A novel DNA joining activity catalyzed by T4 DNA ligase

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ABSTRACT

The use of T4 and *E. coli* DNA ligases in genetic engineering technology is usually associated with nick-closing activity in double stranded DNA or ligation of 'sticky-ends' to produce recombinant DNA molecules. We describe in this communication the ability of T4 DNA ligase to catalyze intramolecular loop formation between annealed oligodeoxyribonucleotides wherein Watson-Crick base pairing is *absent* on one side of the ligation site. Enzyme concentration, loop size, substrate specificity, and base composition were explored in an effort to maximize yield. Amounts of T4 DNA ligase in large molar excess to DNA template and ligated product are necessary to achieve high yields.

INTRODUCTION

T4 DNA ligase has been shown to catalyze the formation of phosphodiester bonds between the 5'-phosphoryl and 3'-hydroxyl end-groups in properly aligned duplex DNA strands (for review, see 1,2). The highly specific requirement for a helical DNA substrate, and ATP, was first demonstrated using extracts from *E. coli* infected with bacteriophage T4 (3,4). Since then a variety of DNA substrates have been used to investigate activities associated with T4 DNA ligase. Examples include blunt-end ligation of chemically synthesized base-paired duplexes (5,6,7), circularization of DNA fragments generated by type II restriction endonucleases (8), and joining synthetic oligodeoxynucleotides containing an AP (apurinic or apyrimidinic) site, gaps, or mismatched bases at the ligation junction (9,10,11,12,13). Terminal cross-linking of DNA strands causing loop formation, catalyzed by T4 DNA ligase, has also been described (14). The rather unconventional ligations mentioned above take place in the presence of base paired 3' and 5' termini. Recently it has been shown that *E. coli* DNA ligase effectively joins some single stranded oligodeoxyribonucleotides in the absence of a complementary template (15). In this paper, we report a novel activity associated with T4 DNA ligase in which 3' and 5' end-groups are substrates for esterification in the *absence* of Watson-Crick base pairing on one side of the ligation junction. Our constructs include two synthetic oligonucleotides of unequal length annealed such that the shorter oligo is completely base paired to the 3' half of the longer oligo. In the presence of highly concentrated T4 DNA ligase, our data strongly suggest that the unpaired 5'-phosphoryl group is covalently linked to the recessed 3'-hydroxyl group to form a stem-loop structure. The data show that the termini of both strands need not be abutted through base pairing for efficient ligation to occur.

MATERIALS AND METHODS

Enzymes and chemicals

Hind III and bacterial alkaline phosphatase (BAP) were purchased from Bethesda Research Laboratories (BRL). T4 polynucleotide kinase was purchased from Stratagene, and T4 DNA ligase was purchased in a highly concentrated form, 2000u/ μ L (Cat. No. 202C, Lots 34, 39, 42, 43) and 400u/ μ L (Cat. No. 202, Lot 32) from New England Biolabs (NEB). The specific activity of T4 DNA ligase ($M_r = 68,000$, 16) at 2×10^6 NEB units/mL is 1.4×10^4 Weiss units/mg protein (N.E.B., personal communication). 1 unit, defined by NEB, equals 0.015 ATP-PP exchange unit (4) and Weiss units will be used throughout this paper. All enzymes were used under the conditions specified by the manufacturer, unless otherwise stated. Cyanoethyl phosphoramidites for DNA synthesis were obtained from Cruachem, Inc., and [γ - 32 P]ATP used in 5'-end-labeling of oligonucleotides was purchased from New England Nuclear (DuPont).

Chemical synthesis of oligonucleotides

Oligonucleotides were synthesized on a BioSearch 8650 DNA synthesizer through standard phosphoramidite methodology (17) and gel purified by electrophoresis on preparative polyacrylamide gels. The DNA was excised, eluted overnight in 0.1 M NH_4CO_3 at 37°C, and concentrated and de-salted using Sep-Pak C₁₈ cartridges (Waters Associates). Sequences are shown in Figure 1.

Preparation of 5'-end-labeled oligomers

Oligonucleotide sequences (ON1 through ON5, ON7, ON6rev) were 5'-end-labeled as previously described (18). Reactions were typically carried out in 10–30 μ L volumes in the presence of [γ - 32 P]ATP at 6000 Ci/mmol, or 2mM ATP (Pharmacia) for phosphorylating ON7, with 10 to 20 units of T4 polynucleotide kinase. Oligonucleotides were separated from the unincorporated nucleotide on Nensorb-20 cartridges (DuPont). Overall efficiencies ranged from 60 to 85 percent.

Ligation reaction

To assay for ligase activity, a 5'- 32 P donor sequence (ON1-ON5, ON6rev) was incubated with the acceptor sequence (ON6 or ON1rev) in 5 mM dithiothreitol, 1 mM ATP, ligase buffer (66 mM Tris-Cl, pH 7.5, 66 mM MgCl_2), and varying amounts of T4 DNA ligase in volumes of 20 or 40 μ L. The reactants were incubated at 65°C for 5 minutes in the ligation mix and annealed at room temperature (24°C, 15–30 minutes) prior to the addition of ligase. Reactions were incubated with T4 DNA ligase at room temperature for at least 2 hours.

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OLIGONUCLEOTIDE	SEQUENCE
ON1	3'- <u>GT</u> TAAATGTGTTTCGAATTATGTAAGGAACGTACGGACGTCCAGCTGAGATC -5'
ON2	3'- <u>GT</u> TAAATGTGTTTCGAATTATGTAAGGAACGTACGGACGTCCAGCTG -5'
ON3	3'- <u>GT</u> TAAATGTGTTTCGAATTATGTAAGGAACGTACGGACGTCC -5'
ON4	3'- <u>GT</u> TAAATGTGTTTCGAATTATGTAAGGAACGTACGGA -5'
ON5	3'- <u>GT</u> TAAATGTGTTTCGAATTATGTAAGGAACGT -5'
ON6	5'- CAATTACACAAGCTTAATACATTCC -3'
ON7	3'- TTATGAATCTTCTCGTGAAATGCCAAACGTACGGACGTCCAGCTGAGATC -5'
ON1rev	3'- CTAGAGTCGACCTGCAGGCATGCAAGGAATGTATTAAGCTTGTGTAATTG -5'
ON6rev	3'- CAATTACACAAGCTTAATACATTCC -5'

Figure 1. Synthetic oligonucleotide sequences used in the ligation reactions. ON1 through ON5, ON7, and ON6rev are donor molecules supplying the 5'-phosphoryl end group. ON6 and ON1rev are acceptor molecules contributing the 3'-hydroxyl end group when base paired to any of the donors (except ON7). Underlined bases indicate region involved in base pairing with ON6 or ON6rev. No significant internal base pairing can be found within the individual oligonucleotides as determined by computer analysis. The program OLIGO, which analyzes DNA/RNA sequences, was purchased from National Biosciences; Hamel, MN.

Polyacrylamide gel electrophoresis (PAGE)

Typically, 8–15% gels were prepared from a 40% (w/v) solution (Amresco) of acrylamide (38%) and bis-acrylamide (2%), 8 M urea (BRL), and 1× TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA). Reaction aliquots were heat denatured at 90°C for 3 minutes in the presence of a formamide loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 50 mM Tris-borate, 1 mM EDTA), chilled on ice, and electrophoresed under standard conditions (19). The *Msp* I digest of pBR322 (NEB) was end-labeled with ³²P and used as a molecular weight standard. Product formation was visualized by autoradiography following exposure to Kodak X-OMATTM AR diagnostic film.

Quantitation of product formation

Following autoradiography, appropriate radioactive bands were excised from the gel and placed into vials containing approximately 10 mLs of Ready SafeTM liquid scintillation fluid (Beckman). Samples were counted on a Beckman LS 2800 Liquid Scintillation instrument. Ligation yields were calculated by counting the dpm in the -T4 DNA ligase control band (denominator) and in the ligated product band (numerator). Multiplying by 100 gives the percentage yield. Ligation efficiencies were not calculated in every case, due to a contaminating phosphatase activity present in various lots of the concentrated ligase.

RESULTS

Ligation of a synthetic, partially double-stranded substrate

The following observation suggested that T4 DNA ligase covalently joins the two strands of the synthetic DNA construct shown in Figure 2. One picomole of 5'-³²P-labeled ON1 annealed to 1.5 picomoles of ON6 was incubated with varying amounts of T4 DNA ligase and the results analyzed by polyacrylamide gel electrophoresis. Autoradiography showed the conversion of ON1 (50mer) into a product 75 bases in length. The appearance of the product band correlated directly with the disappearance of the substrate in the presence of highly concentrated enzyme (Fig. 3A). Very little product was formed when the enzyme concentration was decreased 10-fold (Fig. 3B). No product was observed when either ON6 or the ligase (highest concentration) was omitted (Fig. 3C, D).

Yields increased approximately two-fold when the construct in Figure 2 was changed and the 5'-phosphoryl end-group was

Figure 2. Proposed model depicting the formation of a stem-loop molecule from a partially double-stranded substrate. In the presence of T4 DNA ligase and ATP, joining occurs between the extended 5'-phosphoryl group and the base-paired 3'-hydroxyl group in the absence of any complementarity to align the termini side by side.

Figure 3. Autoradiogram of a 15% denaturing polyacrylamide gel showing the conversion of ON1 (radiolabeled 50mer) to a 75-base product in the presence of ON6 (25mer) and T4 DNA ligase. Product formation after 2, 4, and 20 hours of incubation with (A) 60 units and (B) 6 units of T4 DNA ligase at room temperature. Faint product bands are visible on original autoradiogram at each timepoint in B. No detectable ligation occurs in the absence of ON6 (C) or enzyme (D). This is a 2.5 hour exposure at -70°C with intensifying screens.

recessed. ON1 and ON6 were synthesized in the reverse orientation (ON1rev and ON6rev) to create a substrate (when annealed) with an extended 3'-hydroxyl group and a recessed 5'-phosphoryl group. In a volume of 20 μL, 2 picomoles of ³²P-labeled ON6rev annealed to 3 picomoles of ON1rev were

Figure 4. Autoradiogram comparing product yields when the position of the 5'-phosphoryl end-group is either extended or recessed. Product yield is 24% when the original substrate, ^{32}P -labeled ON1 and ON6, is used (A). Yields double when ^{32}P -labeled ON6rev and ON1rev create a substrate in which the 5' end-group is recessed (B). Aliquots taken before adding ligase (-) and after a 2 hr incubation with 30 units of T4 DNA ligase (+) were electrophoresed on a 15% denaturing polyacrylamide gel. Reactions were performed in parallel using identical buffers and enzyme concentrations.

incubated with 30 units of T4 DNA ligase for 2 hours at room temperature. Results are shown in Figure 4, using ON1 and ON6 under identical conditions as a control.

Characterization of the ligated product

The standard assay for ligase activity measures the conversion of ^{32}P -labeled 5'-phosphomonoesters to diesters. In this form, the linkage is resistant to digestion with bacterial alkaline phosphatase. Although the products from our ligase reactions were phosphatase resistant (data not shown), the assay does not differentiate between a stem-loop structure product and the possible circularization of the ^{32}P -labeled oligonucleotide to its own 3' end. Formally, a circular 50mer might electrophorese in the position of a linear 75mer. A *Hind* III site incorporated into the base-paired region allowed us to predict the cleavage pattern after enzymatic digestion (Fig. 5A). Complete digestion of the 75-base stem-loop (cuts at 1 and 2) would result in a radiolabeled fragment 51 bases in length. A limited digest, however, could release two additional fragments: a labeled 65mer if cleavage occurred at site 1 but not 2, or a 61-base fragment if cleavage occurred at site 2 but not 1. A circularized product was not expected to be a substrate for the enzyme. As shown in Fig. 5B, the mobilities of the major radiolabeled fragments released by digestion are as predicted for the stem-loop structure. The restriction pattern indicates that much of the product is completely digested, while a portion is partially cleaved at site 1, but not site 2. A minor amount of product is undigested. Overnight digestion drives most of the partially cleaved species into the lowest molecular weight band (data not shown).

Effect of single-stranded DNA length on product formation

Four oligonucleotides were synthesized (ON2 through ON5) to examine ligation yields when the length of the unpaired 5' end

Figure 5. (A) *Hind* III cleavage site (arrows) of double-stranded product obtained when ON1 is base-paired to ON6. Following ligation, this stem region is covalently linked through a 25-base loop. Asterisk indicates phosphodiester linkage and internalization of ^{32}P label. (B) Restriction digest of the ligated product with *Hind* III. Prior to enzymatic digestion, the ^{32}P -labeled 75mer was eluted from a 15% polyacrylamide gel in 0.1 M ammonium bicarbonate (1 mL) overnight at room temperature. Following purification by Nensorb chromatography, the oligonucleotide was vacuum-dried in a Speed Vac Concentrator (Savant). Autoradiogram of a 20% polyacrylamide gel shows the intact product in lane 1 and the products obtained after digestion with 10 units of *Hind* III for 1 hour at 37°C in lane 2. From the mobilities of molecular weight markers, we estimate the restriction fragments to be 65 and 51 bases. A faint band corresponding to 61 bases is visible on the original autoradiogram. The gel was exposed to X-ray film overnight at -70°C with intensifying screens.

of the donor molecule is varied. As shown in Figure 1, the oligomers are 5, 10, 15, and 20 bases shorter than ON1 at the 5' end. When substituted for ON1 in the ligation assay, the potential loops formed are 20, 15, 10, and 3 bases in length, respectively. The four oligonucleotides were 5'- ^{32}P end-labeled as described previously. In a volume of 20 μL , 2 picomoles of radiolabeled donor oligonucleotide annealed to 3 picomoles of ON6 were incubated with 30 units of T4 DNA ligase. Aliquots taken before the addition of ligase and following a 2-hour incubation at room temperature were electrophoresed on a 15% denaturing polyacrylamide gel. The results are shown in Figure 6. Ligation yields based on the percent of total ^{32}P label found in the product band were: ON5, 0%; ON4, 7%; ON3, 47%; ON2, 5%; ON1 (control), 27%. The ligation products obtained with donors ON4 and ON2 (lanes 4 and 8) migrated faster than expected (donor length plus 25 bases) compared to molecular weight standards. A possible explanation for this observation will be addressed in the Discussion.

Effect of a competing oligonucleotide on product yields

To demonstrate that the observed ligation products were the result of intramolecular, not intermolecular, joining, a competing donor (ON7) was added in molar excess in a competition assay. The 5' 25 bases of ON7 are identical to the 5' unpaired region of ON1, however, the 3' 25 bases are different and do not base-pair to the acceptor, ON6. Intermolecular ligation of ON6 to the unlabeled competing 50mer would greatly reduce product yields as determined by the fraction of dpm recovered in the product band. One picomole of [5'- ^{32}P] ON1, 1.5 picomoles of ON6,

Figure 6. Autoradiogram (15% gel) of ligation products when ON1 through ON5 are the donor sequences. Odd numbered lanes (1, 3, 5, 7, and 9) represent each of the 5'- ^{32}P -labeled donors, ON5, ON4, ON3, ON2, and ON1 respectively. Fragment size (bases), extrapolated from molecular weight markers, is interpreted to be a) 30, b) 35, c) 40, d) 45, and e) 50. The even numbered lanes (2, 4, 6, 8, and 10) show product formation when these oligos are incubated with ON6 and T4 DNA ligase. Gel was exposed to X-ray film for 3.5 hr at -70°C with intensifying screens.

and increasing amounts of unlabeled ON7 (5–100 molar excess) were mixed and annealed as previously described. Following a 2 hour incubation (room temperature) with 60 units of ligase, aliquots (2 μl of 40 μl total volume) were electrophoresed on an 8% polyacrylamide gel. Results demonstrate that ON7 does not effectively compete for ligation and support our model for intramolecular ligation. In the presence of 100 fold molar excess ON7, an approximate 2 fold decrease in product formation is indeed seen. Reactions run in parallel assayed product when ON7 was radiolabeled instead of ON1. Under identical conditions of annealing, ligation, and electrophoresis, no radiolabeled ligation product was detected (data not shown).

DISCUSSION

In this communication we report a novel activity associated with T4 DNA ligase. The enzyme appears to effectively join a hybridized pair of synthetic oligonucleotides without the benefit of complementarity to align both termini. Our assay monitors the joining of a partially double-stranded substrate radiolabeled at the 5' end. The resulting stem-loop product is analyzed by autoradiography following polyacrylamide gel electrophoresis under denaturing conditions. As shown in Fig. 3, high enzyme concentrations and long incubation times are required for significant product formation. When the substrate was altered such that the 5'-phosphate was recessed instead of extended, product yields doubled.

Four additional oligonucleotides, shorter versions of ON1, were tested for their ability to act as donors in the ligase reaction when base paired to ON6. Experimental results showed no evidence of product formation involving a three-base loop (ON5) which, while sterically possible, is strained, and very little product

Figure 7. Ligation of [^{32}P] ON1 (50mer) to ON6 (25mer) in the presence of increasing amounts of ON7. Product yields, calculated as a fraction of total dpm, were 37%, 38%, 26%, 23%, and 24%, at 5-, 10-, 25-, 50-, and 100-fold molar excess of competing oligo. In the absence of ON7, 42% ligation occurred. No product was detected in the absence of ON6. The autoradiogram was exposed for 2.5 hours at -70°C . The observed increase of ^{32}P label in ON1 with increasing amounts of ON7 is thought to be the titration of a contaminating 5'-phosphatase activity associated with the T4 DNA ligase preparation.

when the potential loop was 10 bases (ON4). Products with 15- and 25-base loops were readily formed (ON3 and ON1), whereas the construct using ON2 (20-base loop) as the donor was a surprisingly poor substrate (see Fig. 6). The preferential joining of ON1 and ON3 to ON6 cannot be explained solely on the basis of chain length. It is possible that base composition at the 5' end of the donor is also a factor. Extensive ligation studies involving oligoribonucleotides and T4 RNA ligase showed pCp to be the more reactive donor when compared to pAp and pGp (20). It is interesting to note that both ON1 and ON3, which react with the highest yields, terminate with a C at their 5' ends. This may explain the appearance of shorter product bands observed in lanes 4 and 8 (indicated by arrows in Fig. 6). The 5' ends of ON2 and ON4 are G and A, respectively, but shorter contaminating oligonucleotides containing a terminal C, arising from premature termination during synthesis of oligomers, may be preferred donor molecules and would yield the slightly shorter products.

The ligation most likely proceeds through an intramolecular joining event. Reduced efficiency (less than 2-fold) was observed when a competing 50mer, which does not hybridize to ON6 but could compete for ligation as a donor, was present during the reactions at 100-fold molar excess. We hypothesize that this small decrease is due to titration of T4 DNA ligase by unproductive protein/DNA binding to the large excess of ON7. Ligation efficiencies remained constant, however, as the substrate (ON1 annealed to ON6) concentration was diluted up to 100-fold (data not shown).

The substrates tested here for T4 DNA ligase catalyzed ligation closely resemble those described by Weiss (14), and to a lesser extent those described in reference (10), with one important exception: we took great care to exclude potential base pairing from one side of the ligation site. All published work we are aware of continue to support the theory that *both* termini need to be base paired to a complementary strand to be a substrate

for T4 DNA ligase. Because of the lack of complementarity in the substrates we have described, the interaction between the end-groups to be ligated is weak, causing proper alignment to be a rare event. It is possible, however, that in the presence of excess ligase, a stable complex is formed between enzyme and nucleic acid. A similar explanation was proposed to account for the joining of complementary oligonucleotides at temperatures above their T_m (21). The final ligation step is now dependent upon those events which bring the two end-groups into close proximity.

Although adenylation of the extended, unpaired 5'-phosphoryl end-group occurs, we hypothesize that the enzyme is better able to recognize and more readily adenylate the recessed, base paired end-group as this form of the substrate more closely resembles a strand break in duplexed DNA. The increased ligation yield of this substrate is still incomplete and may be limited by the accessibility of the 3'-hydroxyl group, however we have no data to support this conclusion.

In this report we provide evidence for the use of T4 DNA ligase and synthetic oligodeoxyribonucleotides in forming stem-loop structures. The termini need not be juxtaposed by base pairing to a complementary sequence for ligation to take place if large amounts of enzyme are present. The process is clearly dependent upon the close proximity of the two strands and there may be some preference for the 5' phosphate to be at the end of a completely annealed sequence and/or present on cytidine. Our results indicate that investigators may need to be cautious in the use of ligase when constructing recombinant DNA molecules, especially when extended unpaired regions are involved. The commercially available 2000 unit/230L (NEB units) is approximately 30 Weiss units/230L. Our findings in Fig. 3 show detectable ligation of an unpaired terminus with as few as 6 Weiss units of T4 DNA ligase, and very high yields with 60 Weiss units (2 230L of the concentrated enzyme). Attention to the position of phosphorylated termini, and the use of the minimum activity of T4 DNA ligase necessary during enzymatic manipulation of DNA will serve to decrease unwanted side products such as those described in this paper. It should also be noted that if DNA amplification is to follow, even very low yield ligation products such as those described here could compete with or dominate the amplified polynucleotides.

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