Published online July 19, 2004

Nucleic Acids Research, 2004, Vol. 32, No. 13 ***e107***
doi:10.1093/nar/gnh103

Protein–DNA footprinting by endcapped duplex oligodeoxyribonucleotides

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Received April 21, 2004; Revised and Accepted June 25, 2004

ABSTRACT

Oligodeoxyribonucleotides (5'-phosphorylated) of varying lengths were capped using a polyamide linker to form thermodynamically stable, endcapped DNA duplexes containing 8–14 bp. We have employed these endcapped DNA duplexes as tools to determine the DNA footprint of T4 DNA ligase. By high-performance liquid chromatography and PAGE analysis of the ligation mixtures of the endcapped DNA duplexes, we have found that by varying the lengths and the position of the nick, we can determine the minimal DNA-binding site as well as the mode of binding (symmetrical or asymmetrical binding) by the enzyme. The results of the study revealed that a 11 bp endcapped duplex was the shortest duplex effectively ligated. Dependence of ligation efficiency on nick position demonstrates that T4 DNA ligase bound asymmetrically to its DNA substrate. The use of a set of thermodynamically stable endcapped deoxyribonucleoside duplexes as a tool to elucidate the DNA footprint provides an efficient strategy for footprinting, which avoids ambiguities associated with chemical and biochemical footprinting methods.

INTRODUCTION

Blunt-end double-stranded oligodeoxyribonucleotides covalently linked at both ends have been investigated as models for natural double-stranded DNA (dsDNA). Placing crosslinks (or endcaps) at each end allows one to construct very short duplexes that if not endcapped would melt and exist as separate single strands in aqueous solution at room temperature. The most common crosslinker, hexaethylene glycol, can be incorporated into oligonucleotides as a phosphoramidite using standard synthetic protocols. For example, Kozerski *et al*. (1) prepared duplex **A** shown in Figure 1 as a model for nicked dsDNA. They used NMR (E.COSY, TOCSY, NOESY and HMQC) to obtain parameters for modeling to show that the duplex assumed typical B-form geometry and that the endcaps did not distort the DNA. The presence of the endcaps allowed these researchers to use a model for nicked DNA containing relatively few base pairs, which made it possible to discern the local geometry about the nick site.

In the same way, Junker *et al*. (2) constructed a doubly endcapped duplex containing a nick terminating in a 3′-phosphoglycolate and a 5′-phosphate. Here, the use of a relatively short sequence stabilized as a duplex by the endcaps allowed the investigators to use NMR to determine the local structure at the lesion and establish that the 3′-phosphoglycolate and the 5′-phosphate were extra-helical.

Prior to the introduction of endcaps, such as the hexaethylene glycol moiety, the most commonly used model for short segments of dsDNA were DNA dumbbells, which contained nucleotide containing loops at each end of the duplex region. For example, dumbbell DNA has been used to study helix–coil transitions. Tying the ends together by loops eliminates the end fraying effects as well as any conformational heterogeneity caused by monomer–dimer equilibrium associated with normal linear DNA duplexes (3,4). However, the use of nucleotide containing loops to close the ends of duplex DNA has a number of disadvantages. In the case of the NMR structural studies, elimination of extraneous nucleotides simplifies the spectra and consequently interpretation of the spectra. Other uses for dumbbell containing oligonucleotides might also benefit from replacement of the loops with simple inert spacers. For example, there is a significant interest in the double-stranded oligonucleotides as therapeutic agents (5–7), where elimination of the loops could potentially be advantageous by eliminating regions that might affect binding to the target protein or by conferring enhanced nuclease stability.

In the present study, our goal was to compare the ability of dsDNA sequences of different lengths to function as substrates for DNA ligase. The option of not linking the ends was untenable because the shorter sequences (e.g. 4mer and 5mer on either side of a nick) would be thermodynamically unstable at the temperature of the ligation. In their analysis of the DNA-joining activity of the Chlorella virus ligase, Odell *et al*. (8) suggested that the low efficiency of ligation of a 4mer was due to the inability of short sequences (4mer) to form a stable hybrid with a 30mer tailed-hairpin strand. Nakatani *et al*. (9) also reported that the formation of a nicked duplex DNA substrate is temperature-dependent and is essential for recognition and ligation by Lig_{Tk}. Such short sequences are essential if one is to determine the limits of the protein–DNA footprint. The use of nucleotide loops to achieve the requisite duplex stability is also unattractive because these loops contain nucleotides that could significantly influence the binding either by direct interaction with the protein or by perturbing

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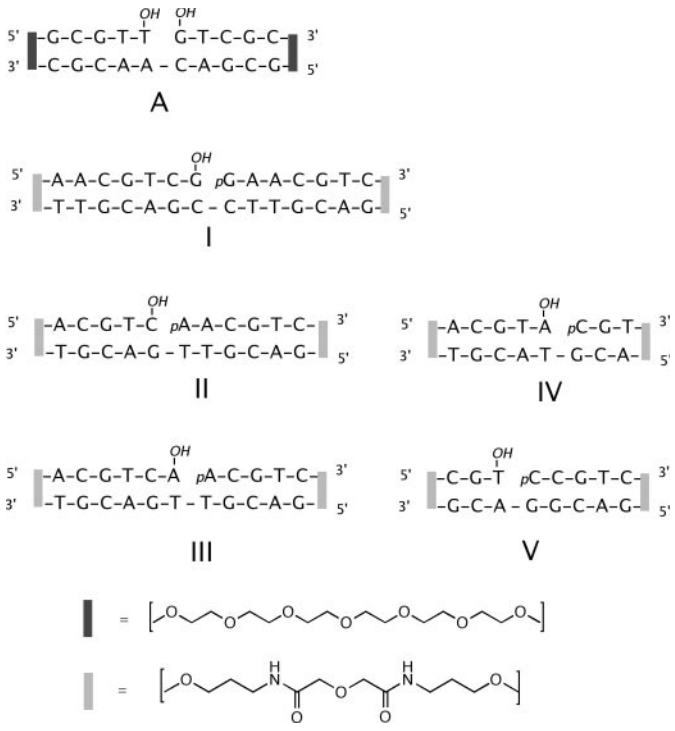

Figure 1. Endcapped nicked DNA duplexes A and I–V.

the ends of the helix. Based on the results of the structural studies with hexaethylene glycol-terminated duplexes, the use of inert endcapped oligonucleotides seem to be a much more reasonable model.

In addition to hexaethylene glycol (10), several other types of non-nucleotide linkers, including triethylene glycol (11), disulfide bridges (12), phosphorothioates (13) and various aromatic moieties (14,15) have been used in the synthesis of closed, non-nucleotide bridged DNA duplexes. However, in conjunction with studies to develop amide-containing endcaps for use in the DNA–protein complex crystallization studies we have adopted a simple hydrophilic amide containing endcap for the present study. The chemical synthesis of this endcap has been reported (16).

Studies of protein–nucleic acid interactions frequently begin with gel-shift assays followed by footprinting studies to establish affinity and to map the bound sequence. Both techniques have proven exceedingly useful, but there are limitations. Some nucleic acid-binding proteins may bind too weakly for effective gel-shift assays. Furthermore, conditions and buffers required for an optimal gel-shift assay may not match those required for maximum binding. In the case of nuclease footprinting, the reagent may be blocked from access to the nucleic acid at sites that are not bound but in close proximity to the protein. In the case of chemical footprinting, the reagents could adversely affect the interaction between the nucleic acid and the protein leading to a reaction at sites normally in contact. These and other limitations have extensively been discussed for the 'gold standard' hydroxyl radical footprinting (17).

In theory, it seems reasonable that endcaps could be used to construct a series of duplexes of varying lengths to probe the outer extremes of a dsDNA-binding site. Too short of sequences would bind less effectively, which should be measurable as either a decreased binding constant or by decrease in the rate or extent of a reaction. There are a number of published studies that demonstrate that endcaps do not adversely affect the binding of nucleic acids as long as the natural recognition sequence is contained in the duplex (18,19).

As a model, we have chosen to study the binding site of T4 DNA ligase since it is widely used and commercially available. We believe that the efficiencies of ligation of these endcapped DNA duplexes would allow us to determine the DNA footprint of the enzyme, which is the shortest endcapped DNA duplex that can be ligated.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (5′-phosphorylated) of self-complementary sequences containing the polyamide endcaps were synthesized by the Midland Certified Reagent Company using conventional phosphoramidite chemistry. All oligonucleotides were purified by PAGE in Tris–Borate–EDTA buffer (pH 8) using 15% denaturing (7 M urea) gel. Oligonucleotides were visualized by UV shadowing, crushed and soaked overnight in 0.5 M ammonium acetate. Oligonucleotides were then desalted using a C18 Sep-pak cartridge. The oligonucleotides were characterized using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Supplementary Material).

Ligations

Endcapped oligodeoxyribonucleotides (5′-phosphorylated) varying in length from 8 to 14 bp were ligated with T4 DNA ligase (NEB). Standard ligation conditions were as follows: (i) 40 μM oligonucleotide, 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA and 2000 U of T4 DNA ligase incubated in a total volume of 100 μl at 25°C for 12 h; and (ii) 200 μM oligonucleotide, 66 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 15% of polyethylene glycol (PEG 6000) and 2000 U of T4 DNA ligase incubated in a total volume of 20 μl at 25°C for 12 h. After ligation, the reactions were quenched with 3 μl of 0.5 M EDTA, pH 8.0.

High-performance liquid chromatography (HPLC) analysis of ligation mixtures

The ligation mixtures were diluted to 400 μl with water. The solution was transferred to a Microcon Concentrator 30 and spun at 12 000*g* for 15 min. This process was repeated. The device was then inverted and spun at 3000*g* for 3 min to recover the purified samples. These samples were analyzed using HPLC on 250 mm × 4.6 mm and 250 mm × 2.1 mm C18 columns with buffers A: 5% CH_3CN in 0.1 M TEAA and B: 10% H_2O in CH_3CN using a gradient of 0–45% B for 60 min.

PAGE analysis of ligation mixtures

Ligation samples were freeze-dried on a SpeedVac concentrator, and dissolved in a buffer of formamide and 10× Tris–Borate–EDTA buffer (9:1) before loading on to the gels (20 cm × 20 cm × 0.15 cm). The gels were pre-run at 280 V

for 1 h and then samples resolved at a constant voltage of 280 V for 3–4 h. The gels were then stained with ethidium bromide for the visualization of the DNA bands.

RESULTS AND DISCUSSION

DNA ligases are repair enzymes that catalyze the closure of a nicked DNA containing a 5′-phosphate and a 3′-hydroxyl (-OH) terminal (20). Ligase is an essential enzyme that plays a critical role in DNA repair, DNA recombination and replication. Hence, the structural determination of the ligase and its DNA substrate is of substantial interest. To date, atomic structures of two ATP-dependent ligases belonging to the bacteriophage T7 and Chlorella virus have been determined using X-ray crystallography but the structure of a ligase bound to a nicked DNA substrate has not been reported (21). Footprinting experiments of the binding sites of bacteriophage T7 and Chlorella virus have been established by the hydroxyl radical method and the exonuclease digestion method, respectively. Both are destructive methods of analysis that roughly indicate the boundaries of the exposed region of the nucleic acid in a protein–nucleic acid complex. However, neither can precisely identify the bases that bind the protein. In the alternative approach described in this paper, we have constructed an endcap (Figure 1) that was designed to exactly span the terminal nucleoside units without warping the helix and without occupying space that might be within the putative protein-binding region.

By a systematic comparison of the ligation of endcapped DNA duplexes of varying lengths (8–14 bp; Figure 1, structures I–V), we can determine the natural footprint of the ligase. In theory this should provide a more accurate assessment of the binding footprint than chemical and enzymatic footprinting methods where access of the reagent/enzyme may be restricted to regions blocked by but not directly bound to the protein. In addition, sequence and/or nucleotide specificity associated with chemical reagents and enzymes may hinder the interpretation of footprinting studies.

Endcapped DNA duplexes offer an advantage over normal DNA duplex substrates since short, nicked DNA duplexes unambiguously exist in the double-stranded form. Assembly of a number of smaller DNA fragments would be expected to affect the overall free energy of association. By utilizing endcaps that are relatively flexible and which do not strongly associate with the terminal bases, it is unlikely that the endcaps would prevent conformational change (e.g. B to A form) or bending associated with protein binding.

Endcapped DNA oligonucleotides of varying lengths (16–28 nt) were synthesized as self-complementary strands that folded to form nicked endcapped DNA duplexes I–V (Figure 1). Ligations of endcapped DNA duplexes I–IV were carried out as described in Materials and methods without the addition of PEG. Samples of the ligation mixtures were analyzed by reverse-phase HPLC. The relative efficiency of ligation was determined by measuring the areas of the peaks corresponding to the unligated material and ligated material. The ligated and unligated DNA duplexes have very close retention time periods as shown in Figure 2 with the ligated DNA duplexes eluding slightly faster than the unligated oligonucleotide. The identity of the ligated endcapped DNA duplexes was confirmed by co-injection with the starting material as shown for DNA duplex I (Figure 2Aiii).

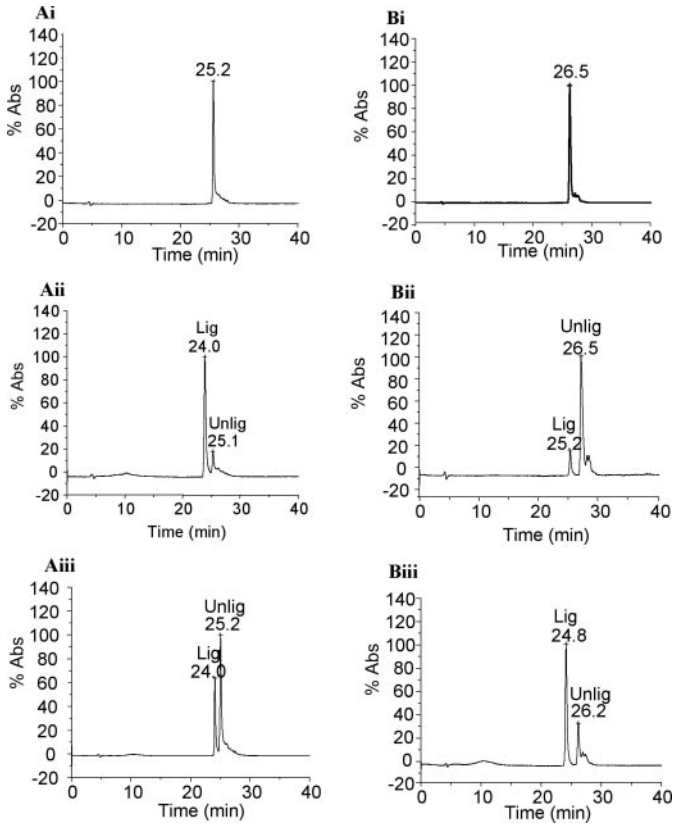

Figure 2. HPLC chromatograms of ligation mixtures of endcapped duplex I (**Ai**) unligated endcapped duplex, (**Aii**) ligation mixture, at 25°C for 12 h, absence of PEG 6000, (**Aiii**) co-injection of unligated and ligated endcapped duplex I (at 25°C for 12 h, absence of PEG 6000). HPLC chromatograms of ligation mixtures of endcapped duplex III (**Bi**) unligated endcapped duplex III, (**Bii**) ligation mixture of endcapped duplex III at 25°C for 12 h, absence of PEG 6000 (**Biii**) ligation mixture of endcapped duplex III at 25°C for 12 h, presence of PEG 6000.

The ligation of the longest endcapped DNA duplex, I (14 bp), occurred with relatively high efficiency, while the shortest duplex, IV (8 bp), was not ligated. The efficiency of ligation was: I (85%) > II (75%) > III (13%) > IV (0%) (Figure 3). In comparison, Erie *et al*. (22) reported that a dumbbell oligonucleotide (24mer) consisting of a 8 bp duplex-stem, constrained by loops of four thymidines, could not be ligated over a broad range of solution conditions and temperatures. They suggested that steric and conformational constraints in small DNA dumbbell oligonucleotides compromised the ligation efficiency of DNA ligase. In a separate study (16), the same research group demonstrated that by increasing the number of thymidines in the loops from four to five, with the presence of PEG, they could ligate the 8 bp, dumbbell oligonucleotide (26mer). The successful ligation was attributed to the additional thymidines in the loops that allow for the terminal residues at the nick to assume a collinear conformation for ligation. Although endcapped DNA duplex IV also consists of 8 bp, it does not contain additional loops at the ends of the duplex and is shorter in length when compared to the 26mer, dumbbell oligonucleotide utilized by Erie and co-workers (22).

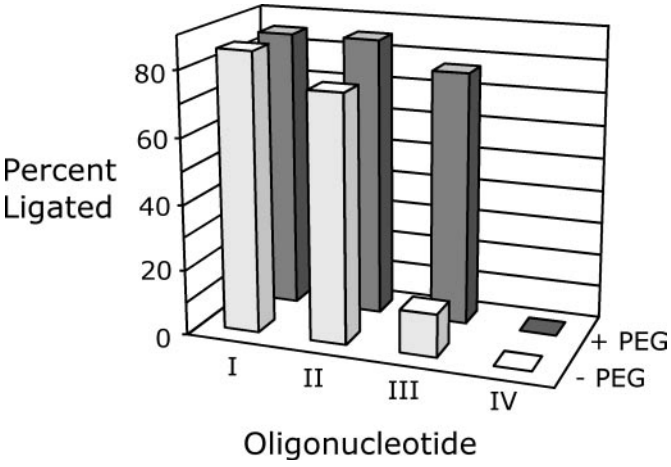

Figure 3. Ligation efficiencies of endcapped DNA duplexes I–IV in the absence and presence of PEG 6000.

In line with our hypothesis, the lack of ligation of endcapped DNA duplex IV is consistent with the loss of critical binding sites. However, it is worthwhile to consider other possible explanations. Particularly, it was important to determine if the endcapped duplex existed in the same form as natural dsDNA (e.g. B-form) duplex at the temperature of the ligation reaction. Accordingly, circular dichroism (CD) and thermal melting (T_m) experiments were carried out on endcapped DNA duplex IV. The thermal melting profile of endcapped DNA duplex IV (1 μM) is shown in Figure 4A.

The thermal melting profile of the endcapped DNA duplex IV is a monophasic, sigmoidal curve that is characteristic of a unimolecular process where the self-complementary strands fold at the endcaps to form a duplex with no other higher order structure. The T_m value of the duplex IV is 54.4°C, an indication that it is stable in the duplex form at room temperature. Thermal melting profiles of duplex IV at 0.3 and 5 μM were similar (data not shown).

A positive signal between 265 and 285 nm and a negative signal between 250 and 260 nm is indicative of a B-DNA helix (23). The CD spectrum of endcapped DNA duplex IV matches this profile and is consistent with a B-DNA helix (Figure 4B). Nevertheless, the CD results do not entirely rule out effects that may be propagated to nearby sites by the endcaps causing re-positioning of the terminal residues at the nick so that they are misaligned in comparison to normal nicked DNA duplex.

The most probable explanation for the low efficiency of ligation of duplex IV is that it is too short to bind tightly to T4 DNA ligase. Our results indicated that among the endcapped DNA duplexes investigated, the minimal length of endcapped DNA duplex that could be ligated was 11 bp in length. The position of the nick in this length duplex was critical. Endcapped DNA duplex II was ligated much more efficiently, 75% yield) than endcapped DNA duplex III (13% yield). In endcapped DNA duplex II, the nick is flanked by 6 nt on the 5′-phosphorylated end and 5 nt on the 3′-OH end whereas in the endcapped DNA duplex III, the nick is flanked by 5 nt on the 5′-phosphorylated end and 6 nt on the 3′-OH end. The significant distinction in the ligation yields of these DNA duplexes as a consequence of shifting the position of the nick by one nucleotide suggested that T4 DNA ligase bound

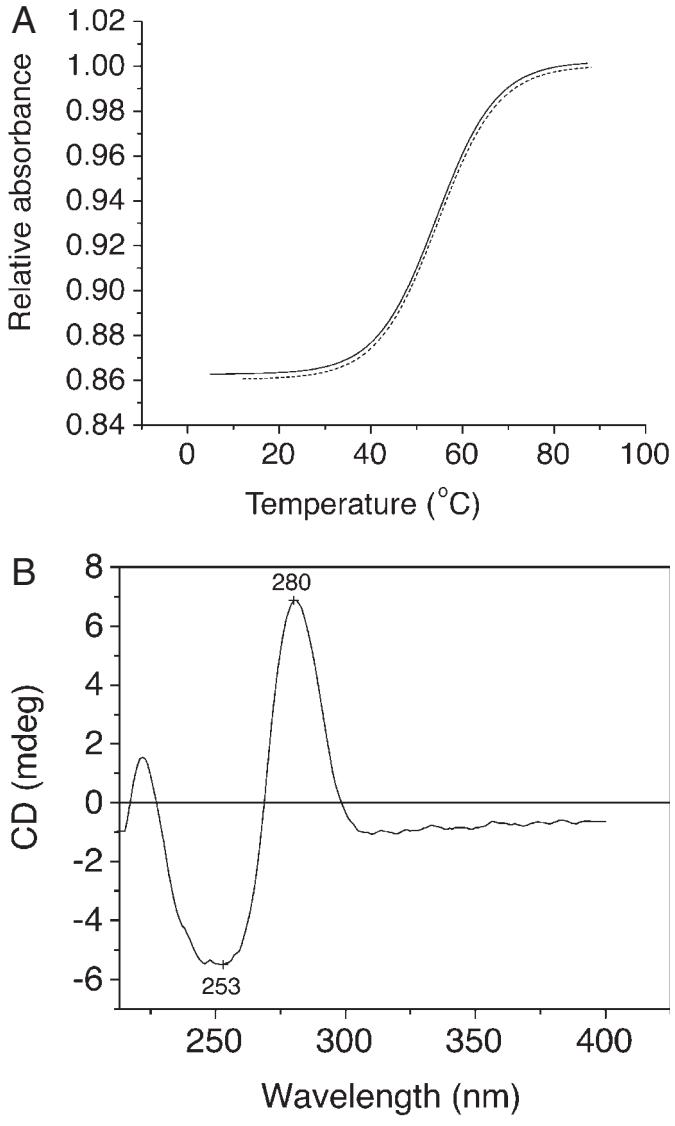

Figure 4. (**A**) Thermal melting profile of endcapped DNA duplex IV at 1 μM in 100 mM sodium phosphate buffer, pH 7.4. Annealing curve (solid line) and melting curve (dashed line). (**B**) CD spectrum of endcapped DNA duplex IV at 1 μM in 100 mM sodium phosphate buffer, pH 7.4.

asymmetrically to nicked dsDNA. Sequence-specific effects, could also conceivably contribute to the differences in ligation efficiency. In particular, bases at the 3′ and 5′ ends of the nick [C-3′-OH to 5′-pA (II) and A-3′-OH to 5′-pA (III)] may affect ligation efficiency. Shilov *et al*. (24) reported that the intensity of stacking interactions of the bases at nicks: purine–purine> pyrimidine–purine > pyrimidine–pyridmidine would affect the stability of the DNA duplex hence affecting ligation efficiency by Phage T4 DNA ligase. In addition, Tong *et al*. (25) in their evaluation of the ligation efficiency of a NAD^+-dependent ligase, noted a general trend in the ligation rates of A > C > G ⩾ T at the 3′-OH end, when adenine, the base at the 5′-phosphate side of the nick was kept constant. Extrapolation from these findings would suggest that endcapped DNA duplex III might have a higher ligation rate than II, opposite in order of the relative ligation rates that we observed. We believe that under our experimental conditions, differences in

the observed ligation yields are most likely due to a more extended binding site on the 5′-phosphate side of the nick, i.e. asymmetrical binding with sequence-specific effects being a secondary factor.

Doherty and Dafforn (21) reported that T7 DNA ligase binds asymmetrically to nicks extending 7–9 nt on the 5′-phosphate side of the nick and 3–5 nt on the 3′-OH side. The Chlorella virus DNA ligase has been reported by Shuman *et al*. (26) to bind to nicks extending 11–12 nt on the 5′-phosphate side of the nick and 8–9 nt on the 3′-OH side. Using our endcapped oligodeoxyribonucleotide substrates and through the comparison of ligation yields, our study provided evidence for the asymmetric binding of T4 DNA ligase to nicked DNA substrate. Although the ligation of varying lengths of oligodeoxyribonucleotides (3′-OH substrates, 6mer to 10mer) to a 9mer oligodeoxyribonucleotides (5′-phosphorylated) by T4 ligase was reported by Pritchard and Southern (27), their study did not investigate the ligation of varying lengths of oligodeoxyribonucleotides (5′-phosphorylated substrates) to a 3′-OH substrate.

In light of our results on the asymmetry of the ligation site, we synthesized an additional endcapped duplex, V. Like duplex IV, which was not ligated, duplex V contains only 8 bp, but unlike duplex IV the nick is re-positioned to include the greater number of bases on the 5′-phosphate side of the nick (Figure 1).

Ligation could not be observed by HPLC analysis, but we assayed this and other ligations by PAGE analysis as well. PAGE is a useful method that is commonly employed for the analysis of ligated products (9mer to 40mer) (11,28). Ligated and unligated duplexes are well separated by PAGE. This is shown for duplex II in Figure 5A, where the ligated product runs faster than the unligated. As shown in Figure 5B and C, ligated products are not observed for either duplex IV or V. In the case of duplexes II and V, a band appears that runs slightly slower than the unligated duplex. This may be due to a small amount of the adenylated intermediate (29). Similarly, Odell *et al*. (8) observed no ligated product but appearance of an adenylated intermediate when a 4mer oligodeoxynucleotides (3′-OH) was investigated for ligation by Chlorella virus ligase to an 18mer strand (5′-phosphorylated).

Thermal melting and CD experiments were also carried out on endcapped DNA duplex V to confirm that it, like the other duplexes, existed in a stable B-form duplex. The thermal melting temperature was found to be 60.6°C and the CD profile matched that observed for duplex IV.

Effect of PEG 6000 on the efficiency of ligation

Pheiffer and Zimmerman (30) have reported that macromolecular crowding with the use of volume excluders such as PEG 6000 enhanced cohesive-end and blunt-end ligation of DNA. A similar study by Hayashi and co-workers (31) confirmed the findings that both intermolecular and intramolecular ligations were improved in the presence of PEG 6000. It was of interest to determine if addition of PEG 6000 would result in enhanced ligation of endcapped DNA duplexes I–III. Reversed phase HPLC analysis was carried out on ligation mixtures of duplexes I–III to examine the effects of PEG 6000 on the ligation yields. The HPLC chromatograms of the ligation of duplex III in the presence and absence of PEG are shown in Figure 2B.

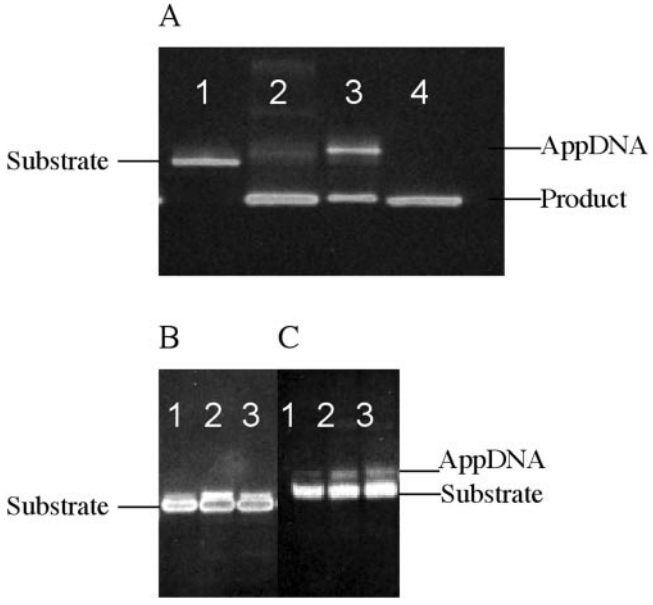

Figure 5. PAGE analysis of ligation mixtures (**A**) endcapped DNA duplex II: lanes 1, unligated duplex; lane 2, ligation mixture, at 25°C for 12 h, presence of PEG 6000; lane 3, ligation mixture, at 25°C for 12 h, absence of PEG 6000, lane 4, purified ligated duplex isolated from HPLC analysis. (**B**) Endcapped DNA duplex IV: lanes 1, unligated duplex; lane 2, ligation mixture, at 25°C for 12 h, presence of PEG 6000; lane 3: ligation mixture, at 25°C for 12 h, absence of PEG 6000. (**C**) Endcapped DNA duplex V: lanes 1, unligated duplex; lane 2, ligation mixture, at 25°C for 12 h, presence of PEG 6000; lane 3, ligation mixture, at 25°C for 12 h, absence of PEG 6000.

Addition of PEG did not lead to measurable ligation of duplexes IV and V, the ligation yield of duplex II increased slightly from 75% in the absence of PEG to 85% with the presence of PEG, and more significantly, the ligation yield with duplex III increased from 13 to 77% (Figure 3).

The presence PEG 6000 may lead to stabilization of the enzyme or enzyme–subtrate complex or by virtue of macromolecular crowding, which facilitates interaction of the endcapped DNA duplexes and the ligase. But the effect was not great enough to allow ligation of the shorter duplexes.

CONCLUSIONS

We have discovered a novel use of endcapped DNA duplexes and have shown that endcapped, nicked dsDNA can be utilized to determine the DNA footprint of T4 DNA ligase. By comparing the ligation efficiencies of endcapped, nicked DNA of (i) varying lengths (ii) varying nick positions, we can establish not only the DNA-binding site but also the mode of binding (symmetrical and asymmetrical) of the enzyme to the DNA substrate. The results of our study indicated that among the endcapped DNA duplexes (8–14 bp) investigated, the shortest endcapped DNA duplex that was ligated was 11 bp. We did not further investigate the ligation of endcapped DNA duplexes with 9 and 10 bp since the ligation efficiencies of endcapped DNA duplexes II and III indicated that the 11 bp substrate lies at the threshold of ligation by T4 DNA ligase. In addition, the significant difference in the ligation yields of endcapped DNA duplexes II and III suggested that T4 DNA ligase binds to its

substrate asymmetrically. Use of a hairpin strand as a substrate to investigate DNA length requirements of Chlorella virus DNA ligase has been reported (21,32) but the hairpin loop may provide additional length for binding of the enzyme and hence compromised the accuracy of the DNA footprint.

The use of endcapped DNA duplexes as a tool to elucidate the DNA footprint of a protein offers a number of advantages: the procedure is relatively fast, employing easily synthesized oligonucleotides substrates and detection and quantification can be done without radiolabeling. The commercial availability of the phosphoroamidite form of a simple flexible endcap, e.g. hexaethylene glycol allows for the incorporation of endcaps into oligonucleotides with no further chemistry. This makes the routine synthesis of a set of different endcapped DNA duplexes for biochemical studies relatively simple and easy. The limitation of the procedure, as described here is that it depends on enzymatic transformation of the substrate oligonucleotides to a product that can be distinguished by HPLC or PAGE. On the other hand there is no reason why this procedure could not be extended to gel-shift assays. A series of endcapped duplexes would provide its own set of internal controls (long and short extremes) that may make interpretation of results relatively straightforward.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

Discussions with Dr Alan Friedman concerning the potential of amide containing endcapped oligonucleotides for studying protein–nucleic acid interactions were greatly appreciated.

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