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# Making AppDNA using T4 DNA ligase

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**Abstract**

5′,5′-Adenylyl pyrophosphoryl DNA (AppDNA) contains a high-energy pyrophosphate linkage and can be exploited as an activated DNA substrate to derive new DNA enzymes for carrying out various DNA modification reactions. For this reason, enzymatic synthesis of AppDNA is highly desirable. AppDNA is a known intermediate in DNA ligase mediated DNA ligation reactions, but rarely accumulates under normal reaction conditions. Here we report that T4 DNA ligase can quantitatively convert 5′-phosphoryl DNA donor into AppDNA in the absence of acceptor DNA but in the presence of a template DNA that contains at least one unpaired nucleotide opposite the 5′-phosphoryl DNA donor site. This adenylylation behavior of T4 DNA ligase is not observed with *Thermus aquaticus* (*Taq*) and *Escherichia coli* DNA ligases. We further found that a donor-template duplex of 11-bp in length is required by T4 DNA ligase for the formation of AppDNA.


*Keywords:* T4 DNA ligase; DNA adenylylation; AppDNA; DNA ligation; DNA modification; Deoxyribozymes

## 1. Introduction

Although DNA is best known for its double-helical character and its universal role as the genetic material, it can also form complex tertiary structures and perform

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enzymatic function in single-stranded form [1,2]. To date, over 100 DNA sequences have been reported for facilitating many different types of chemical transformations [3–12] (for recent reviews, see [11,13–15]). In spite of having very limited chemical functionalities, deoxyribozymes that perform catalysis with surprising proficiency have been described in a number of studies. For example, a small DNA enzyme known as 10–23 performs site-specific RNA cleavage with a very impressive $k_{cat}$ of $\sim$10 $min^{-1}$ [7]. The lack of a 2′-hydroxyl group in DNA that exists in RNA does not appear to render a detrimental effect to catalytic performance. Furthermore, the catalytic capabilities of DNA can be enhanced through the use of metal ions [16] and small-molecule cofactors [17] as well as through the modification of aromatic bases with chemical functionalities useful for catalysis [18]. Compared to ribozymes, deoxyribozymes are easier to prepare, and more resistant to chemical and enzymatic degradation; therefore, they can be quite useful in a variety of biotechnological applications. The RNA-cleaving DNA enzyme 10–23 and its derivatives have been used to cleave various RNA molecules both in vivo and in vitro (for a recent review, see [19]).

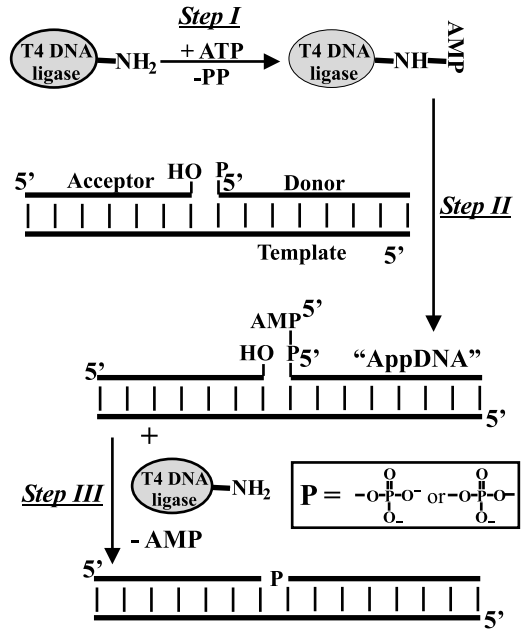
Considerable efforts have been made to create DNA enzymes to promote self-processing reactions and to catalyze various modification reactions on separate DNA or RNA substrates. These reactions are of particular interest to those who seek new means to manipulate nucleic acids and to create catalytic DNA systems for DNA-related applications [1,2,12,13]. DNA enzymes have been made for DNA phosphorylation [8], DNA capping [9], and DNA ligation [11]. The first DNA enzyme of the list was created to mimic the function of polynucleotide kinase and the last two were made to perform similar reactions catalyzed by T4 DNA ligases. The successful creation of these deoxyribozymes suggests that DNA-modifying DNA enzymes can be created relatively easily by in vitro selection.

We are interested in creating more DNA enzymes that can process DNA molecules that contain 5′-5′-adenylyl pyrophosphate structure unit (which is referred to as AppDNA in this report). Since AppDNA contains a high-energy pyrophosphate linkage, they can be exploited as an activated DNA substrate to derive new deoxyribozymes (or ribozymes) for various DNA modifications including DNA ligation, circularization, and DNA coupling to peptides or proteins. Efficient DNA enzymes performing these functions are highly desirable because they can be used to build unique catalytic systems for practical applications. To create these DNA enzymes, however, we need to find ways to generate AppDNA in large quantities. One way to make AppDNA is through chemical synthesis by coupling adenosine 5′-phosphorimidazolide and 5′-phosphorylated oligonucleotide [20–22]. Since adenosine 5′-phosphorimidazolide is not very stable in aqueous solution, it usually has to be used at high concentrations. In addition, the chemical method does not give rise to the quantitative conversion from 5′-phosphorylated DNA to AppDNA. An alternative way to make AppDNA is through enzymatic synthesis. For instance, a deoxyribozyme has been described for DNA-adenylylation [9]. However, it can only be used to perform self-capping reaction on a specific DNA oligonucleotide and cannot be used to generate the App cap on generic oligonucleotides. Since AppDNA is used as an intermediate by all DNA ligases in DNA ligation reaction [23,24], it might be possible to find ways to use DNA ligases to produce AppDNA molecules.

All DNA ligases and RNA ligases are known to use a common, three-step mechanism for DNA ligation, which is shown in Scheme 1 using ATP-dependent T4 DNA ligase as an example [23]. In step I, the DNA ligase acquires the adenylyl group (AMP) from ATP and places it on the ε-amino group of an internal lysine residue. The ligase-adenylylate covalent complex is formed with the release of pyrophosphate. In step II, the newly adenylylated ligase transfers its adenylyl group to the 5′-phosphate of the donor DNA to produce AppDNA. In step III, the ligase catalyzes the nucleophilic attack by the 3′-hydroxyl group of the acceptor DNA on the second phosphate group of the App unit and concludes the DNA ligation with the concomitant release of AMP.

AppDNA does not normally accumulate in a DNA ligation reaction. Under some controlled conditions, however, this intermediate can be obtained in small amounts. For example, AppDNA was observed when nicked DNA substrates were briefly treated with excess *Escherichia coli* DNA ligase at 0 °C [25] or with purified T4 ligase-adenylylate complex at pH 5.6 and 0 °C [26]. AppDNA intermediates were also found in T4 DNA ligase-mediated blunt-end DNA ligation reaction [27] and in a solution containing supercoiled circular DNA, large amounts of *E. coli* DNA ligase and excess AMP [28].

Utilizing DNA ligases to make AppDNA in relatively large quantity has been described in two instances. Shuman [29] reported that when a DNA substrate containing 1-nt gap was incubated with excess *Vaccinia* virus DNA ligase, nearly half




Scheme 1. Catalytic mechanism by T4 DNA ligase. T4 DNA ligase (as well as other DNA ligases) uses a three-step mechanism for DNA ligation. Ligase-AMP covalent complex is formed in step I, 5′,5′-adenylyl pyrophosphoryl DNA (AppDNA) is formed in step II, and 3′,5′-phosphodiester bond is formed in step III. P stands for a phosphate unit.

the DNA substrate was converted into AppDNA. Another successful approach by Modrich and Lehman [30] used 2′,3′-dideoxy-terminated acceptor to force *E. coli* DNA ligase to produce AppDNA. Since the modified DNA substrate does not have the ligation-essential 3′-hydroxyl group, it is impossible for the DNA ligase to proceed beyond the DNA-adenylylation step, leading to the accumulation of large amounts of AppDNA intermediate.

We speculate that DNA ligases might also be able to generate AppDNA when the acceptor strand is omitted completely from the ligation mixture. We found that T4 DNA ligase can indeed quantitatively adenylylate any 5′-phosphoryl DNA in the absence of an acceptor DNA but in the presence of a DNA template that contains at least one unpaired nucleotide at its 3′ end. We further found that *Thermus aquaticus* (*Taq*) and *E. coli* DNA ligases did not have the same capability. Since acceptor-free adenylynation has not been documented for any DNA ligase and this approach represents the simplest way to make AppDNA by enzymatic manipulation, we have conducted a detailed characterization of this unique adenylylation activity of T4 DNA ligase.

## 2. Materials and methods

*Materials.* Standard DNA oligonucleotides as well as 3′-phosphorylated DNA, 2′,3′-dideoxy-terminated DNA and 2′-hydroxyl-3′-deoxy-terminated DNA were prepared by standard automated chemical synthesis (Keck Biotechnology Resource Laboratory, Yale University). All synthetic DNA oligonucleotides were purified by 10% preparative denaturing polyacrylamide gel electrophoresis (PAGE) containing 7 M urea, followed by elution and ethanol precipitation. Purified oligonucleotides were dissolved in water and their concentrations were determined using standard spectroscopic method. Nucleoside 5′-triphosphates and [$\gamma$-$^{32}$P]ATP were purchased from Amersham Pharmacia. T4 DNA ligase and T4 polynucleotide kinase (PNK) were purchased from MBI Fermentas; *E. coli* and *Taq* DNA ligases were purchased from New England Biolabs. All other chemical reagents were purchased from Sigma.

*DNA phosphorylation using PNK and [$\gamma$-$^{32}$P]ATP.* Donor DNAs were labeled at the 5′-terminus with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase (T4 PNK) using manufacturer-supplied protocols. T4 PNK-mediated end-labeling solution containing 2.5 μCi/μl [$\gamma$-$^{32}$P]ATP was further incubated with 1 mM ATP for 30 min at 37 °C to insure high-yield phosphorylation. The above-phosphorylated DNA was purified by 10% preparative denaturing PAGE and quantitated by determining the absorbance at 260 nm. The concentrations were calculated using Biopolymer Calculator program (it can be accessed at http://paris.chem.yale.edu/extinct.frames.html).

*DNA adenylylation reactions.* Unless stated otherwise, DNA adenylylation reaction conditions were as follows: The final concentration of each DNA oligonucleotide was 0.2 μM. DNA mixture containing donor and template DNA oligonucleotides (as well as acceptor DNA if applicable) was heated in water to 90 °C for 1 min, cooled to room temperature, and combined with 1/10th volume of 10× ligase buffer. The 10× ligase buffer contained 500 mM tris(hydroxylmethyl)aminomethane (Tris; pH 7.8 at 23 °C), 200 mM sodium chloride, 100 mM magnesium

chloride, 10 mg/ml bovine serum albumin (BSA), 5 mM adenosine 5′-triphosphate (ATP). T4 DNA ligase was added to the solution to a final concentration of 0.1 Weiss U/μl [31] and the reaction mixture was incubated at 37 °C for 16 h. Aliquots were removed from the reaction mixture at various times and quenched by mixing with equal volume of 40 mM disodium ethylenediaminetetraacetate ($Na_2EDTA$; pH 8.0 at 23 °C). The mixture was then combined with gel loading buffer, heated at 90 °C for 3 min. The adenylylated DNA and unreacted DNA substrate were separated by 10% denaturing PAGE and quantitated using a Molecular Dynamics Storm 820 Phosphorimager with ImageQuant software. Each experiment was carried out in duplicate or in triplicate and the data typically exhibited less than 15% variation.

## 3. Results

### 3.1. Different ways to produce AppDNA using T4 DNA ligase

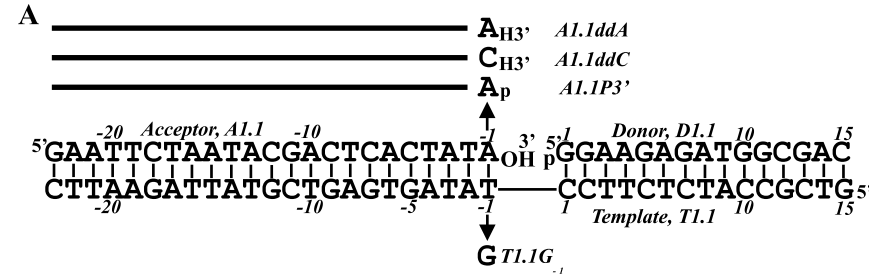
According to the three-step mechanism depicted in Scheme 1, any strategy that permits the first two steps but obstructs the third step should cause the accumulation of AppDNA. The simplest approach is to use an acceptor DNA in which the 3′-hydroxyl group is eliminated or altered. Thus we tested several modified acceptor DNA molecules to determine whether T4 DNA ligase is capable of making AppDNA in large quantity in the presence of these acceptor molecules.

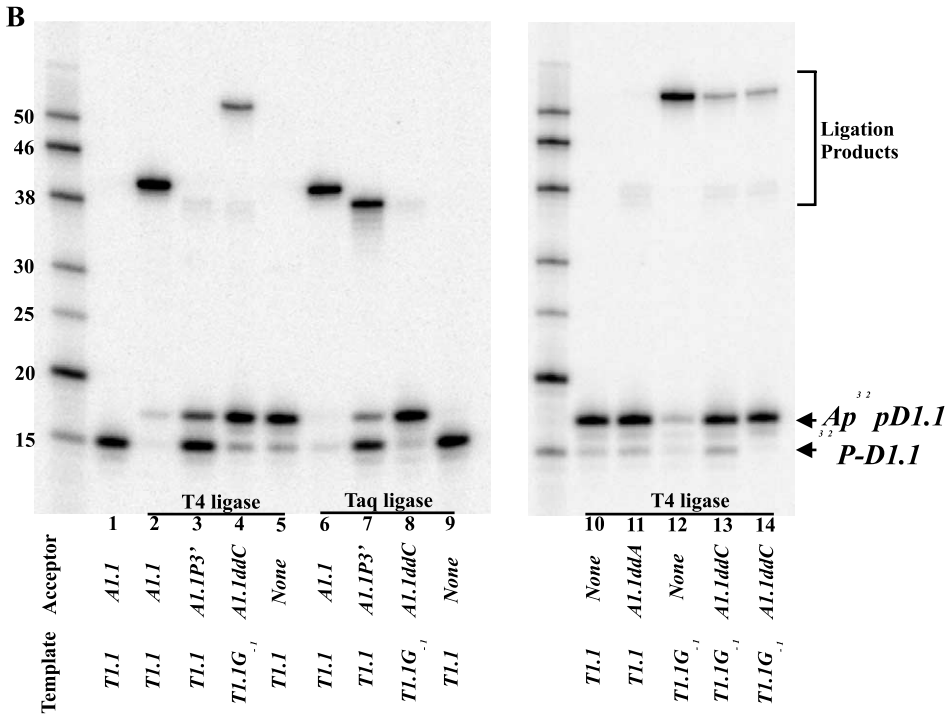
The main nicked duplex, shown in Fig. 1A, was made of 23-nucleotide (nt) A1.1, 15-nt D1.1, and 38-nt T1.1. When equimolar A1.1, T1.1, and 5′-$^{32}$P-labeled D1.1 were annealed and treated with T4 DNA ligase (lane 2, Fig. 1B), *Thermus aquaticus* (*Taq*) DNA ligase (lane 6) and *E. coli* DNA ligase (data not shown), 5′-$^{32}$P-labeled

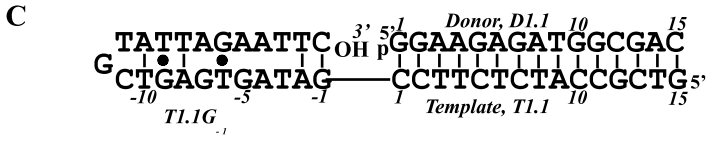
Fig. 1. Examining adenylylation activities of T4 DNA ligase and *Taq* DNA ligase with various DNA constructs. (A) DNA constructs used for the study. For clarity, nucleotides in the donor (D1.1), acceptor (A1.1), and template (T1.1) oligonucleotides are numbered as follows: Nucleotides of D1.1 (15 nt) are numbered from 1 to 15 in the 5′–3′ direction, A1.1 (23 nt) is labeled −1 to −23 in the 3′–5′ direction, while each nucleotide in T1.1 is given the same number as its base-paring partner in D1.1 or A1.1. (B) PAGE analysis of various DNA oligonucleotide mixtures incubated with T4 DNA ligase or *Taq* DNA ligase. The far left lane of each gel is a home-made single-stranded DNA ladder consisting of seven 5′-$^{32}$P-labeled synthetic oligonucleotides with their indicated sizes. *Ligases* – lane 1: no ligase; lanes 2–5 and 10–14: T4 DNA ligase; lanes 6–9: Taq DNA ligase. *Donor* – D1.1, 5′-$^{32}$P-labeled: all lanes. *Templates* – lanes 1–3, 5–7, 9–11: T1.1; lanes 4, 8, 12–14: T1.1G$_{-1}$ (T1.1 with T–G substitution at −1 position). *Acceptor* – lanes 1–2, 6: A1.1; lanes 3 and 7: A1.1P3′; lanes 4, 8, 13–14: A1.1ddC (A1.1 with 2′,3′-dideoxyC substitution at −1 position); lane 11: A1.1ddA; lanes 5, 9, 10, 12: no acceptor. All DNA concentrations were 0.2 μM while ligase concentration was 0.1 U (Weiss)/μl. All the samples were incubated at 37 °C for 16 h. The reactions were stopped by the addition of EDTA to a final concentration of 15 mM and the reaction products were directly analyzed on 10% PAGE. Lane 14 is identical to lane 13 except that DNA in the later was treated with alkaline phosphatase after T4 ligase treatment. (C) A putative structure formed by T1.1G$_{-1}$ which makes its 3′-hydroxyl available for ligation to D1.1.

donor DNA D1.1 was successfully ligated to non-radioactive acceptor DNA A1.1. As a result, 38-nt DNA band was observed.

Our first approach was to use a 3′-phosphate on the acceptor DNA to block the DNA ligation. When the 3′-hydroxyl group of A1.1 was replaced by the 3′-phosphate (A1.1P3′, Fig. 1A), AppDNA was found at 28% and 12% in the reaction mixtures containing T4 DNA ligase (lane 3 in Fig. 1B, 16-h incubation at 37 °C) and *Taq* ligase (lane 7, 16-h incubation at 50 °C), respectively. Extending the reaction



time did not significantly increase the adenylylation yield (data not shown). These results suggest that 3′-phosphorylated acceptor cannot force DNA ligases to produce AppDNA in high yields.

It is interesting to note that an abnormal ligation product was also observed at ~40% in the *Taq* ligase mediated reaction. The DNA appeared to be one nucleotide shorter than the normal ligation product. It is known that DNA ligases possess gap-ligating activity [32,33]. We speculated that the formation of the above DNA might be due to the deletion of the terminal nucleotide from 3′-phosphorylated A1.1, followed by gap ligation by *Taq* DNA ligase. The speculated nucleotide deletion may be caused by *Taq* DNA ligase itself or by some contaminating nucleases in the commercial enzyme (our preliminary assessment indicated that this activity might be associated with *Taq* DNA ligase itself). Although it was interesting, the issue was not pursued in this study.

Modrich and Lehman have shown that a dideoxy-terminated DNA acceptor can cause the accumulation of AppDNA in *E. coli* DNA ligase-catalyzed DNA ligation reactions [30]. Dideoxy-terminated DNA acceptors also have been used to make AppDNA with *S. cerevisiae* DNA ligase Cdc9p [34]. We repeated the same approach with both T4 and *Taq* DNA ligases. A modified A1.1, A1.1ddC, was chemically synthesized that contained a terminal 2′,3′-dideoxyC (ddC was chosen due to the availability of the amidite for automated DNA synthesis). A new template molecule $T1.1G_{-1}$ was also made, which is the modified version of T1.1 with a T–G mutation at the −1 position to match the A–C change in the acceptor sequence (see Fig. 1A). When A1.1ddC was annealed with $T1.1G_{-1}$ and 5′-$^{32}$P-labeled D1.1 and treated with T4 DNA ligase (lane 4, Fig. 1B) or *Taq* DNA ligase (lane 8, Fig. 1B), 72% and 90% of AppDNA was observed, respectively, following a 16-h incubation at either 37 °C (T4 ligase) or 50 °C (Taq ligase). Thus similar to the results obtained using *E. coli* DNA ligase [30] and *S. cerevisiae* DNA ligase Cdc9p [34], AppDNA also can be obtained in high yields with T4 and *Taq* DNA ligases when a dideoxy-terminated acceptor is used.

Interestingly, another DNA product was also observed at 18% in the T4 DNA ligase-mediated reaction (lane 4, Fig. 1B). It appeared to be more than 50-nt long. Since we noticed that $T_{-22}$ and $C_{-23}$ of $T1.1G_{-1}$ could form intramolecular base pairs with $A_{-2}$ and $G_{-1}$ (Fig. 1C), we speculated that this product was due to the direct ligation of D1.1 and $T1.1G_{-1}$. Such an arrangement may make D1.1-$T1.1G_{-1}$ a suitable substrate for DNA ligation. The projected length of the ligated DNA product is 53 nucleotides. Several experiments were conducted to test this speculation. First, when D1.1 and $T1.1G_{-1}$ were incubated in the absence of A1.1ddC, the yield of this DNA product was expected to increase because no A1.1ddC was present to disrupt the formation of the speculated two internal base-pairs. When treated with T4 DNA ligase, 5′-$^{32}$P-labeled D1.1 was indeed converted into the ligated DNA quantitatively in the presence of $T1.1G_{-1}$ (lane 12). Second, the increase of A1.1ddC concentration was expected to inhibit the internal base-pair formation and therefore decrease the ligation yield. When the ratio of D1.1:$T1.1G_{-1}$:A1.1ddC was changed from 1:1:1 (lane 4) to 1:1:5 (lane 13), the ligation percentage indeed dropped from 18% to 4%. The third evidence came from the experiment using D1.1, T1.1, and 2′,3′-dideoxyA-terminated A1.1 (A1.1ddA; Fig. 1A). Primer extension was used to make this DNA oligonucleotide because direct chemical synthesis was not possible.

The corresponding 22-nt oligonucleotide (i.e., A1.1 lacking the 3′-terminal A) was extended by a reverse transcriptase (SuperScript II from Gibco/BRL) to incorporate the required dideoxyA at the 3′-end in the presence of 2′,3′-dideoxyadenosine 5′-triphosphate (ddATP) and T1.1 as the template. When 5′-$^{32}$P-labeled D1.1 was annealed with T1.1 and A1.1ddA and then incubated with T4 DNA ligase and ATP (lane 11), no ligation product was observed. Lastly, the treatment of the ligation solution in lane 13 with alkaline phosphatase did not result in the loss of radioactivity in either the adenylylation product or the suspected ligation product (lane 14), which indicated that the originally exposed 5′-$^{32}$P was now recessive in both the adenylylation product and the suspected ligated DNA. In comparison, the trace amount of the radioactivity seen with the remaining 5′-$^{32}$P-labeled D1.1 (lane 13) was lost (lane 14). The data were consistent with our speculation that the 2-bp interaction residing in T1.1G$_{-1}$ resulted in the unexpected DNA ligation (Fig. 1C). T4 DNA ligase is known to be capable of ligating nicked DNA duplexes in which the acceptor DNA contains only six nucleotides that pair with the DNA template [31]. It was quite surprising to find that T4 DNA ligase could even ligate an internal duplex that has only two Watson–Crick base pairs next to the ligation junction.

Finally, we tested the ability of T4, *Taq*, and *E. coli* DNA ligases to adenylylate donor DNA in the presence of template DNA but in the absence of acceptor DNA. When the DNA mixture containing 5′-$^{32}$P-labeled D1.1 and T1.1 was incubated with T4 DNA ligase and ATP, more than 90% AppD1.1 was produced (lane 5, Fig. 1B) following a 16-h incubation at 37 °C. However, when the same DNA mixture was treated with *Taq* DNA ligase (lane 9) or *E. coli* DNA ligase (data not shown), no AppDNA was produced, indicating that T4 DNA ligase may act quite differently on DNA substrate than *Taq* and *E. coli* DNA ligases.

The existence of the "Ap-p" linkage in AppDNA was confirmed by two experiments. First, AppDNA is known to be resistant to the degradation by alkaline phosphatase [35]. When AppDNA produced with $^{32}$p-D1.1 was treated with alkaline phosphatase, the radioactivity was not lost (see lane 14). Second, AppDNA is known to be the substrate of deadenylylated ligases in the absence of ATP [9,23,30]. When mixed with A1.1 and T1.1 in the presence of deadenylylated T4 DNA ligase (deadenylylated using the method described in [9]) but *in the absence of ATP*, purified AppD1.1 (the AppDNA produced with D1.1) was converted into 38-nt DNA product (data not shown). In a control experiment, no 38-nt ligated DNA product was observed when we mixed 5′-$^{32}$P-labeled D1.1, A1.1, and T1.1 with the T4 DNA ligase under same conditions.

### *3.2. Necessity of 3′-overhanging nucleotides in template DNA*

The DNA adenylylation activity exhibited by T4 DNA ligase towards 15-nt D1.1 was observed in the presence of 38-nt T1.1, an oligonucleotide that binds D1.1 to form a DNA duplex containing a 23-nt overhanging (or protruding) single-stranded sequence (from position −1 to position −23). Deletion of this entire single-stranded segment resulted in various blunt-end ligation products, along with small amount of AppDNA (data not shown). This observation is consistent with what is known as blunt-end ligation activity of T4 DNA ligase [27]. Therefore, the existence of

overhanging nucleotides at the 3′-end of template DNA is essential to halting T4 DNA ligase at the DNA adenylylation step.

We tested a series of T1.1 derivatives carrying different sizes of overhanging nucleotides to determine the minimal number of overhanging nucleotides that are required for the production of AppDNA (Fig. 2A). The four new oligonucleotides,

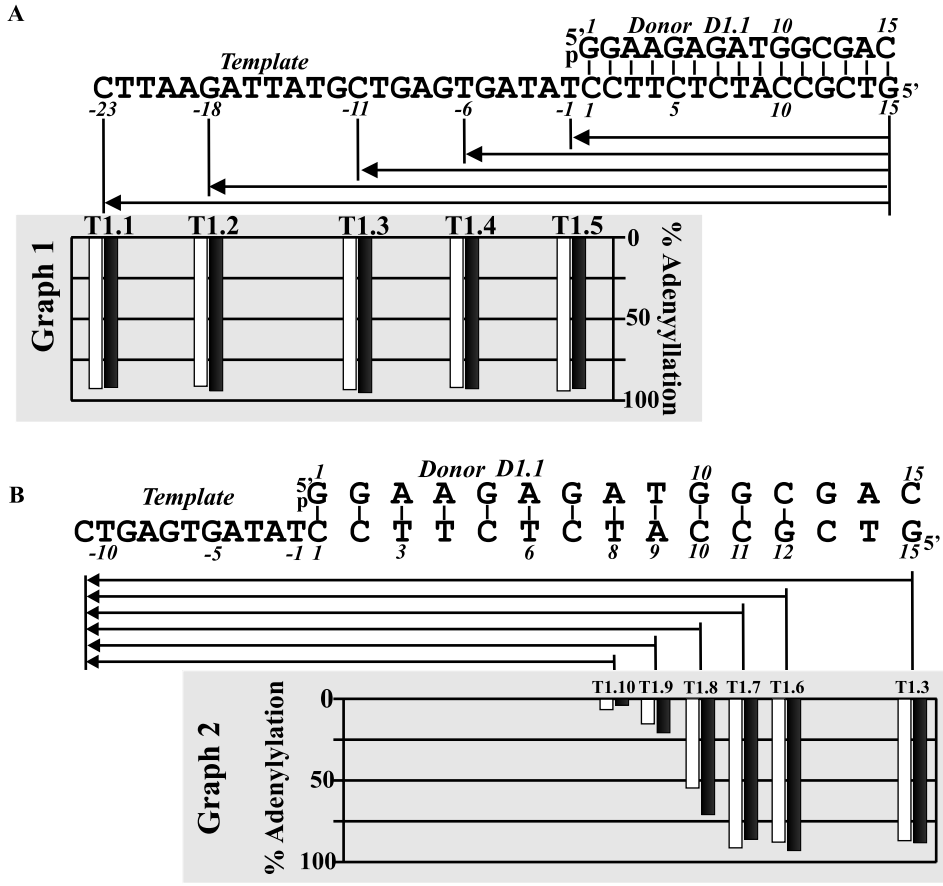



Fig. 2. Sequence requirement by T4 DNA ligase for DNA adenylylation activity. (A) Adenylylation activity of T4 DNA ligase toward protruding duplexes formed between D1.1 and 3′ deletion sequences of T1.1. T1.2, T1.3, T1.4, and T1.5 are the four deletion sequences with nucleotides trimmed from 3′ end beyond $G_{-18}$, $C_{-11}$, $T_{-6}$, and $T_{-1}$, respectively. The *y*-axis (downward direction) shows % adenylylation. (B) Adenylylation or ligation activity of T4 DNA ligase toward duplexes formed between D1.1 and various 5′ deletion sequences of T1.3. Five deletion sequences were used that correspond to T1.3 with nucleotides trimmed from 5′ end beyond $G_{12}$ (T1.6), $C_{11}$ (T1.7), $C_{10}$ (T1.8), $A_9$ (T1.9), and $T_8$ (T1.10). The DNA adenylylation conversion was assessed in the absence of an acceptor DNA (Graph 2). White and black bars are the results from two independent experiments and the *y*-axis in each graph is plotted in downward fashion. Reaction conditions: All DNA concentrations were 0.2 μM. Ligase concentration was 0.1 U (Weiss)/μl. All the samples were incubated at 37 °C for 16 h.

T1.2–T1.5, are T1.1 deletion mutants trimmed at 3′-end beyond $G_{-18}$, $C_{-11}$, $T_{-6}$, and $T_{-1}$, respectively. The ability of these five oligonucleotides as template DNAs to support T4 DNA ligase mediated adenylylation activity on D1.1 was found to be essentially equal (see the embedded Graph 1 in Fig. 2A, duplicates are shown as black and white bars). Therefore, it was apparent that T4 DNA ligase can adenylylate 5′-phosphorylated donor DNA as long as at least one protruding nucleotide exists at the 3′-end of the template oligonucleotide.

### 3.3. Minimal base pairs for DNA adenylylation

Several 5′-deletion mutants of T1.3 were synthesized which carried nucleotide deletions from 5′-end beyond G12 (T1.6), C11 (T1.7), C10 (T1.8), A9 (T1.9), and T8 (T1.10), respectively (Fig. 2B). The DNA adenylylation activity of T4 DNA ligase towards 5′-phosphorylated D1.1 in the presence of these template DNAs was determined and the percentage of adenylylation is shown in Graph 2 of Fig. 2B (data from two independent experiments are shown as white or black bars). Our data indicated that the DNA substrate had to contain at least 11 bp for T4 DNA ligase to render robust adenylylation activity. The DNA substrate containing 10 bp (i.e., duplex made of D1.1 and T1.8) supported the adenylylation at ~60%, but DNA templates that contain 9 or less nucleotides complementary to D1.1 failed to support the substantial level of DNA adenylylation by T4 DNA ligase.

### 3.4. Mismatch tolerance

T4 DNA ligase is known to be capable of accepting base-pair mismatches within DNA duplexes for DNA ligation [34,36–39]. We tested the tolerance for mismatches within overhanging DNA duplexes for DNA adenylylation activity. Several D1.1 derivatives were synthesized, which carried mutations at one, two, or three locations within the original 15-nt D1.1 (Fig. 4). The modified acceptor molecules were assessed individually for DNA adenylylation by T4 DNA ligase with T1.3 as the DNA template (Fig. 3).

When the 5′-terminal G1 of D1.1 was replaced by T1 (D1.2), C1 (D1.3), or A1 (D1.4), AppDNA was observed at 83%, 74%, and 60%, respectively following a 16-h incubation with T4 DNA ligase, ATP, and T1.3 (under the same conditions, 94% of AppDNA was found in the reaction solution containing D1.1). The yield was decreasing in a manner sensitive to base identities. Thus, correct base pairing at the reaction center appeared to be beneficial but not essential for adenylylation.

The mismatch at the second nucleotide at the 5′-end of the donor D1.1 (D1.5; G2–C2) had much more effect on the DNA adenylylation as only 29% of AppDNA was produced under the assay conditions. The adenylylation yields started to recover when the single mismatch was made further down the donor DNA chain, and AppDNA was produced at 62% and 89% for the mismatch at the third (D1.6; A3–T3) and fourth (D1.7; A4–T4) positions, respectively. Single mutations made at the fifth position (D1.8; G5–C5) or beyond (e.g., D1.9; G11–C11) did not affect DNA adenylylation yield.

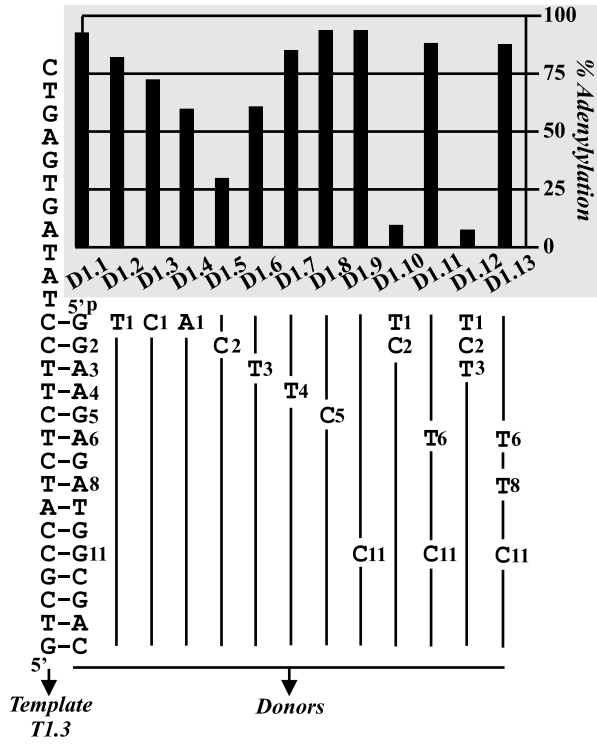



Fig. 3. Adenylylation activity of T4 DNA ligase toward duplexes containing various mismatch base-pairs. DNA adenylylation activity was determined using T4 DNA ligase and protruding duplexes either containing single mismatch base-pair at 5′-end (D1.2, D1.3, and D1.4, where original G1 was mutated to T, C, and A, respectively), near 5′-end (D1.5, G2–C2; D1.6, A3–T3), and at internal positions (D1.7, A4–T4; D1.8, G5–C5; D1.9, G11–C11), containing double mismatches at 5′-end (D1.10, G1G2–T1C2) and at internal positions (D1.11, A6 and G11–T6 and C11), and containing triple mismatches at 5′-end (D1.12, G1G2A3–T1C2T3) and at internal positions (D1.13, A6, A8 and G11–T6, T8, and C11). Adenylylation yield was plotted. Reaction conditions: DNA concentrations were 0.2 μM, ligase concentration was 0.1 U (Weiss)/μl. All the samples were incubated at 37 °C for 16 h. Solid lines indicate no changes of mutated sequences relative to D1.1.

Double mismatches made at the 5′-end of the donor DNA dramatically lessened DNA adenylylation level. AppDNA was observed only at 10% for D1.10 (G1–T1, G2–C2). On the other hand, mismatches at two separate internal locations five bases away from the reaction center (e.g., D1.11; A6–T6, G11–C11) had no effect on DNA adenylylation. As expected, triple mutations at the 5′-end of A1.1 had very severe effect on DNA adenylylation and only 6% AppDNA was seen with D1.12 (G1–T1, G2–C2, and A3–T3). However, mutations made at three internal locations away from the 5′-end of the donor did not significantly reduce adenylylation.

### 3.5. Optimal reaction conditions

We examined the influence of temperature, pH, and the concentrations of ATP, magnesium and T4 DNA ligase on the DNA adenylylation. The results are given in Fig. 4. D1.1 and T1.3 were used as the donor and template DNA in all the experiments.

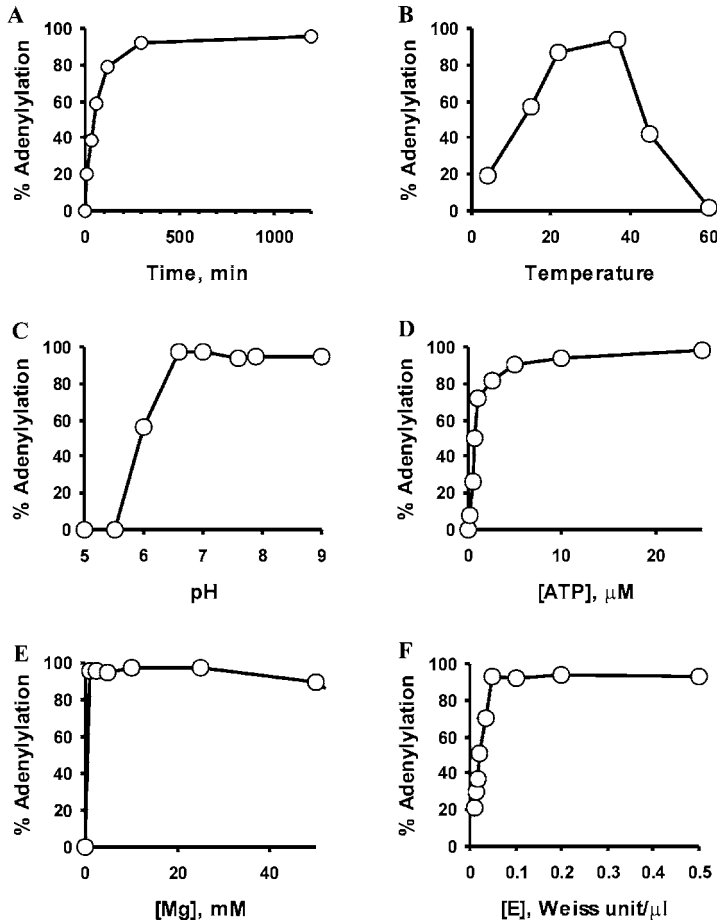



Fig. 4. Examination of adenylylation conditions. Adenylylation conditions were examined using the duplex of D1.1–T1.3. The adenylylation yields were determined as function of incubation time (A), temperature (B), pH (C), ATP concentration (D), $MgCl_2$ concentration (E), and T4 DNA ligase concentration (F). All data points are the average of triplicates. The general reaction conditions were as follows if not otherwise indicated in the plots: all DNA concentrations were 0.2 μM while ligase concentration was 0.1 U (Weiss)/μl. ATP and $MgCl_2$ concentrations were 0.5 and 10 mM, respectively, with incubation being carried out for 16 h at pH 7.8 and 37 °C. The reactions were stopped by the addition of EDTA to a final concentration surpassing $MgCl_2$ concentration.

DNA adenylylation reached more than 90% after 5-h incubation and further increasing the reaction time only slightly enhanced the reaction yield (Fig. 4A). The optimal temperature was found to be 37 °C, although reaction temperature of 23 °C also resulted in the formation of AppDNA at nearly 90%. Increasing the incubation temperature beyond 37 °C decreased the yield. When temperature reached 60 °C, the adenylylation activity of T4 DNA ligase was essentially abolished (Fig. 4B).

No AppDNA was observed at pH 5.0 and 5.5. At pH 6.0, ~50% of the donor DNA D1.1 was converted into its AppDNA. The maximum yield of DNA adenylylation was reached at pH 7.0 and was sustained two pH units above pH 7.0 (Fig. 4C). A very low concentration of ATP was required for full adenylylation activity (Fig. 4D). We observed that divalent metal ions were also essential for the acceptor-free DNA adenylylation mediated T4 DNA ligase. When magnesium was used, as little as 1 mM was sufficient to support full adenylylation activity of T4 DNA ligase (Fig. 4E). Other metal ions including calcium and manganese were found equally effective (data not shown).

We also assessed the relationship between the adenylylation yield of phosphorylated D1.1 and T4 DNA ligase concentration. When pre-annealed D1.1–T1.3 overhanging DNA duplex at 0.2 μM was incubated with various concentrations (in Weiss units) of T4 DNA ligase under our standard assay conditions, we observed a relationship shown in Fig. 4F. The percentage conversion increased linearly with the increase of the concentration of T4 DNA ligase in the range of 0.01–0.05 U/μl, with the maximum conversion being reached at 0.05 U/μl. Further increasing the enzyme concentration did not result in more AppDNA.

Using the data in Fig. 4F, we calculated that T4 DNA ligase only has a catalytic efficiency of $5 \times 10^{-3}$ pmol $min^{-1}$ $U^{-1}$ for the above DNA adenylylation reaction. In comparison, this enzyme has a ligation efficiency of ~3 pmol $min^{-1}$ $U^{-1}$ determined when the acceptor DNA A1.1 was present. Therefore, T4 DNA ligase has about 500-fold reduced catalytic performance in the acceptor-free system for the forced DNA adenylylation as compared to the normal DNA ligation.

### *3.6. DNA concentrations*

The experiments described thus far were all performed at 0.2 μM of relevant DNA duplexes formed between equimolar donor and template DNAs. To determine whether the yield of DNA adenylylation is affected by DNA concentration, D1.1–T1.3 duplex concentrations were varied from 0.05 to 1 μM. It is evident from Fig. 5A that the adenylylation yield was not affected by DNA concentration when T4 DNA ligase was in excess.

The ratio of the donor (D) to template (T) was also changed by holding the donor concentration at 0.2 μM and varying the template concentration between 0.1 and 10 μM (Fig. 6B). Providing excess template had no effect on DNA adenylylation until the template concentration was too high (more than 20-fold excess). At 10 μM (T:D at 50), the template DNA had reduced the yield of DNA adenylylation. We speculated that the inhibition may be due to the non-specific interaction between T4 DNA ligase and single stranded template DNA at high concentrations, which may prevent the DNA duplex from freely accessing the active site of T4 DNA ligase for binding and for the subsequent adenylylation.

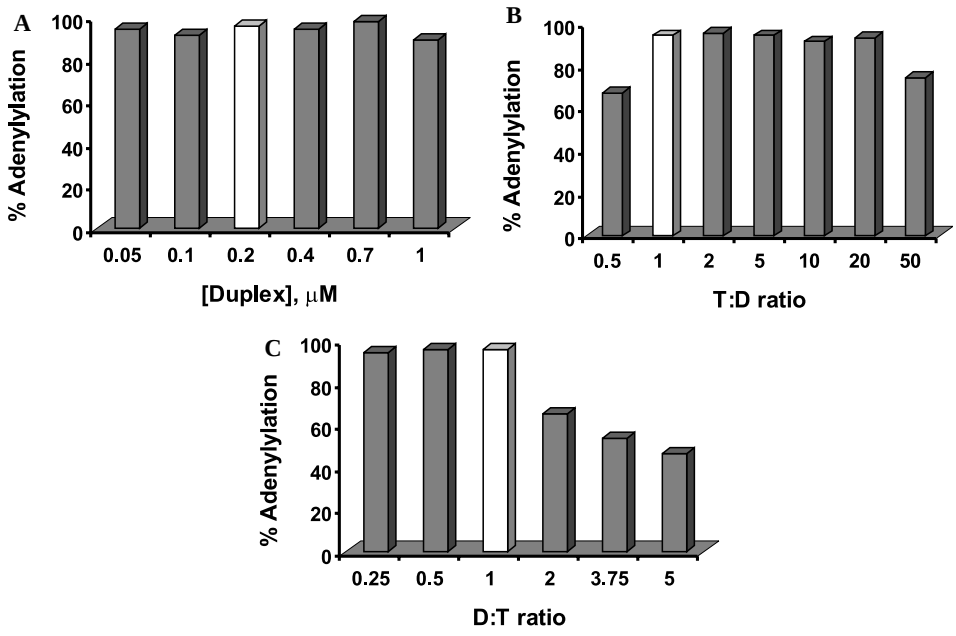
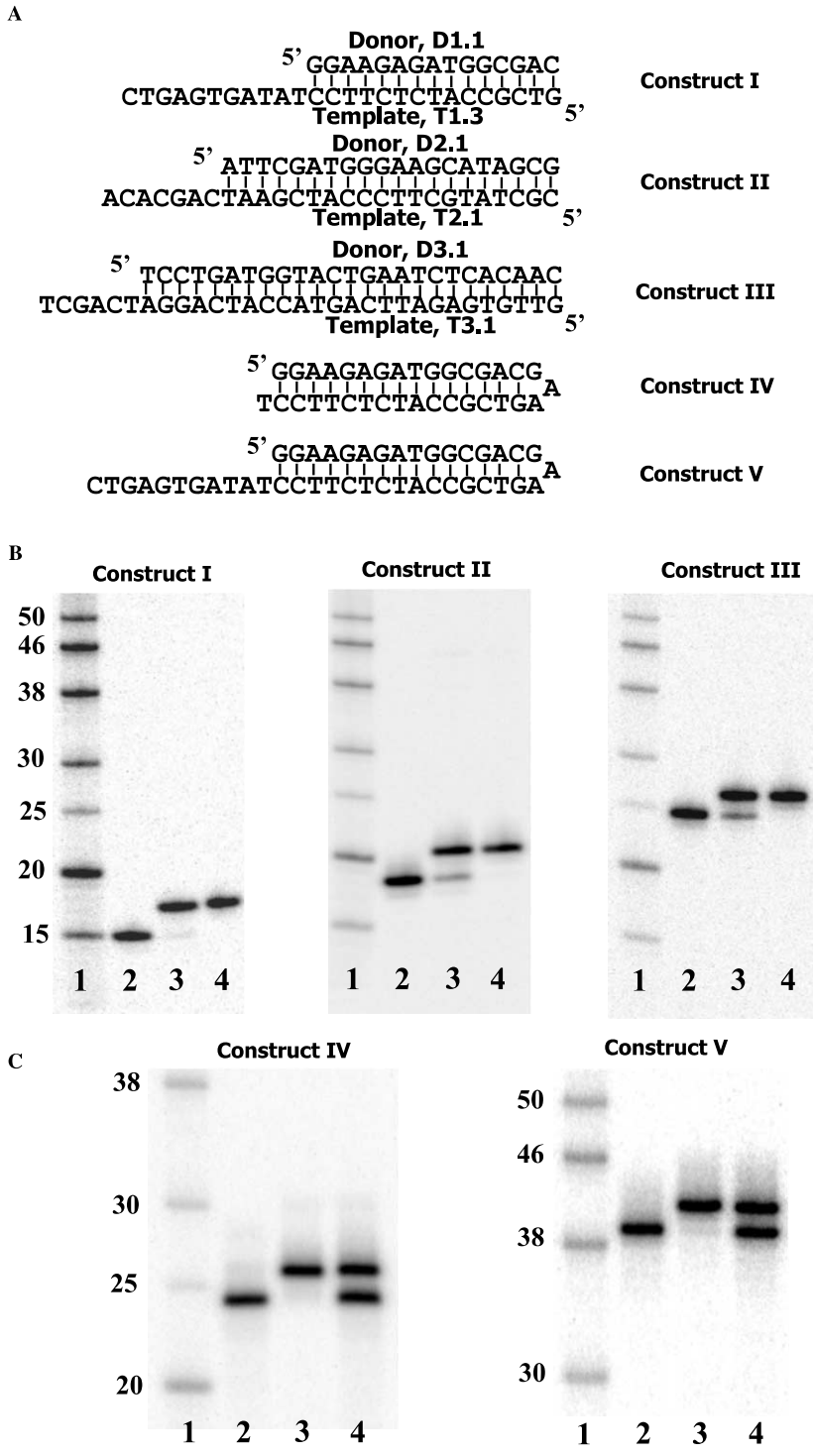



Fig. 5. Relationship between adenylylation yield and DNA concentration. Adenylylation yields were determined using the duplex of D1.1–T1.3. All data points are the average of triplicates. (A) Adenylylation yield vs. T1.3–D1.1 duplex concentration in the range of 0.05–1 μM. (B) Adenylylation yield vs. the ratio of template (T) to donor (D). The ratio was changed by holding the donor concentration at 0.2 μM and varying the template concentration between 0.1 and 10 μM. (C). Adenylylation yield vs. the ratio of donor to template. The template DNA concentration was constant at 0.2 μM and the donor DNA concentration was varied between 0.05 and 2 μM. Ligase concentration was 0.1 U (Weiss)/μl. All the samples were incubated at 37 °C for 16 h at pH 7.8, with ATP and $MgCl_2$ concentrations at 0.5 and 10 mM, respectively.

Fig. 5C shows the data obtained with the constant template DNA concentration (0.2 μM) and the donor DNA concentration being varied between 0.05 and 2 μM (D:T ratio at 0.25–5 as shown). When D:T ratio was 0.25, 0.5, and 1, quantitative adenylylation conversion was observed because all the donor DNA molecules existed in the duplex form. When the donor concentration was twice as much as that of the template (D:T = 2), in theory only half the donor molecules were in the duplex form and therefore can be adenylylated by T4 DNA ligase. Under our experimental conditions, 68% AppDNA was observed. When D:T ratio was increased further to 3.75 and 5, 27% and 20% AppDNA was expected to form by theoretical calculation, respectively; 56% and 48% AppDNA was observed. We presume that the donor molecules within the DNA duplex could undergo exchange with the uncomplexed donors in solution. Higher donor concentrations will likely bring about more such exchanges, thereby resulting in more AppDNA. This explained the higher AppDNA conversion than expected when excess donor DNA molecules were in solution.


A
Donor, D1.1
5' GGAAGAGATGGCGAC
CTGAGTGATATCCTTCTCTACCGCTG 5'
Template, T1.3
Construct I
Donor, D2.1
5' ATTCGATGGGAAGCATAGCG
ACACGACTAAGCTACCCTTCGTATCGC 5'
Template, T2.1
Construct II
Donor, D3.1
5' TCCTGATGGTACTGAATCTCACAAC
TCGACTAGGACTACCATGACTTAGAGTGTTG 5'
Template, T3.1
Construct III
5' GGAAGAGATGGCGACG A
TCCTTCTCTACCGCTGA
Construct IV
5' GGAAGAGATGGCGACG A
CTGAGTGATATCCTTCTCTACCGCTGA
Construct V
B
Construct I
Construct II
Construct III
50
46
38
30
25
20
15
1 2 3 4
C
Construct IV
Construct V
38
30
25
20
50
46
38
30
1 2 3 4

### 3.7. Generic DNA adenylylation

The main DNA duplex used thus far consisted of a 15-nt donor DNA beginning with a G-residue and template DNA that provided 11-nt overhanging fragment (Construct I in Fig. 6A). To demonstrate that the results obtained above were independent of oligonucleotide sequences, four more constructs were tested (Fig. 6A). Construct II consists of a donor DNA that has 20 nucleotides starting with an A-residue and a 27-nt template affording 7-nt overhanging single-stranded domain. Construct III has six protruding nucleotides next to 25-bp region and the adenylylation center has a T-residue. Construct IV and V are two intramolecular versions of construct I where the donor and the template sequences reside within the same DNA molecule. Construct IV has one overhanging nucleotide while construct V has 11-nt unpaired nucleotides.

The adenylylation reactivity of these constructs was assessed under our standard assay conditions (Figs. 6B and C). For constructs I, II, and III, lane 2 shows the results from a reaction that contained the $^{32}$P-phosphorylated donor DNA and non-radioactive template DNA in the absence of T4 DNA ligase. Lane 3 resulted from a reaction that contained the same DNA mixture but treated with T4 DNA ligase for adenylylation. Lane 4 is the alkaline digestion sample using the mixture from lane 3 (Fig. 6B). It was evident that all the DNA donors have been successfully adenylylated by T4 DNA ligase. For intramolecular constructs IV and V, lanes 2 and 3 show the relevant DNAs treated without and with T4 DNA ligase, and lane 4 is an equal mixture of solutions from lanes 2 and 3 (Fig. 6C) to show that AppDNA and unadenylylated DNA can be resolved on the gel. The data demonstrated that T4 DNA ligase was capable of adenylylating donor DNA in our acceptor-free system in a manner independent of the donor sequence identity.

## 4. Discussion

Elimination of the ligation-essential 3′-hydroxyl from the acceptor DNA without changing other chemical moieties on the last nucleotide in the acceptor DNA was first used by Modrich and Lehman to produce AppDNA with *E. coli* DNA ligase [30]. Dideoxy-terminated DNA acceptor also has been used to make AppDNA using

Fig. 6. DNA adenylylation with different DNA molecules. (A) Sequences of five different protruding DNA duplex constructs tested for DNA adenylylation. (B) PAGE analysis for constructs I–III. Lane 1: a home-made DNA ladder containing oligonucleotides with sizes indicated; lane 2: 5′-$^{32}$P-phosphorylated donor DNA and non-radioactive template DNA in the absence of T4 DNA ligase; lane 3: the same DNA mixture treated with T4 DNA ligase; lane 4: the alkaline digestion of the mixture from lane 3. (C) PAGE analysis for constructs IV and V: Lanes 2 and 3 are 5′-$^{32}$P-phosphorylated DNA without and with T4 DNA ligase treatment, respectively; lane 4 is an equal mixture of solutions taken from lanes 2 and 3. Reaction conditions: all DNA concentrations were 0.2 μM while ligase concentration was 0.1 U (Weiss)/μl. All the samples were incubated at 37 °C for 16 h.

*S. cerevisiae* DNA ligase Cdc9p [34]. We confirmed that both T4 and *Taq* DNA ligases can successfully produce AppDNA with the dideoxy-terminated acceptor as well. It is interesting to note that dideoxy-terminated acceptor cannot cause the accumulation of AppDNA by human DNA ligases I and II [40] and by *Chlorella* virus DNA ligase [41], suggesting that each DNA ligase can be quite distinct in substrate recognition and catalysis.

In this study, more acceptor DNA molecules were tested for facilitating the DNA adenylylation by T4 DNA ligase. The acceptors examined include DNA molecules ending with a 3′-phosphate, a 2′-hydroxyl-3′-deoxy-terminus (data not shown), or one or more unpaired nucleotides (data not shown). T4 DNA ligase appeared to be capable of making some AppDNA when each of these DNAs was used as the acceptor but failed to produce AppDNA in high yields. Quite surprisingly, we found that when the acceptor DNA was taken out of the reaction mixture (therefore the DNA template contains a short unpaired, single-stranded region), T4 DNA ligase can quantitatively convert the phosphorylated DNA donor into AppDNA. We found that the number of the unpaired nucleotides can be chosen at random without affecting the DNA adenylylation yield. Even with a single overhanging nucleotide, the template can still support robust adenylylation activity. Therefore, the existence of a short single-stranded region on the template next to the reaction center is essential for the DNA adenylylation. In sharp contrast, both *E. coli* ligase and *Taq* ligase do not have the same ability. It remains to be determined whether this unusual adenylylating behavior is the sole property of T4 DNA ligase.

The method that we described in this report uses widely accessible T4 DNA ligase and the acceptor-free duplex DNA system to modify any DNA oligonucleotide with an App structure in high yields and at low cost. The facile way of generating the high-energy linkage at the 5′ end of DNA now permits the exploration of creating various deoxyribozymes that catalyze various nucleophilic reactions involving activated AppDNA as a substrate. In addition, since AppDNA is a key intermediate in the ligation cascade catalyzed by all DNA ligases, it may find use in the efforts of determining crystal and solution structures of various DNA ligases bound to DNA containing this key intermediate.

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