<td>57</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>46</td>
<td>18</td>
<td>23</td>
<td>11</td>
</tr>
</tbody>
</table>

For a geometric model, A·T and G·C should be equivalent, and an especially strong temperature effect would not be anticipated.

The data presented in Table 1 demonstrate a preference for 3' termini in A·T pairs over G·C; this preference is especially pronounced when ε is acting by itself. To examine temperature effects, we carried out exonuclease assays at 26, 30, and 37 °C. The exonuclease activity with duplex DNA was compared with that found for the unpaired single-strand oligonucleotide.

Examples of the data are presented in Fig. 3 for assays with pol III core with A opposite template T (Fig. 3A) and C opposite template G (Fig. 3B). There is a notable increase in exonuclease activity with increased temperature. A more complete set of data is collected in Table II. There is a very large thermal effect on exonuclease for both ε and pol III core for correctly paired G·C and A·T and for the G·T mispair. There is very high exonuclease activity with the single-strand DNA substrate but only a small thermal effect.

The data presented in Table II demonstrate that the principle predictions of the melting mechanism are fulfilled. Therefore, we conclude that the melting capacity of the 3' terminus is the primary recognition determinant for the editing exonuclease.

Steady-state Kinetic Parameters of Exonuclease Activity: V\textsubscript{max} discrimination—To assess the relative contribution of K\textsubscript{m} and V\textsubscript{max} to editing specificity, we carried out a steady-state kinetic analysis of pol III core. A similar analysis was not possible for ε alone because the K\textsubscript{m} value was too high, as noted previously (12). A 35-mer oligonucleotide template was used to obtain substrate concentrations higher than those obtainable with M13. The 35-mer was annealed to oligonucleotides generating a correct 3'-terminal C·G pair and an incorrect T·G pair. Conditions were determined that satisfied Michaelis-Menten kinetics: reaction velocity was directly proportional to enzyme concentration, and reaction velocity was constant over the time period measured (4 min for C·G substrate and 2 min for T·G substrate).

Reaction velocities were measured at substrate concentra-
Editing Specificity of E. coli DNA Pol III

A

Fraction of Substrate Remaining

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

Time (min)

2

4

6

8

B

Fraction of Substrate Remaining

0.1

0.2

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

Time (min)

2

4

6

8

FIG. 3. Effect of temperature on pol III core exonuclease. Exonuclease assays were carried out at 26 °C (■), 30 °C (■), and 37 °C (□). A, substrate containing 3′-terminal G·C pair. B, substrate containing 3′-terminal A·T pair.

TABLE II

Temperature effect on exonuclease of pol III core and ε

A. Temperature effect on rate constants for exonuclease of pol III core and ε

<table>
<thead>
<tr>
<th>Primer 3′ terminus</th>
<th>kₚ × 10⁴ for core</th>
<th>kₚ × 10⁴ for ε</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26 °C 30 °C 37 °C</td>
<td>26 °C 30 °C 37 °C</td>
</tr>
<tr>
<td>G·C</td>
<td>1.4 3.6 10</td>
<td>1.2 2.1 3.0</td>
</tr>
<tr>
<td>A·T</td>
<td>2.5 9.2 40</td>
<td>5.0 11 27</td>
</tr>
<tr>
<td>G·T</td>
<td>14 33 65</td>
<td>16 31 54</td>
</tr>
<tr>
<td>Single-stranded</td>
<td>280 340 410</td>
<td>210 220 250</td>
</tr>
</tbody>
</table>

B. Relative kₑₒₓ data normalized to single-strand velocity at each temperature

<table>
<thead>
<tr>
<th>Primer 3′ terminus</th>
<th>Pol III core</th>
<th>ε subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>26 °C 30 °C 37 °C</td>
<td>26 °C 30 °C 37 °C</td>
</tr>
<tr>
<td>G·C</td>
<td>1 2.1 4.8</td>
<td>1 1.6 2.0</td>
</tr>
<tr>
<td>A·T</td>
<td>1 2.9 11</td>
<td>1 2.1 4.5</td>
</tr>
<tr>
<td>G·T</td>
<td>1 1.9 3.1</td>
<td>1 1.7 2.7</td>
</tr>
</tbody>
</table>

† Rate constants at 26, 30, and 37 °C were obtained as for Table I.
‡ Rate constants were normalized to values at 26 °C for each substrate, then normalized to the velocity of single-strand DNA degradation at each temperature.

FIG. 4. Exonuclease velocity as a function of substrate concentration. Velocities were measured at various substrate concentrations using substrates with a 3′-terminal correct C·G pair (○) or a 3′-terminal incorrect T·G pair (□).

TABLE III

Kinetic parameters of pol III core exonuclease

The exonuclease velocity was measured at substrate concentrations ranging from 25 to 850 nM on substrates with either C·G or T·G terminal pairs. Vₘₐₓ and Kₘ were obtained from a linear least squares fit to an Eadie-Hofstee plot (V/[S] versus 1/[S]).

<table>
<thead>
<tr>
<th>Primer terminus</th>
<th>Vₘₐₓ/Kₘ × 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fmol/s nM</td>
</tr>
<tr>
<td>C·G</td>
<td>2.8 ± 1.3 420 ± 80 6.7</td>
</tr>
<tr>
<td>T·G</td>
<td>17 ± 4.5 460 ± 90 37</td>
</tr>
</tbody>
</table>

The increased Vₘₐₓ for removal of the mismatched T·G supports the concept that the source of editing specificity is the greater melting capacity of a mispaired 3′ terminus. If the ε subunit in pol III core binds with similar affinity to a matched and mismatched terminus, it can be readily shown (and is intuitively clear) that Vₘₐₓ will be proportional to the fraction of melted 3′ ends:

\[ Vₘₐₓ = \left( \frac{kₚ}{kₚ + kₙ} \right) k_s E \]

where kₚ and kₙ are the rate constants for formation of melted and annealed 3′ ends, respectively, kₛ is the rate of single-strand exonuclease activity, and E is the enzyme concentration. Since the fraction of melted 3′ termini is greater for mismatched bases, Vₘₐₓ values should be higher for mispairs. If the source of editing specificity was direct binding recognition of a mispaired 3′ terminus, discernible Kₘ differences would be expected. Thus our kinetic data are completely consistent with a melting mechanism.

DISCUSSION

Specificity of Exonucleolytic Editing—Recent structural work has demonstrated that exonucleolytic editing is a complex process requiring a communication between different domains or subunits of the DNA polymerase. For pol I, the exonuclease domain is not only separated from the polymerase...
domain by some 30 Å, but the exonuclease site can accept only single-strand DNA (13, 15, 22). Thus for pol I, the editing "decision" appears to depend entirely on events at the polymerase site. Indeed, kinetic studies have indicated strongly that editing specificity in DNA replication is achieved mainly by delayed DNA chain elongation from a mispaired 3' terminus (16). This lag in polymerase action presumably allows more time for a mismatched terminal base to melt and slide into the exonuclease site (14).

For pol III, the exonuclease active site is also presumably spatially separated from the polymerase site, because the two biochemical activities reside on distinct subunits (8, 10, 12). To examine the question of intrinsic editing specificity by the exonuclease subunit, we have carried out a detailed comparison of the exonuclease activity of pol III core and ε subunit. We have found that ε has intrinsic specificity for any 3' terminus with a mismatched base. The specificity of ε is qualitatively similar to that of pol III core. However, the addition of the polymerase subunit markedly increases the activity of the exonuclease for all duplex substrates; this effect probably derives from the contribution of the α subunit to effective recognition of the 3' terminus (12).

Mechanism of Exonucleolytic Editing—The initial experiments with pol I and T4 polymerase strongly indicated a melting mechanism for exonuclease action (19, 20). The structural analysis of pol I defined a surprisingly extreme form of melting mechanism; the exonuclease site could accept only single-strand DNA (13, 22). Our work with the editing exonuclease of pol III strongly supports a melting mechanism for both pol III core and the isolated ε subunit. Thus the major difference between pol I and pol III appears to be the intrinsic editing specificity of ε itself. As noted below, this intrinsic specificity is unlikely to be sufficient to explain the complete contribution of editing to the fidelity of replication by pol III. However, this feature of the exonuclease probably allows pol III to achieve the very high editing precision needed for an enzyme dedicated to chromosomal duplication. Although we do not know whether a melting mechanism will be a universal property of editing exonucleases, the idea is attractive, because a melting mechanism allows a ready evolutionary transition from a free single-strand nuclease to an editing subunit or domain designed to excise misinserted nucleotides.

Contribution of Editing to Replication Fidelity—An important biological question is whether the intrinsic specificity of the exonuclease is sufficient to explain the contribution of editing to the fidelity of DNA replication. An accurate estimate of the editing concentration in vivo is complicated by the interplay of fidelity systems (4). The most defective mutations in the dnaQ gene coding for ε confer a very large increase in mutation rate, up to 10^6-10^10-fold (23, 24). However, this number is an overestimate, because the mismatch repair system becomes ineffective at high mutation rates (25, 26). The best current guess for the contribution of exonucleolytic editing in vivo is in the 10^-6-10^-9 range (4, 25). This number is in the same range as a rough estimate obtained for pol III in vitro by comparing the overall fidelity of pol III with base insertion data for pol I (27).

Our work on the exonuclease specificity of pol III core and ε indicates a much lower intrinsic discrimination for the editing exonuclease than the editing contribution to DNA replication inferred in vivo. Our data in Table I were obtained under conditions in which the relative rates of exonuclease activity (k_{exo}) should represent the relative specificities for terminal nucleotides (V_{exo}/K_{exo}). This conclusion is supported by the detailed kinetic data of Table III. For pol III core, these specificity ratios range from 2- to 30-fold.

The probable explanation for the quantitative discrepancy between editing in vivo and exonuclease in vitro is that intrinsic exonuclease specificity must be augmented by a contribution from the polymerase site, most likely a kinetic amplification provided by inefficient polymerization from a mismatched 3' terminus. This delayed chain elongation would allow the exonuclease more time to act at a mispaired 3' terminus before the next base insertion event (16, 28). As noted above for pol I, this feature is the major determinant of editing specificity (16). A similar kinetic amplification of editing has been noted recently for phage T7 DNA polymerase (29). A modest kinetic amplification from the polymerase step would boost the specificity of pol III into the range expected from in vivo work. Thus pol III probably partitions its specificity determinants for editing between the intrinsic discrimination of the exonuclease site and the kinetic contribution of the polymerase site.

REFERENCES