

Template-independent ligation of single-stranded DNA by T4 DNA ligase

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blunt-end ligation; circularization of oligonucleotides; competitive PCR; nontemplated ligation; rolling-circle amplification

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T4 DNA ligase is one of the workhorses of molecular biology and used in various biotechnological applications. Here we report that this ligase, unlike *Escherichia coli* DNA ligase, *Taq* DNA ligase and Ampligase, is able to join the ends of single-stranded DNA in the absence of any duplex DNA structure at the ligation site. Such nontemplated ligation of DNA oligomers catalyzed by T4 DNA ligase occurs with a very low yield, as assessed by quantitative competitive PCR, between 10^{-6} and 10^{-4} at oligonucleotide concentrations in the range 0.1–10 nM, and thus is insignificant in many molecular biological applications of T4 DNA ligase. However, this side reaction may be of paramount importance for diagnostic detection methods that rely on template-dependent or target-dependent DNA probe ligation in combination with amplification techniques, such as PCR or rolling-circle amplification, because it can lead to nonspecific background signals or false positives. Comparison of ligation yields obtained with substrates differing in their strandedness at the terminal segments involved in ligation shows that an acceptor duplex DNA segment bearing a 3'-hydroxy end, but lacking a 5'-phosphate end, is sufficient to play a role as a cofactor in blunt-end ligation.

DNA ligases play a pivotal role in the replication, repair, and recombination of DNA [1–3]. They catalyze the formation of a phosphodiester bond between juxtaposed 3'-hydroxy and 5'-phosphate termini in double-stranded (ds) DNA, and can be classified according to their adenylation cofactor requirement as either ATP-dependent or NAD⁺-dependent ligases [1,3–5]. DNA ligases have become indispensable tools for *in vitro* DNA manipulation in a wide range of applications in molecular biology [6–8], in the detection of specific nucleic acid sequences (DNA or RNA) or protein analytes [9–11], in DNA nanotechnology [12,13], and in DNA computation [14–16].

T4 DNA ligase, the prototype of ATP-dependent DNA ligases [17–19], is the most commonly used DNA ligase. One factor that contributed to the widespread use of T4 DNA ligase is the fact that it catalyzes efficiently the joining of blunt-ended dsDNA [20,21], in

contrast with all other DNA ligases studied so far. It has been shown that T4 DNA ligase seals dsDNA substrates containing an abasic site or a gap at the ligation junction, joins branched DNA strands, and forms a stem-loop product with partially double stranded DNA [22–25]. Furthermore, it has been demonstrated that the moderate fidelity that this ligase typically exhibits [26,27] can be significantly lowered by changing the reaction conditions, thus permitting sequence-independent ligation reactions at ligation junctions [28]. The examples mentioned above illustrate that T4 DNA ligase displays some unusual catalytic properties with respect to joining substrates that lack a complementary template or stable base pairing at the site of ligation.

Here we report on the ability of T4 DNA ligase to join the ends of single-stranded (ss) DNA. Whereas T4 RNA ligase has long been known to catalyze such a

Abbreviations

qcPCR, quantitative competitive PCR; RCA, rolling-circle amplification.

nontemplated ligation on ssDNA substrates [29,30], this property of T4 DNA ligase has, to our knowledge, not been reported. Unlike T4 DNA ligase, bacterial DNA ligases that we tested did not have any detectable ssDNA ligation activity.

Our findings have important implications for the development of diagnostic or DNA computational methods that rely on template-dependent or target-dependent ligation in conjunction with nucleic acid-based amplification. Recently, the appearance of nonspecific signals has been reported in ligase-based DNA detection assays in some experiments [31,32]. Our data provide experimental evidence that template-independent ssDNA ligation may be a source of nonspecific signals in such ligase-based technologies. Comparison of the ssDNA ligation yields with ligation yields of substrates in which either one or both termini consist of a short blunt-ended duplex suggests a cofactor role for 3'-hydroxy groups in blunt-end ligation.

Results

Incubation of ssDNA with T4 DNA ligase results in DNA circularization as detected by PCR or rolling-circle amplification (RCA)

To detect very low yields of potential ssDNA ligation product, we performed exponential amplification reactions with samples obtained after incubation of a 5'-phosphorylated oligonucleotide with T4 DNA ligase (Fig. 1 gives the experimental outline; Table 1 shows sequences of oligonucleotides). As shown in Fig. 2A, PCR amplification of T4 DNA ligation samples of oligonucleotide **I** produced several distinct product bands together with a broad distribution of products, visible as a smear (lanes 3–5). This result was not unexpected if circularization had occurred because an RCA-like reaction can proceed on a circular DNA template under typical conditions used for PCR [33]. Indeed, the distinct bands observed represent dsDNA products differing by unit-circle lengths, as typically observed for RCA products using a pair of primers [33–36]. On the other hand, a smear indicates an RCA reaction with a single primer [37]. Thus, the amplicons observed probably originate from a combination of both RCA formats during PCR.

To investigate the possibility that a contamination of the T4 DNA ligase used was responsible for the apparent circularization of **I**, batches of this enzyme from other suppliers (Fermentas and Invitrogen) were used. Incubation of **I** with the same units of T4 DNA ligase from those suppliers, followed by PCR

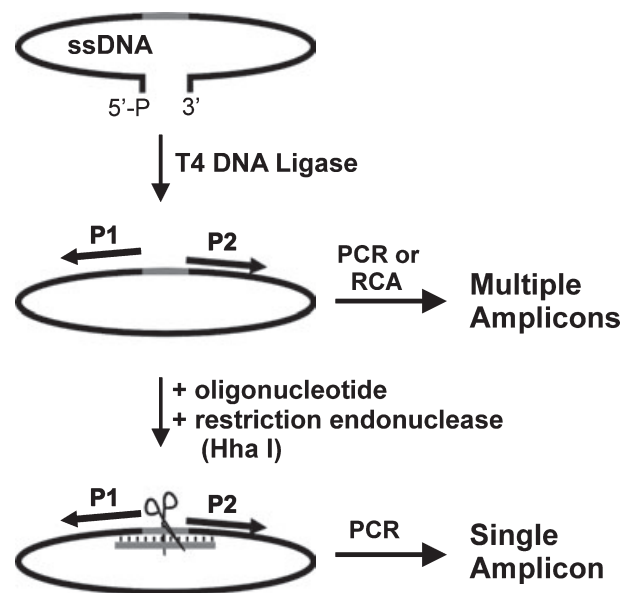


Fig. 1. Schematics explaining the protocols used in the study to detect ssDNA ligation. A ssDNA oligomer (≈ 80 nucleotides) carrying a phosphate group at its 5' end is incubated with T4 DNA ligase. The fraction of the resulting circularized product is then detected via PCR or RCA. Amplification was either performed directly after the ligation reaction or after cleavage of the DNA circle by a restriction endonuclease at a site distant to the ligation point.

amplification, gave semiquantitatively similar results (Supplementary Fig. S1) to those shown in Fig. 2A.

Using the same primer pair as in the PCRs, we then performed RCA reactions on ligated **I**. As can be seen from Fig. 2B, concatemers of various lengths are formed during this isothermally performed amplification (lanes 3–5). The short concatemers have the same gel electrophoretic mobility as the discernible bands obtained by PCR, verifying the RCA-like reaction during PCR. Note that the mobility of each concatemer, when compared with the DNA marker, does not correspond precisely to its specific length but is slightly decreased. The reason for the retardation in mobility is the presence of one or more A-tracts, which cause DNA bending [38], within the dsDNA products obtained. PCR and RCA reactions with unligated **I** resulted, like the negative controls without **I**, in either formation of a primer-dimer product (Fig. 2A, lanes 1 and 6) or no amplicon (Fig. 2B, lanes 1 and 6).

To validate further that the detected products resulted from amplification of circular **I**, PCR and RCA products were treated with different restriction endonucleases, the recognition sequence of which had each been incorporated once into **I**. As expected, the amplicons were converted into three fragments: a longer fragment u (corresponding to unit-circle length in the

case of blunt-end cutters), as a result of cleavage between adjacent sites for the specific restriction endonuclease, and two shorter fragments *a* and *b* resulting from cleavage sites next to each terminus (Fig. 3). Whereas the ladder-type RCA product, which consists of concatemers with defined ends, leads to clean fragmentation on restriction cleavage (lanes 5, 8, and 11), an additional smear is observed for the cleaved PCR product (lanes 4, 7, and 10), because this amplicon is composed of a mixture of concatemers with a wide distribution of products varying in length. Together, the data shown in Figs 2 and 3 provide clear evidence for the presence of circularized **I** in the ligation samples.

It has been previously reported that vaccinia virus DNA ligase can ligate ssDNA composed of T₃₀, but not the other three homopolymers or mixed-sequence oligonucleotides [39]. To check whether T4 DNA ligase exhibits a similar sequence preference, oligonucleotides with different nucleotides at both termini were used. Incubation of oligonucleotides **II** or **III** (Table 2) with T4 DNA ligase, followed by PCR amplification, led to similar products to those shown for oligonucleotide **I**, except that the distinct bands now displayed a gel electrophoretic mobility equivalent to their lengths due to the absence of any bent region within the amplicons (data not shown). Although all these PCR amplicons could only be semiquantitatively compared, it became apparent that ligation yields with **I–III** did not differ substantially, a fact later confirmed by quantitative PCR (Table 2).

Incubation of ssDNA with *Escherichia coli* DNA ligase, *Taq* DNA ligase, or Ampligase does not result in any detectable ligation product

We tested whether other DNA ligases would also result in template-independent ssDNA ligation. Oligonucleotide **I** was incubated with either *E. coli* DNA ligase, *Taq* DNA ligase, or Ampligase. Ligation

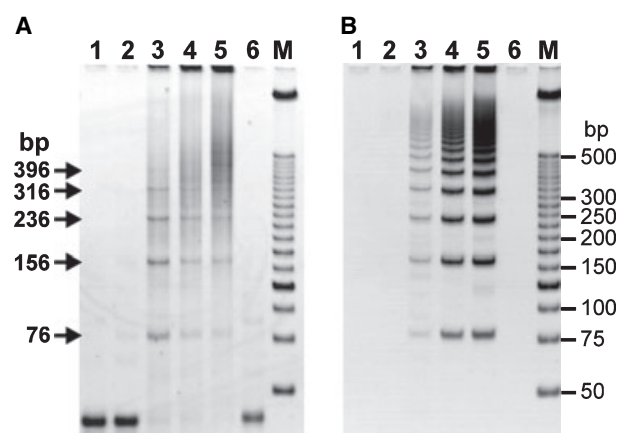


Fig. 2. Analysis of amplicons obtained with oligonucleotide **I** after incubation with T4 DNA ligase. PCR amplification (A) or RCA (B) of ligation samples with 0.01 nM, 0.1 nM, 1 nM, or 10 nM oligonucleotide **I** present during ligation (lanes 2–5). Lanes 1 and 6 are controls in the absence of **I** or with 10 nM **I** in the absence of ligase respectively. Here and below, M denotes a 25-bp DNA ladder (Invitrogen).

reactions were carried out at optimal temperatures given by the supplier (see Experimental procedures). In each case, amplification reactions of uncleaved or *HhaI*-cleaved ligation sample by PCR did not result in an amplicon that would signify ligation product (Fig. 4, lanes 5–7). Larger quantities of ligase and prolonged incubation times did not lead to a PCR amplicon as well (not shown).

Determination of ssDNA ligation yields by quantitative competitive PCR (qcPCR)

Quantitative assessment of yields of ligation reactions required the occurrence of a single PCR product. The complete cleavage of any circularized oligonucleotide by a restriction endonuclease before PCR amplification should yield such a single amplicon (Fig. 1). Because the restriction endonuclease *HhaI* reportedly cleaves

Table 1. Oligonucleotides used in this study. P, phosphate; b, biotin. The recognition site for the restriction endonuclease *HhaI* is shown in bold and sequence segments identical or complementary with the primers (P1, P2) are underlined.

Oligo	Sequence (5'–3')
I	P- <u>TTTGTCCATTCTGTGTCAGCTACTTGTCTCCATC</u> GCGC <u>CTTCCAGCGTATCGTTTTACCTGCATTTTCGCACCTCTGTTT</u>
II	P-CTATCCATTCTGTGTCAGCTACTTGTCTCCATC GCGC <u>CTTCCAGCGTATCGTTTTACCTGCATTTTCGCACCTCTACTC</u>
III	P-CTATCCATTCTGTGTCAGCTACTTGTCTCCATC GCGC <u>CTTCCAGCGTATCGTTTTACCTGCATTTTCGCACCTCTACTC</u>
IV	CACAGGAATGGATAG-b
V	GAGTAGAGGTGCGAA
H17	TGGAAG GCGC GATGGAG
C63	<u>CCTTCCAGCGTATCGTTTTACCTGCACCTCTGTTTTTGTGTCAGCTACTTGTCTCCATCGCG</u>
P1	<u>TTCCAGCGTATCGTTTTACCT</u>
P2	<u>CGATGGAGACAAGTAGCTGAC</u>

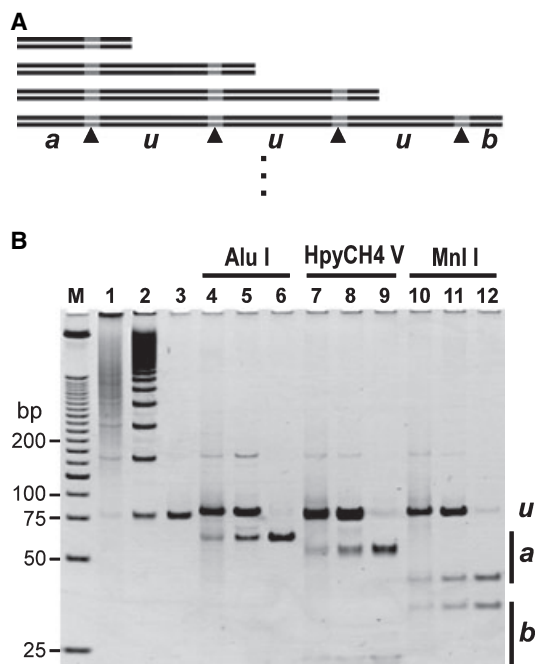


Fig. 3. Analysis of amplicons by restriction endonuclease cleavage. (A) General schematics of amplicons obtained at RCA performed with a pair of primers. Cleavage of products at sites (marked in gray) specific for a restriction endonuclease leads to fragments *a*, *b*, and *u*. Lengths of fragments *a* and *b* depend on the distances between the 5'-terminus of each primer and the cleavage site. For restriction endonucleases generating blunt ends, fragments *u* correspond to unit-circle length. (B) Amplification products before (lanes 1–3) and after restriction endonuclease cleavage (lanes 4–12). Uncleaved or cleaved amplicons correspond to PCR product (lanes 1, 4, 7, and 10) or RCA product (lanes 2, 5, 8, and 11) of **I** obtained directly after T4 DNA ligation. For comparison, the PCR product of a sample of **I** obtained after T4 DNA ligation and *HhaI* restriction digestion (lane 3) was also cleaved by the corresponding restriction endonuclease (lanes 6, 9, and 12). Calculated lengths of fragments *a* and *b* are 60 bp and 16 bp (*AluI*), 54 bp and 22 bp (*HpyCH4V*), and 41 bp and 34 bp (*MnlI*), respectively.

ssDNA substrates [40], we incorporated the recognition sequence for this enzyme into the substrate oligonucleotides beforehand. Incubation of ligation samples of oligonucleotide **I** with *HhaI* and subsequent PCR amplification, however, still resulted in significant

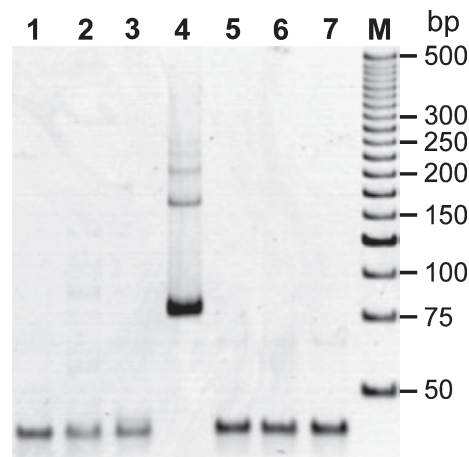


Fig. 4. Investigation of ssDNA ligation activity of different DNA ligases. Oligonucleotide **I** (10 nM) was incubated with ligase, cleaved by *HhaI*, and subjected to PCR amplification. Ligases were T4 DNA ligase, *E. coli* DNA ligase, *Taq* DNA ligase, and Ampligase (lanes 4–7), respectively. Lanes 1–3 are controls in the absence of both **I** and T4 DNA ligase, and in the absence of either T4 DNA ligase or **I**, respectively.

quantities of lower-oligomeric products besides the desired 76-bp-long amplicon, suggesting incomplete digestion of the ssDNA template by the restriction enzyme (not shown). Thus, to render all circularized **I** linear, we hybridized oligonucleotide **H17** to the DNA segment of **I** encompassing the recognition sequence of *HhaI* before restriction digestion. With this modification, essentially a single PCR amplicon was obtained (Fig. 5, lanes 3–5), allowing us to proceed with qcPCR to determine the efficacy of the template-independent ligation reactions.

To estimate the ligation yield we chose to use qcPCR [41]. This method is relatively simple and results in reliable quantitation of target samples when certain prerequisites are met, of which equal amplification efficiency of target and competitor and avoidance of heteroduplexes during amplification are the most crucial [41–44]. As competitor, we used oligonucleotide **C63** which was identical in sequence with circularized and *HhaI*-cleaved oligonucleotide **I** except for two

Table 2. Ligation yields of various substrates as determined by qcPCR. Values are means \pm S.D. from triplicate determinations. n.d., Not determined.

Substrate concentration (nM)	Ligation yields with substrate					
	I	II	III	II/IV	II/V	II/IV/V
0.1	$(1.9 \pm 0.1) \times 10^{-5}$	$(1.8 \pm 0.1) \times 10^{-6}$	$(3.0 \pm 0.2) \times 10^{-6}$	$(1.2 \pm 0.1) \times 10^{-4}$	n.d.	$(3.7 \pm 0.3) \times 10^{-2}$
1	$(3.7 \pm 0.2) \times 10^{-5}$	$(4.6 \pm 0.4) \times 10^{-6}$	$(6.1 \pm 0.3) \times 10^{-6}$	$(3.0 \pm 0.5) \times 10^{-4}$	$(2.5 \pm 0.5) \times 10^{-6}$	$(5.9 \pm 1.0) \times 10^{-2}$
10	$(1.1 \pm 0.1) \times 10^{-4}$	$(1.5 \pm 0.2) \times 10^{-5}$	$(2.6 \pm 0.4) \times 10^{-5}$	$(1.4 \pm 0.3) \times 10^{-3}$	$(2.4 \pm 0.2) \times 10^{-5}$	n.d.

small deletions outside the primer-binding sites (Table 1). The use of primer pair **P1/P2** and a constant input of ligation sample with increasing input of competitor at PCR resulted in typical qPCR gel patterns with two product bands (Fig. 6A). Bands of intermediate mobility representing a heteroduplex were either completely absent or barely detectable and thus could be neglected. Data were analyzed by plotting the logarithm of the product ratio of the standard to the target against the logarithm of the quantity of the competitor added (Fig. 6B), from which the amount of initial target template was derived [41,44]. In all qPCR experiments performed, the data points obtained were lying on a straight line ($r^2 > 0.98$) with a slope close to 1, so that equal amplification of target and competitor can readily be assumed [42]. In support of this assumption, qPCR experiments carried out with dilutions (up to 50-fold) of a ligation sample resulted in calculated x coordinate values at the equivalence point which differed exactly by the logarithm of the dilution factor.

Yields of ligation were determined for samples in which the ligation reaction had been performed at an oligonucleotide concentration of 0.1 nM, 1 nM, or 10 nM. Within this concentration range, the calculated ligation yield of oligonucleotide **I** increased about six-

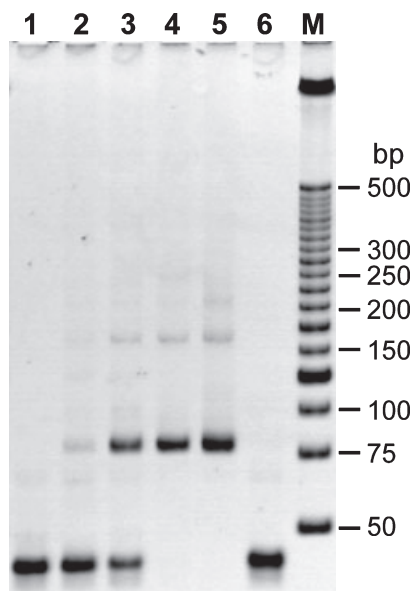


Fig. 5. Analysis of PCR products obtained with oligonucleotide **I** after incubation with T4 DNA ligase and cleavage by *HhaI*. Concentrations of oligonucleotide **I** at ligation were 0.01 nM, 0.1 nM, 1 nM, or 10 nM (lanes 2–5), respectively. Lanes 1 and 6 are controls in the absence of **I** or with 10 nM of **I** in the absence of ligase, respectively.

fold, from 1.9×10^{-5} at 0.1 nM to 1.1×10^{-4} at 10 nM (Table 2). Ligation yields with oligonucleotides **II** and **III** were slightly lower than the yields obtained with oligonucleotide **I** (Table 2). We conclude that there is no significant sequence preference of ssDNA ligation catalyzed by T4 DNA ligase.

Determination of the ligation yield of substrates containing short, blunt-ended dsDNA at one terminus or at both termini

To investigate the dependence of the efficiency of non-templated DNA ligation on the strandedness of the DNA substrate at the ligation point, we determined

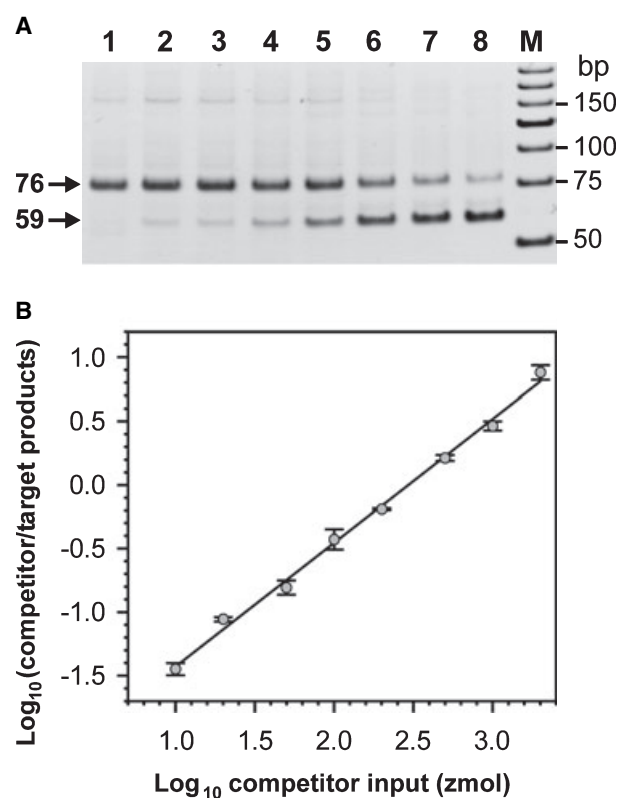


Fig. 6. Determination of ligation yields by qPCR. (A) Co-amplification of 8 fmol **I**, which had previously been incubated with T4 DNA ligase and cleaved by *HhaI*, with serial amounts of competitor C63. Ligated **I** leads to an amplicon 76 bp in length (upper bands), whereas the competitor results in a product 59 bp in length (lower bands). Each amplicon contains one centrally located A_6 tract leading to some gel retardation in comparison with the mobility of the DNA size marker. Amounts of competitor added to each reaction (lanes 1–8) were 10, 20, 50, 100, 200, 500, 1000 and 2000 zfmol, respectively. (B) Double logarithmic plot of the ratio of competitor/target products as a function of competitor input. The standard curve was generated by linear regression of data points from three independent experiments yielding $y = 0.97x - 2.40$ ($r^2 = 0.996$).

ligation yields for substrates **II/IV** and **II/V**, each consisting of one ssDNA and one blunt-ended dsDNA terminus, and substrate **II/IV/V**, consisting of two blunt-ended dsDNA termini. To ensure that only the 5'-phosphate and 3'-hydroxy groups of **II** participated in the ligation, thus avoiding unwanted ligation products, oligonucleotide **IV** was tagged with a biotin moiety at its 3' end and oligonucleotide **V** lacked a 5'-phosphate (Table 1). The lengths of both oligonucleotides (15 nucleotides) were chosen to ascertain the presence of stable duplexes during ligation and to avoid their hybridization to **II** during subsequent PCR amplification reactions. As shown in Table 2, the yield of dsDNA–ssDNA ligation was about 70–90 times higher than ssDNA ligation, when the DNA duplex was located at the donor site, i.e. the 5'-phosphoryl terminus (substrate **II/IV**). In contrast, no elevated yield in comparison with the ssDNA ligation was observed, when the acceptor was composed of a blunt-ended duplex (substrate **II/V**). For the substrate with two blunt-ended dsDNA termini (**II/IV/V**), the ligation yield was about four orders of magnitude higher than with the single-stranded substrate **II**.

Discussion

We show here that T4 DNA ligase is capable of ssDNA ligation, although with low efficiency. Such an activity has not been previously reported for this enzyme. In contrast, it has been repeatedly stated that this enzyme lacks any activity on ssDNA substrates [39,45,46]. Our findings, however, do not contradict the data on which these previous statements were based, because the efficiency of ssDNA ligation we report here is below the sensitivity of the direct detection methods used previously. Our data are supported by findings of Shiba *et al.* [47], who obtained additional products after PCR amplification of template-directed ligation products obtained with ssDNA strands after incubation with T4 DNA ligase. They hypothesized that these products may result from template-independent ligation, but did not provide any evidence for this hypothesis.

The ssDNA ligation observed must be an inherent property of T4 DNA ligase and not due to the presence of other enzymes or components in the ligation mixture, because batches of this enzyme from different suppliers produced essentially the same results. As the specific source and purification of this enzyme varies with each supplier, it is highly unlikely that all batches of T4 DNA ligase we used would contain the same contaminant in a similar quantity and/or with similar activity that would lead to our experimental data.

Because of the design of the ssDNA substrates, it is also unlikely that any short segment in the oligonucleotides that we used served intramolecularly or intermolecularly as a bridging splint for a template-directed ligation reaction. In fact, oligonucleotides **I** and **II** do not even contain a dinucleotide sequence complementary to the two terminal nucleotides that are joined, let alone a longer sequence that could form a stable duplex consisting of matched and mismatched base pairs at the ligation point. Because **I** and **II** differ completely in the sequence of their terminal segments, whereas the remainder of the sequence is identical, the presence of unusual but stable duplexes containing non-Watson-Crick base pairs can also be excluded.

It should be noted that under typical assay conditions (i.e. in the absence of macromolecular crowding agents), only T4 DNA ligase catalyzes the joining of blunt-ended dsDNA with a detectable efficiency [20,48,49]. So far, however, little is known about the actual mechanism of this template-independent dsDNA ligation. Rossi *et al.* [50] proposed a general model of T4 DNA ligase activity which involves two different protein–DNA complexes with substrates containing nicked or blunt-ended dsDNA. According to this model, the adenylated enzyme first scans dsDNA for substrates through successive transient complexes. When a 5'-phosphate group is encountered, the AMP moiety is transferred from the enzyme to the DNA and the deadenylated enzyme stalls on it in a stable complex until a suitable 3'-hydroxy end becomes available to complete the ligation reaction.

Our data with the four substrates **II**, **II/IV**, **II/V** and **II/IV/V**, which differ in the strandedness of the terminal DNA segments involved in ligation, give rise to the following conclusions about the nontemplated ligation reaction catalyzed by T4 DNA ligase: (a) the enzyme has a considerably higher affinity for a donor site comprising dsDNA than one comprising ssDNA; (b) if the donor is single-stranded, the strandedness of the acceptor plays no role in the ligation reaction; and (c) when both donor and acceptor are composed of blunt-ended dsDNA, the acceptor appears to be a cofactor for the ligation reaction. The last conclusion is of most interest. We hypothesize that T4 DNA ligase forms a more stable complex with both duplex termini than with the complex of the enzyme with the duplex donor itself, thus mediating juxtaposition of 5'-phosphoryl and 3'-hydroxy termini. Consistent with the model of Rossi *et al.* [50], the donor may already be activated before such complex formation. Of course, in contrast with the ligations performed in our study, regular blunt-end ligation requires the formation of two phosphodiester bonds, as each of the two termini

serves as both donor and acceptor. The nonlinear dependence of blunt-end ligation on the T4 DNA ligase concentration and the stimulation of blunt-end ligation by T4 RNA ligase led to the conclusion that two ligase molecules are involved in blunt end joining [51]. According to our results, the previously raised possibility of co-operation of two ligase molecules, one to hold the termini in juxtaposition and one to catalyze the phosphodiester bond formation [51], however, appears to be less likely and not required for blunt-end ligation. With regard to ssDNA ligation, random fluctuation of the flexible oligonucleotide chain probably accounts for juxtaposition of donor and acceptor groups.

The intramolecular ligation of partially double-stranded DNA substrates by T4 DNA ligase has been previously demonstrated [25]. In contrast with our design, however, the substrates used did not have blunt-ended acceptor or donor groups, excluding quantitative comparison with our data. Nevertheless, in agreement with our results, Western & Rose [25] observed a significantly higher ligation yield for a substrate with a double-stranded donor than for the corresponding substrate containing a single-stranded donor group.

The occurrence of template-independent ssDNA ligation events may lead to nonspecific signals or false positives in diagnostic assays that rely on target-dependent or template-dependent ligation followed by an amplification method, so that the accuracy of the results, especially at low concentration of template, may be severely compromised. A number of such ligase-based approaches of various formats, involving linear or circularized probes, have been described [9,11,52–54]. One recent example is the LigAmp assay for the detection of single-base mutations [31]. Although the specificity of this assay is quite high, nonspecific signals were observed with this method in some experiments. In fact, Shi *et al.* [31] point out the possibility that these signals may have arisen from template-independent oligonucleotide ligation and emphasize the need to identify the sources contributing to the nonspecific signals. Besides for diagnostic methodologies, high reliability of template-directed DNA ligation is imperative in DNA computation [15]. Thus, in certain applications, it is necessary that signals resulting from unwanted ligation events, such as template-dependent dsDNA ligation of substrates containing one or more mismatches and template-independent ligation, can be clearly distinguished from those arising from the correct template-dependent ligation. Alternatively, the formation of unwanted ligation products should be minimized as much as possible or even completely suppressed. Our data

indicate that template-independent ligation products may be avoided by using ligases such as *E. coli* DNA ligase, *Taq* DNA ligase or Ampligase.

Finally, we should mention that our data were obtained in conditions under which ligation reactions are generally performed. In some analytical detection methods using T4 DNA ligase, different reaction conditions (e.g. elevated temperature, different concentrations of ATP and/or salt) have been reported [26,32,55,56]. Further studies are thus warranted to investigate how the template-independent ligation reaction we report here is affected by differences in reaction conditions.

Experimental procedures

Materials

All oligodeoxyribonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). DNA concentrations were determined spectrophotometrically at 260 nm using the absorption coefficients provided by the supplier. Oligonucleotides **I–III** were obtained chemically 5'-phosphorylated and PAGE-purified, and oligonucleotides **IV** and **V** were purchased HPLC-purified (see Table 1 for sequences). Using mfold [57], **I–III** were designed not to form any stable secondary structure, especially at the point of ligation. For instance, oligonucleotide **I** carries three consecutive thymines at both termini, but contains only single adenines, separated by three or more nucleotides, in the remainder of its sequence. In addition, several four-base recognition sequences for restriction endonucleases and suitable sequences for PCR amplification were incorporated into oligonucleotides **I–III**. Enzymes were purchased from New England Biolabs (Beverly, MA, USA) except Ampligase, which was obtained from Epicentre (Madison, WI, USA). In some experiments, T4 DNA ligase from Invitrogen (Carlsbad, CA, USA) or from Fermentas (Hanover, MD, USA) was used.

DNA ligation

Substrates containing short dsDNA at one or both termini (**II/IV**, **II/V** and **II/IV/V**) were prepared before ligation by heating the corresponding oligonucleotides (1 μ M each) in 20 μ L annealing buffer consisting of 10 mM Tris/HCl (pH 7.4 at 25 °C), 0.1 mM EDTA and 100 mM NaCl at 90 °C for 90 s, followed by cooling to 10 °C at a rate of 1 °C per min. Ligation reactions on DNA oligomers **I–III** or complexes **II/IV**, **II/V** and **II/IV/V** were performed for 2 h in 100 μ L reaction volumes containing 1 \times the ligation buffer provided by the supplier for the corresponding ligase, 0.1–10 nM substrate, and 10 U DNA ligase (Weiss units in the case of T4 DNA ligase) at 16 °C (T4 DNA ligase and *E.coli* DNA ligase) or 45 °C (*Taq* DNA ligase and

Ampligase). The specific 1 × ligation buffers used for ligation reactions with T4 DNA ligase were as follows: 50 mM Tris/HCl (pH 7.5 at 25 °C), 10 mM MgCl₂, 1 mM ATP, 10 mM dithiothreitol, 25 µg·mL⁻¹ BSA (New England Biolabs); 40 mM Tris/HCl (pH 7.8 at 25 °C), 10 mM MgCl₂, 0.5 mM ATP, 10 mM dithiothreitol (Fermentas); and 50 mM Tris/HCl (pH 7.6 at 25 °C), 10 mM MgCl₂, 1 mM ATP, 1 mM dithiothreitol, 5% (w/v) poly(ethylene glycol) 8000 (Invitrogen). After ligation, samples were isolated by a standard procedure, i.e. purified by phenol and chloroform extraction, precipitated by the addition of 2 vol. cold ethanol and centrifugation, and dissolved in buffer containing 10 mM Tris/HCl (pH 7.4) and 0.1 mM EDTA. Samples were then either subjected to PCR (or RCA) or cleaved by *HhaI* restriction endonuclease. To perform the restriction digestion, first two equivalents of oligonucleotide **H17** were added to the ligation samples in 195 µL buffer containing 50 mM potassium acetate, 20 mM Tris/acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.9 at 25 °C (1 × NEBuffer 4). The mixture was heated at 90 °C for 1 min, followed by cooling to 10 °C at a rate of 1 °C per min. Subsequently, 2 µL 100 µg·mL⁻¹ BSA and 3 µL *HhaI* restriction endonuclease (20 U·µL⁻¹) were added, and the samples incubated at 37 °C for 16 h, followed by incubation at 65 °C for 20 min. Samples were then isolated as described above.

PCR

Reactions were performed in 1 × ThermoPol buffer (New England Biolabs) containing 200 µM each dNTP, 0.5 µM each primers **P1** and **P2**, 2 µL ligation sample (uncleaved or *HhaI*-cleaved), and 0.02 U·µL⁻¹ *Taq* DNA polymerase. In qPCR experiments, 2 µL of a standard solution of oligonucleotide **C63** were also added to each tube. Amplification was typically carried out with an initial denaturation step at 94 °C for 60 s, followed by 37 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The last cycle was followed by an extension step at 72 °C for 2 min.

RCA

Reactions were performed in 35 µL volume containing 20 mM Tris/HCl (pH 8.8 at 25 °C), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2.5 mM MgSO₄, 0.1% Triton X-100, 1 mM each dNTP, 0.4 µM each primers **P1** and **P2**, 2 µL ligation sample, and 10 U *Bst* DNA polymerase. Amplification was carried out at 60 °C for 90 min.

Analysis of amplicons

Amplicons and their respective restriction digests were resolved by 12% nondenaturing PAGE [29 : 1 (w/w)

acrylamide/bis-acrylamide], run for 2–3 h (12.5 V·cm⁻¹) in 1 × TBE buffer (90 mM Tris/borate, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, illuminated at 302 nm, and scanned with a CCD camera. PCR products were quantified using the IS-1000 digital imaging system (Alpha Innotech Corporation, San Leandro, CA, USA). To compare molar amounts of products in qPCR experiments, the integrated peak areas of the 59-bp-long band originating from amplification of competitor oligonucleotide **C63** were corrected by a factor corresponding to the ratio of the fragment lengths of target to competitor PCR products [58].

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Analysis of PCR amplicons obtained with oligonucleotide **I** after incubation with T4 DNA ligase from other vendors.