DNA End Joining by the Klenow Fragment of DNA Polymerase I*

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DNA end joining is a type of illegitimate recombination characterized by the joining of two DNA ends that lack homology. Using oligonucleotides as substrate, we found that an exonuclease-free derivative of the Klenow fragment of Escherichia coli DNA polymerase I can mediate DNA end joining in vitro. DNA sequence analysis of product DNA indicated that overlap products were formed between direct repeat sequences at the termini of the oligonucleotides. Formation of recombinant products was dependent on the strandedness of the substrate DNA, and the rate of product formation was dependent on the size of the potential overlap. With one to three complementary bases available for pairing at the 3′ termini, there was an absolute requirement that one of the oligonucleotides be double-stranded, whereas with four complementary bases, products were also formed in reactions with single-stranded oligonucleotides. When noncomplementary nucleotides were added to the terminus of one of the oligonucleotides, product formation was delayed but not blocked. These data indicate that a DNA polymerase can mediate DNA double-strand break rejoining in the absence of other proteins.

DNA-dependent DNA polymerases provide the pivotal function of DNA synthesis in the cellular processes of DNA replication, recombination, and repair. Escherichia coli DNA polymerase I has roles in all three of these processes (1). Polymerase I is the most extensively studied DNA polymerase. It has distinct regions for binding single- and double-stranded DNA as well as domains for dNTP binding, polymerization, and 3′-5′ exonuclease (2). There are significant amino acid sequence similarities between various DNA polymerases in the critical polymerization and 3′-5′ exonuclease domains, suggesting a general structure for DNA polymerases (3). In fact, mammalian DNA polymerase can complement E. coli polymerase I defects in some replication and repair assays (4, 5).

DNA double-strand breaks can be produced in cells as a consequence of normal cellular processes or after exposure of cells to DNA-damaging agents. It is essential for the integrity of the genome that double-strand breaks be repaired quickly. DNA double-strand breaks can be repaired by homologous recombination, which can restore the original sequence, or by illegitimate recombination, in which two ends are joined, typically resulting in a change of sequence (reviewed for mammalian cells in Ref. 6). Failure to rejoin a double-strand break can result in the loss of DNA sequences, and misrepair of a double-strand break can lead to the addition or deletion of nucleotides, resulting in mutation. This repair process does not ensure that the ends joined came from the same molecule and can thus lead to formation of gross chromosomal rearrangements (e.g. translocations, insertions, inversions, and exchange-type aberrations) (7, 8). Chromosomal rearrangements are seen in cancer cells (9, 10) and can lead to genomic instability (11–13) and cell death (14, 15). This indiscriminate end joining mechanism is a primary pathway for the repair of double-strand breaks in mammalian cells (16–18).

Several model systems have been developed to study the rejoining of restriction enzyme-produced DNA double strand breaks with noncomplementary ends (16, 19, 20). Three types of end joining products have been described in these systems: insertions (16), "fill-in" (21), and "overlap" (20). Insertions have one or more additional nucleotides in the junction between the joined ends and occur in approximately 10% of the junctions formed in mammalian cells (16, 22). Fill-in products are those in which the protruding single strands produced by the restriction enzymes are preserved. Overlap products are those in which bases are deleted from the protruding single-stranded ends; typically, there are one to six complementary bases at the junction of the two ends. Overlap products have been observed in human cells (23). Chinese hamster ovary cells (24), monkey cells (16, 22), Xenopus cell extracts (20, 21), Saccharomyces cerevisiae (25, 26), Schizosaccharomyces pombe (27), and E. coli (28). Although these overlap products do not have extensive homology, they typically have more complementary bases at the junction site than would be expected for random joining, thus supporting the assertion that they are not produced by a blunt-ended or single-stranded ligation process.

Several DNA polymerases that either lack 3′-5′ exonuclease activity or have repressed exonuclease activity, including the Klenow fragment of E. coli polymerase I and the Taq DNA polymerase of Thermus aquaticus, are known to add bases to the 3′ hydroxyls of blunt-ended DNA duplexes in vitro (29, 30). Once a base has been added to a blunt end, Klenow and Taq DNA polymerases can use a 3′ protruding single strand as a template for additional polymerization (31, 32). This demonstrates that the fill-in products of illegitimate recombination could be the result of DNA synthesis on a discontinuous template. The other major products of illegitimate recombination, overlap products, result from the complementary pairing between short, directly repeated DNA sequences.

We investigated the DNA polymerase-mediated joining of single- and double-stranded oligonucleotides and constructed a model based on our findings in which both fill-in and overlap products are mediated by DNA synthesis. The model is based on simple in vitro experiments and has yet to be demonstrated in vivo.
Materials and Methods

Reaction Conditions—Oligonucleotides were annealed by mixing equal molar amounts and heating to 65 °C. The mixture was then cooled slowly to ambient temperature. The reaction conditions were 50 pmol of a top left oligonucleotide (single- or double-stranded) and 500 pmol of a bottom right oligonucleotide with 10 units of Klenow fragment (7 pmol), 100 μM deoxynucleotide triphosphates, 25 mM Tris-HCl (pH 8.0), 10 mM MgCl2 in 15-μl reactions carried out at ambient temperature. Biotinylated oligonucleotides were purchased from the Molecular Resource Center (University of California, San Francisco, CA). All other oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 391 DNA synthesizer. In time course experiments, the Klenow fragments were used as a template for U.S. Biochemical Corp. Sequenase 2.0 sequencing reactions. Because DNA sequencing product was readily obtainable, we concluded that recombination products were formed by using the top left oligonucleotides as a primer and the bottom right oligonucleotide as a template. The sequences we saw supported this conclusion and showed the exact junctions formed between the top left oligonucleotides and the bottom oligonucleotides were end-labeled with 32P, they were the only molecules detected by the film. The results of these time course experiments showed that substrate DNA was converted into product DNA over time. Fig. 3C shows the results of sequencing reactions performed on the product DNA. Because there was less product or it was not homogeneous, readable sequence could not be obtained for the 1-base pair overlap (Fig. 2, ONE BP) or for the 4-base pair overlap with one noncomplementary base (Fig. 2, FOUR BP ONE NON-COMPLEMENTARY). Only the 32P end-labeled oligonucleotides contained biotin; thus, we could selectively isolate those molecules by using streptavidin-coated paramagnetic beads. Furthermore, the primer used in the sequencing reactions was identical to the first 17 bases of the bottom right oligonucleotide and was thus complementary to any DNA synthesized when the bottom right oligonucleotide was used as a template.

Results

In Vitro End Joining Reactions—To assess the ability of DNA polymerases to produce overlap products, we set up reactions using discontinuous oligonucleotides as substrate. The reactions used in these experiments consisted of substrate oligonucleotides, an exonuclease-free derivative of the Klenow fragment of E. coli polymerase I (U.S. Biochemical Corp.), deoxynucleotide triphosphates, and a reaction buffer. Reaction products were separated on denaturing polyacrylamide gels.

Table 1

| Oligonucleotides          | Sequencing primer |
|---------------------------|-------------------|
| Top left oligonucleotides | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| Top left complements      |                   |
| 1a                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1b                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1c                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1d                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| Bottom right oligonucleotides |                   |
| 1a                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1b                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1c                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| 1d                        | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
| Sequencing primer         | 5′-GTAACATACGCGCATAGACAGATTGCCG-3′ |
right oligonucleotides.

The first four entries in Fig. 2 show the results of reactions in which the top left oligonucleotide was double-stranded and the top left and bottom right oligonucleotides had one, two, three, or four complementary nucleotides at their 3' termini. In each reaction an 18-mer was converted into 46–49-mers, depending on the substrates used and whether the reaction mixture contained mismatched bases. The predominant products were the blunt-ended molecules, and the biotinylated DNA was removed for sequence analysis.

In addition, we constructed top left complement 2b so that annealing to top left 2 resulted in a blunt-ended molecule, and we used this molecule in reactions with bottom right 2a. The results of these reactions and the alignment of the top and bottom oligonucleotides are summarized in Figs. 2, 4, and 5. The results demonstrate that unpaired bases 3'-terminal to the direct repeat sequence on the template DNA and possibly those 5'-terminal to the complement of the primer DNA do not prevent product formation. The predominant product was the same as that formed when noncomplementary bases were not present (see Fig. 2, THREE BP). A mismatched base internal to a stretch of four complementary bases impeded but did not prevent product formation (see Fig. 2, FOUR BP ONE NON-COMPLEMENTARY). The joining of a blunt-ended to a single-stranded oligonucleotide with a terminal thymidine residue resulted in synthesis across the break without overlap formation. This is similar to what was observed in a previous study (32) with blunt-ended substrates. It was not known if the blunt-ended substrate 2 would join in a blunt to 3' fashion, form a three-base pair overlap, or form a single-base pair overlap with the terminal bases. The predominant product formed was the single-base pair overlap.

Fig. 4 shows a graph of the rate of formation of product DNA (defined as the percentage of oligomers greater in size than the substrate DNA) for the reactions in which the oligonucleotides had up to four complementary bases at the 3' termini. For overlaps of one to three base pairs, the larger the size of the overlap, the less time was required for the formation of detectable amounts of product. This effect saturated between three and four complementary bases, which had equally rapid product formation. With single-stranded oligonucleotides and four complementary bases at the 3' termini, product formation was slower, but was substantially delayed compared with the identical reaction in which the top left oligonucleotide was double-stranded. The rates of product formation for the one-base pair overlap, the two-base pair overlap, and the blunt substrates were similar (see Fig. 4). The predominant product formed for the blunt substrate could form by multiple mechanisms. It may have formed by using a single thymidine to adenine pairing of the terminal nucleotides. Alternatively, if a nontemplate-derived adenine, the preferred base for such an addition from Klenow fragment (30), had been added to the blunt end, there would be two complementary bases at the 3' termini, or product formation may have occurred by a single base pairing between a nontemplate-derived adenine and the internal thymidine. It is important to keep in mind when looking at Fig. 4 that because the oligonucleotides often differed in sequence as well as in size of overlap, it is possible that properties other than the size of the overlap affected the rate of product formation.

Fig. 5 shows the rate of formation of product DNA for the reactions in which there were noncomplementary guanine residues at the 3' terminus of the bottom right oligonucleotides. In general, noncomplementary bases inhibited product formation. The four guanine residues may be less inhibitory than one or two, but they did allow for an additional G to C base pairing with the 3'-terminal G of the bottom right oligonucleotide that may have facilitated the reaction; however, this would have resulted in greater displacement of the top left complement oligonucleotides. Alternatively, this result may be due to G-G base pairing (34). Rapid product formation was not observed when a bottom right oligonucleotide without four consecutive Gs was used (Fig. 5). These results demonstrate that a DNA polymerase can join two oligonucleotides in the presence of unpaired bases. More specifically, they show that noncomplementary bases 3'-terminal to the direct repeat sequence on the

**Fig. 1. Schematic design of experiment.** A, a top left oligonucleotide was made double-stranded by annealing it to a complementary oligonucleotide. B, a 10-fold excess of a bottom right oligonucleotide was added to the reaction mixture. C, DNA polymerase and deoxynucleotide triphosphates were added. Samples were removed at various times and analyzed for DNA synthesis by using the top left strand as the primer.
FIG. 2. Reactant structures and products formed. In the left column are the substrates shown in Figs. 4 and 5. In the right column are the products detected by sequence analysis after isolation of biotinylated DNA. We were not able to obtain readable sequence for the 1-base pair overlap and four noncomplementary 2e reactions, most likely because of too little product or nonhomogeneous product.
template DNA do not prevent product formation, and they may indicate that unpaired bases on the DNA complementary to the primer also do not prevent product formation.

Model of DNA Polymerase-mediated End Joining—These results led us to propose the following model of DNA polymerase-mediated end joining. The initial step in the joining of DNA ends by a DNA polymerase would be the binding of the polymerase to a region of double-stranded DNA (Figs. 6 and 7). If 3'-recessed ends are present, they would be extended to produce blunt-ended molecules (Fig. 6A). An additional, nontemplate-derived base could be added to the end before the reaction would proceed down one of two pathways (Fig. 6B). The polymerase could "bridge" the break, proceed to a different end by binding to a 3' protruding single strand (Fig. 6C), and use it as a template for additional DNA synthesis (Fig. 6D). This would produce the fill-in products observed in the model systems. Evidence for the catalysis of this reaction in vitro was presented by Clark (30) and King et al. (32). Alternatively, the polymerase could align two 3'-ends at from one to several complementary bases (Fig. 7B) before additional polymerization produced a stable intermediate (Fig. 7C). This would produce the overlap products observed in the model systems. Unpaired bases at the template 3' terminus or on the 5' end of the DNA complementary to the primer DNA would not prevent end joining. However, because of the multiple means of producing identical products, we have not conclusively shown that unpaired bases at the 5' ends of the DNA complementary to the primer would not prevent product formation. DNA proofreading would remove noncomplementary bases present at 3' termini. Additions to these schemes, including fold-back, slippage, and misalignment, could produce the plethora of DNA rearrangements observed in illegitimate recombination processes.

Fig. 3. Reactant structures and products formed. One guanine residue at the 3' terminus of the bottom right oligonucleotide. A, top left 2, with complement 2a, in reaction with bottom right 2b. B, image of denaturing gel. C, sequencing gel.

Fig. 4. Rate of product formation for oligonucleotides with up to four complementary bases at the 3' termini. Open triangle, top left 1:top left complement 1a in reaction with bottom right 1. Inverted closed triangle, top left 1:top left complement 1b in reaction with bottom right 1. Closed circle, top left 2:top left complement 2a in reaction with bottom right 2a. Open square, top left 3:top left complement 3 in reaction with bottom right 3. Open inverted triangle, top left 3 in reaction with bottom right 3. Closed triangle, top left 2:top left complement 2b in reaction with bottom right 2a.

Fig. 5. Rate of product formation for oligonucleotides with up to four noncomplementary guanine residues at the 3' terminus of the bottom right oligonucleotide. Open triangle, three complementary bases at the 3' terminus and no noncomplementary bases. Open circle, one noncomplementary guanine residue. Closed triangle, two noncomplementary guanine residues. Closed inverted triangle, four noncomplementary guanine residues. Open square, four noncomplementary GGTC residues.

DISCUSSION
The results of this study indicate that an exonuclease-free derivative of the Klenow fragment of E. coli polymerase I can mediate illegitimate recombination between oligonucleotides in vitro to produce overlap products between directly repeated DNA sequences similar to those observed from mammalian cells. This suggests that DNA polymerases can produce products identical to those associated with the repair of double strand breaks in mammalian cells (6, 24).

Sequence data from model systems show that 3' and 5' protruding single strands can be joined to blunt-ended DNA with preservation of the protruding single strand. This process
uses a novel priming activity facilitated by a putative align-
ment protein (21). Previous work indicates that DNA poly-
erase can function as this alignment protein and can perform this
reaction in vitro (31, 32). Overlap products from the Xenopus
system exhibit mismatch correction at the termini of 3' pro-
truding single strands (35), which suggests the involvement of
DNA polymerase proofreading in the repair process (36). Fur-
thermore, junction formation is inhibited by aphidicolin and
requires all four deoxyribonucleotidetriphosphates (37). Many
of the mutations induced by restriction enzyme-generated dou-
ble strand breaks at an endogenous adenine phosphoribosyl-
transferase gene in Chinese hamster ovary cells appear to be
polymerase-mediated errors (24) and are similar to DNA poly-
merase misalignment mutations seen in vitro replication
studies (38). Thus, it appears that DNA polymerases are active
at the 3' termini of break-rejoining events, both in extract
systems and in vivo on a mammalian chromosome. Conse-
quently, we would like to know which steps in the joining of
noncomplementary ends can be performed by DNA poly-
merases on their own and which functions must be either facili-
tated or performed by other enzymes. Our data indicate that
under specific constraints DNA polymerases can carry out
alignment reactions in vitro to produce the overlap products
characteristic of illegitimate recombination. Furthermore, non-
complementary bases terminal to the template direct repeat
sequence do not prevent overlap formation. Therefore, in sim-
ple in vitro reactions, DNA polymerases can produce the major
products of illegitimate recombination.

If a DNA polymerase does function as the putative alignment
protein of noncomplementary end joining (21, 32, 37), the join-
ing of two noncomplementary ends would not require any novel
enzyme activities other than the increased flexibility of the
DNA polymerases described (see Figs. 6 and 7) and a DNA
ligase to complete repair. Nevertheless, this does not preclude
the involvement of other proteins. A specific protein that may
be involved is the Ku antigen, which may protect ends before
joining (39–41) and which also has helicase activity (42). There
may also be proteins that facilitate end joining by holding ends
in proximity to each other. Proteins involved in chromatin structure, such as histones, would be likely candidates to play such a role. Furthermore, proteins that may be involved in the detection and signaling of double strand breaks, such as the 350-kDa DNA-activated kinase (43), would be expected to be part of a mammalian DNA double strand break repair complex. If end joining in vivo proceeds by a mechanism similar to the one we have proposed, extension would be likely to involve more than the intrinsic helicase of the polymerase and to be facilitated by an additional DNA helicase, providing a role for DNA helicases in DNA repair in addition to their established roles in excision repair (44) and transcription-coupled repair (45, 46). Alternatively, the 5' to 3' exonuclease associated with some DNA polymerases may hydrolyze the other strand until the polymerase disassociates, to allow ligation to produce closed duplex DNA.

Illegitimate recombination is the major source of genome rearrangements in somatic cells. The products of this process can result in mutation, gene deletion, and chromosomal rearrangements. DNA polymerases can carry out many of the reactions that are central to this process. In our model, short complementary stretches of DNA that are not stable are converted by DNA synthesis into stable nicked intermediates that can be ligated to complete repair. The data presented here indicate that a DNA polymerase may play a central role in the rejoining of spontaneous or induced double strand breaks to produce the major molecular product of illegitimate recombination, the overlaps formed between direct repeat sequences.

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