**Bridging the Gap**

**JOINING OF NONHOMOLOGOUS ENDS BY DNA POLYMERASES**

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DNA double strand breaks with noncomplementary ends can be joined by mechanisms of nonhomologous recombination. In some systems a DNA end with a 3'-protruding single strand (PSS), which does not have a recessed 3'-hydroxyl that can allow for fill-in DNA synthesis, is joined to a blunt end with preservation of the 3'-PSS. It has been proposed that this process occurs via single strand ligation or is facilitated by an alignment protein. We were interested in testing the hypothesis that a DNA polymerase could function as this putative alignment protein. To characterize polymerase activities in this type of reaction, we incubated short double-stranded oligonucleotides that had an excess of one of the strands with an exonuclease-free Klenow fragment of Escherichia coli polymerase I, Taq DNA polymerase from Thermus aquaticus, or an exonuclease-free Stoffel fragment of Taq DNA polymerase. Products were analyzed by using biotinylated oligonucleotides separated by denaturing polyacrylamide gel electrophoresis. To further assess the effect of DNA polymerases on the joining of 3'-PSS ends to blunt ends, we incubated linear plasmid DNA with the polymerases and subjected the DNA to Southern blot and sequence analysis. We determined that these DNA polymerases can use a 3'-PSS end as a template after priming off the 3'-hydroxyl of a blunt end. This implies that the joining of noncomplementary ends in eukaryotic cells could proceed by a similar mechanism.

The joining of two DNA ends is a defining step in the process of nonhomologous recombination, which occurs in the cellular processes of integration of extrachromosomal DNA, repair of spontaneous or damage-induced DNA breaks, and formation of gross chromosomal rearrangements. The joining reactions of these processes can result in deletion, insertion, or both deletion and insertion of nucleotides and frequently involves very short stretches of homology (one to six bases) at the junction (1, 2). The resultant changes in chromosome structure can result in cell death (3), mutation, gene amplification, cell transformation (4, 5), or genomic instability (6-8).

Model systems that start with DNA ends produced by restriction enzymes, which produce ends of defined structure, have been developed to study noncomplementary end joining. One system uses cultured monkey cells transformed with SV40 DNA (9-11), and another system exposes plasmid DNA to Xenopus laevis cell extracts (12, 13). Transformation of Escherichia coli with linear DNA with noncomplementary ends has been used to demonstrate that the end-joining products in *E. coli* are very similar to the products from higher eukaryotes (14). DNA sequence analysis of the junctions formed during end-joining reactions in these model systems has established patterns of junction formation.

There are two distinct types of products observed in these systems. The observation of each is typically dependent on the structure of the ends being joined (reviewed in Ref. 15). The first is typified by strand resection either before or after alignment at up to six complementary nucleotides (11). This type of product is predominant in the joining of DNA ends of the same polarity (i.e. 3' to 3' and 5' to 5'). The second major type of product is observed in the joining of a 3'-PSS to either a 5'-PSS or a blunt end and preserves the protruding single-stranded ends. This process has been shown to use a novel priming activity facilitated by a putative alignment protein (15).

We investigated whether a DNA polymerase could function as a putative alignment protein in DNA end joining. The inspiration for this investigation came from two sources. The first was the structure of DNA polymerases (16), which have distinct double- and single-stranded DNA binding regions, a property an alignment protein would be likely to have. The second came from studies that showed that polymerases that lack 3'-5'-exonuclease activity, including Klenow and Taq DNA polymerases, are known to add bases to the 3'-hydroxyls of blunt ended DNA molecules (17, 18). Polymerases that contain 3'-5'-exonuclease activity carried out very little of this nontemplate addition unless the exonuclease activity was repressed by addition of dNMPs (17).

To test the hypothesis that a DNA polymerase that had added a single base to a blunt end molecule could use a 3'-PSS as a template for the polymerization of additional nucleotides, we incubated short oligonucleotides with an exonuclease-free Klenow fragment of *E. coli* polymerase I (KF), the Taq DNA polymerase from *Thermus aquaticus* (Taq), or an exonuclease-free Stoffel fragment of Taq DNA polymerase (SF). In addition we used linearized plasmid DNA with restriction enzymes to produce fragments with 3'-PSS and blunt ends, incubated in vitro with the same DNA polymerases and analyzed junction formation on Southern blots.

MATERIALS AND METHODS

**Treatment of Oligonucleotides with DNA Polymerases and Detection of Biotinylated DNA**—A biotinylated M13 reverse-sequence-primer was purchased from U. S. Biochemical Corp. The following oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) 391 DNA synthesizer: 1, 5'-CGGTGTTGTTCTGGATGAAATCTG-3'; 2, 5'- CGGCCTTGGTTGTTCTGGATGAAATCTG-3'; 3, 5'-CGGTGTTGTTCTGGATGAAATCTG-3'; 4, 5'-CTGGCTGTTTCCTGTGTGAAATCTG-3'; 5, 5'-CTGGCTGTTTCCTGTGTGAAATCTG-3'.

†The abbreviations used are: PSS, protruding single strand; KF, Klenow fragment; SF, Stoffel fragment; bp, base pairs.)

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GAAAGCTGTTCTG-3' and 4'-CGGTTTCTCTGTTGAAAAGCTCCTC-3'.
(The bases complementary to the M13 sequencing primer are underlined.)

Reactions were carried out in 10-μl solutions with 1 pmol of M13 biotinylated primer, 10 pmol of complementary oligonucleotide, and either 5 units of Taq or SF or 2.5 units of KF. The M13 primer was annealed to the oligonucleotides by heating to 65 °C for 2 min, and then the reaction was cooled slowly to ambient temperature. Taq was purchased from Boehringer Mannheim, SF was purchased from Perkin-Elmer, and KF was purchased from U. S. Biochemical Corp. Reactions with Taq were carried out at 50 °C in 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 0.1 μg/ml gelatin, and 1.5 mM MgCl₂ (5 μg for SF). Reactions with KF were carried out at ambient temperature in 25 mM Tris-HCl (pH 8.0) and 10 mM MgCl₂.

Concentrations of nucleotide triphosphates were 100 μM each dATP, dGTP, and ddTTP. The ddTTP was used to accumulate products in a single band and facilitate detection. The oligonucleotides were designed so that the first da in the template would be encountered after the addition of 8 bases and thus would prevent products of >25-mer from forming. Reactions were terminated by the addition of 6.6 μl of 95% formamide and freezing to -20 °C. The oligonucleotides were separated by electrophoresis on a Life Technologies, Inc. S2 sequencing gel system on 0.4-mm denaturing 15% polyacrylamide gels. DNA from the gels was transferred by capillary action onto BioTrace HP membrane (Gelman Sciences, Ann Arbor, MI). The biotinylated derived DNA was detected by using the Clontech (Palo Alto, CA) Gene-Tect detection system that uses a streptavidin-alkaline phosphatase conjugate. A light-generating reaction with Lumi-Phos (Boehringer Mannheim) and exposure to XAR5 film (Kodak) was used in place of the color precipitation reaction.

FIG. 1. Detection of Taq-treated biotinylated DNA from a denaturing polyacrylamide gel. In all reactions the biotinylated M13 was sequenced directly by using the solid-phase method (20). The DNA was pur- chased from Boehringer Mannheim. pBR322 was linearized simultaneously with PvuII, which produces blunt ends, and AatII, which produces 2'-PSS ends. AatII was purchased from New England Biolabs; all other restriction enzymes were purchased from Boehringer Mannheim. After restriction digestion with the appropriate enzymes, fragments were separated in agarose gels. The agarose containing the desired fragments was cut out, and the DNA was extracted by electroelution. 0.6 μg of plasmid DNA was treated under the same conditions used for the oligonucleotides in 17-μl volumes. After polymerase reactions, enzymes and salts were removed from the DNA using Promega (Madison, WI) DNA clean-up kits. The DNA was then digested with additional restriction enzymes. Control DNA was treated identically except DNA polymerase was not added. The DNA was analyzed by agarose gel electrophoresis and Southern blot hybridization. AatII-to-PvuII DNA fragments were used as a probe and labeled with 32P by using a random primed DNA labeling kit from Boehringer Mannheim. Hybridization and washing were carried out as described in Ref. 19. Junction DNA for sequencing was obtained by using the polymerase chain reaction (PCR). Using the Perkin-Elmer GeneAmp kit, we amplified the Taq-treated fragment DNA (0.1 μg) with primers straddling the junction. The DNA was sequenced directly by using the solid-phase method (20), 35S, and the Sequenase 2.0 DNA sequencing kit from U. S. Biochemical Corp.

RESULTS AND DISCUSSION

To determine if DNA polymerases could use a 3'-PSS as a template to bridge the gap formed by a double-stranded oligonucleotide and the 3'-hydroxyl of a single-stranded oligonucleotide, we incubated oligonucleotides with DNA polymerases and separated the products on polyacrylamide gels. The oligonucleotides consisted of a 5'-biotinylated 16-mer annealed to a...

FIG. 2. Detection of KF-treated biotinylated DNA from a denaturing polyacrylamide gel. The biotinylated M13 reverse-sequencing primer was annealed to oligonucleotide 3 or 4 and incubated with KF. Oligonucleotides 3 and 4 are identical except for the last two nucleotides and when annealed to the primer result in 2-base 5'-overhangs and 8-base 3'-overhangs. The vertical numbers indicate the size of the biotinylated DNA in nucleotides.
Fig. 3. Southern blot and sequence analysis of Taq-treated plasmid DNA. A, Southern blot analysis. All lanes contain pBR322 or pBR322-derived DNA that had been cut with PstI and NdeI, which produce 1-, 312-, and 3,049-bp fragments from pBR322 (lane 1) and 1,312- and 908-bp fragments from a plasmid that had been cut with AatII and PvuII and the ends joined (lane 2). Lane 3 contains fragment DNA from pBR322 that had previously been cut with AatII and PvuII. Restriction digestion of this fragment with PstI and NdeI produced fragments of 1,312, 677, and 231 bp (off the gel). Lane 4 contains the same DNA as lane 3 except that the DNA had been incubated with Taq for 4 h. B, sequence analysis. Junction DNA was amplified by PCR and sequenced.

25- or 26-mer with internal bases complementary to the 16-mer. We used a 10-fold excess of the longer oligonucleotide, which was considered the template. The 16-mer was considered the primer, and extension to the end of the template produced a model blunt end. The excess of the template served as a model 3'-PSS. In Fig. 1 it is apparent that, as observed previously (17, 18), Taq can add a single nucleotide to a blunt end molecule, because the 16-mer was quickly extended to a 20-mer (the end of the template), and an additional base was added forming a 3'-PSS.

Fig. 4. Model of polymerase-mediated end joining. A, the polymerase reaches the end of a template, terminating in a blunt end; B, the polymerase adds a base to the end of the blunt molecule; C, a 3'-PSS with a complementary terminal base is aligned in the active site of the polymerase; D, the DNA polymerase synthesizes DNA by using the 3'-PSS as a template.
21-mer. Furthermore, additional bases can be added, producing products up to 25-mer. This addition is facilitated by the existence of a terminal dT residue on the 3' end of the template oligonucleotide, because products >21-mer were not detected with oligonucleotide 2, which has a 3'-terminal dG, but were easily detected with oligonucleotide 1, which has a terminal dT (Fig. 1). The dT would be complementary to an added dA, which is the predominant base added to a blunt end for both Taq and KF (17, 18).

Fig. 2 shows the results obtained by incubating KF with oligonucleotides 3 and 4. With both of these oligonucleotides, the primer was quickly extended to the end of the template (18-mer), and an additional base was added (19-mer). Incubations with oligonucleotide 3, which has a 3'-PSS with a terminal dT, more rapidly produced products >19-mer than did incubations with oligonucleotide 4, which has a 3'-PSS ending in a terminal dC.

To verify that base additions beyond the blunt end were derived from the template, we incubated KF, Taq, and SF with all four oligonucleotides. With oligonucleotides 1 and 2, all three polymerases paused after the addition of four and five bases respectively (Fig. 2). With oligonucleotides 3 and 4, KF and SF paused after the addition of two and three bases (shown for KF in Fig. 2). Incubation of oligonucleotides 3 and 4 with Taq for longer than 3 h resulted in loss of signal (data not shown). This is probably due to the 5'-3' exonuclease activity of this polymerase, because the signal did not deteriorate after incubation with SF, which lacks this exonuclease. At the incubation temperatures we used, Taq and SF could bridge the gap only if the 3'-hydroxyl of the template oligonucleotide was a dT. KF could bridge the gap with a dT, a dC, or a dG at the 3'-position (data not tested). Because oligonucleotides that had a 3'-terminal dT, which could complement an added dA, resulted in quicker formation of 24-mers, it is easy to conclude that this reaction is facilitated by a complementary base.

Several of the bands in Figs. 1 and 2 are doublets (see Fig. 1, 20- and 21-mers and Fig. 2, 19-mers). The doublets are likely to be the result of the slight change in mobility of similarly sized bacteriophage λ DNA. This explanation is consistent with the fact that double bands are particularly noticeable at positions corresponding to bases added to a blunt end, which are less likely to be template derived than are bases added at other positions. Furthermore, additional bands may result from the pausing of polymerases at certain positions (Fig. 1, 23-mers at 4 h for oligonucleotide 1; Fig. 2, 22-mers at 12 min for oligonucleotide 3). The pausing may result from the gap in the template and mismatches between the template and the primer, because DNA polymerases are inhibited by primers with mismatches (21, 22). Taq, SF, and KF added ddT less efficiently than they did the dNTPs, resulting in more intense bands for 24-mers than 25-mers (Figs. 1 and 2).

To determine the extent of junction formation, we digested pBR322 plasmid DNA with AatII and PvuII and gel-purified the fragment containing the ampicillin-resistant marker and the origin of replication. The DNA was then incubated with DNA polymerases and digested with additional enzymes that would produce uniquely sized fragments from junctions formed when the AatII/PvuII ends were joined. The products were separated on agarose gels, transferred to membranes, and probed with pBR322-derived DNA (Fig. 3A). The known rejoin (lane 2) was obtained by transforming E. coli with Taq-treated AatII-to-PvuII DNA fragments and recovering circularized plasmid DNA, which contains a 908-bp fragment resulting from the joining of these two sites (verified by sequencing). In Fig. 3A, lane 4, a band at 908 bp shows that in Taq-treated DNA, a continuous strand of DNA extends for hundreds of bases on either side of the original double strand break. This DNA was not present in untreated samples (Fig. 3A, lane 3). Analogous results were obtained with KF (not shown). To demonstrate that the junction formed preserves the original sequence of the PSS, we amplified Taq-treated fragment DNA by PCR and sequenced it using the dideoxy method. The sequence demonstrates that the PSS is preserved (Fig. 3B).

From these results we conclude that Taq, SF, and KF are able to synthesize DNA across a double strand break using a 3'-PSS as a template and can thus bridge the gap formed between these ends. Fig. 4 illustrates a model for end joining mediated by a DNA polymerase, based on the results we obtained. In this model the polymerase binds to a double-stranded region of DNA and adds a nucleotide at a blunt end or a 5'-PSS converted to a blunt end. The double-stranded DNA is held in the double-stranded DNA-binding region of the polymerase, and then the single-stranded DNA-binding region binds a 3'-PSS. The polymerase can then add additional bases by using the 3'-PSS as a template. These results are further supported by experiments described in Ref. 23.

Our work and that of Clark (23) demonstrate that a DNA polymerase can function as the putative alignment protein of noncomplementary end joining. However, this does not preclude the involvement of proteins that may facilitate end joining by holding ends in proximity to each other. Proteins involved in chromatin structure, such as histones and poly(ADP-ribose) polymerase, would be likely candidates to play such a role.

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