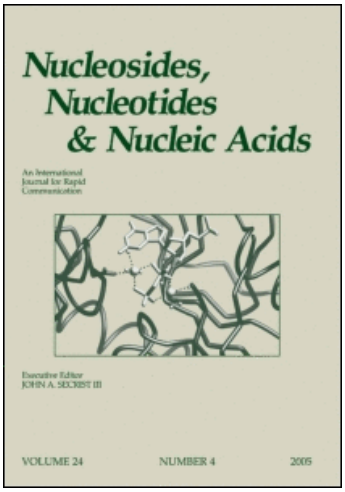

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NOVEL APPROACH TO THE LIGATION OF SINGLE-STRANDED DNA FRAGMENTS BY T_4 DNA LIGASE - DNA MOBILE MULTIPLE-RESTRICTION FRAGMENTS: "UNI-LINKERS" FOR CLONING OF GENES

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ABSTRACT

A group of uniquely designed single-stranded oligodeoxyribonucleotides that form hairpin loops were synthesized. These oligonucleotides can be ligated to other synthetic single-stranded fragments differing in length and design without the need for external annealing templates. A novel approach to building a limitless variety of mobile multiple-restriction DNA fragments termed "uni-linkers" and which are open only at one end, is described. The latter were used as "multi-stage" linkers in cloning experiments. The cloning of the cDNA for a carrot proline-rich protein, DC-5, into a plant vector is presented as an example. Other possible applications are also discussed.

INTRODUCTION

Oligodeoxynucleotides with internal self-complementarity have been shown to favour the formation of intramolecular hairpin structures when certain requirements are met [1, 2]. A short stretch of unpaired nucleotides flanked by palindromic purine-rich sequences result in an internal plane of symmetry that stimulates internal base-pairing to form a loop structure. Roy et al. [3] have shown that longer purine-rich arms shifted the equilibrium towards the loop formation. Alternatively, it was shown by Miller and coworkers [4] that increasing the number of pyrimidines in the center of symmetry led to stabilization of the duplex form. The optimum number of unpaired nucleotides in a stable loop structure was found to be 3 to 4 [5]. In addition, hairpin loops have long been recognized for their ability to prime second strand synthesis, irrespective of their chain length, and were extensively utilized in cDNA library construction [6]. More recently, these properties were applied to sequence determination of restriction DNA fragments by ligation to synthetic hairpin loops and subsequent sequencing [7].

We are interested in developing procedures for random ligation of single-stranded DNA fragments, a provision which would lead to a number of new avenues such as dideoxy nucleotide sequencing of synthetic single-stranded fragments and the enzymatic generation of double-stranded DNA fragments from single-stranded counterparts using universal-type primers. In our hands, attempts at using the described "splinker"-type fragments [7] for ligation to synthetic single-stranded fragments failed and led always to exclusive dimerization of the hairpin species. In this paper we describe a general approach utilizing the aforementioned principles and properties in designing and building two universal synthetic DNA fragments which lend themselves as substrates for single-stranded DNA ligation protocols using T4 DNA ligase. The use of these in the general production of linker-type mobile multiple-restriction-site fragments (termed "uni-linkers") is also described. The universal fragments, 27 and 29 nucleotides long respectively (Fig. 1), are uniquely designed to form a hairpin structure along 9 palindromic bases in the case of (1), and 10 in the case of (2), leaving 5 non-complementary nucleotides exposed at their 3' ends.

While maintaining the structural requirements necessary for loop formation, as seen in Fig. 1, we incorporated in these particular examples two restriction sites common to both sequences, BamHI and SmaI. In addition, each fragment contained a 3' unique sequence which can be converted into a PstI or SalI site respectively. By design, five nucleotides are included in the 3' protruding end to prevent dimerization in the presence of DNA ligase. The 3' protruding ends permit ligation to single-stranded fragments which end with either 5'----TGCAG-3' or 5'----TCGAC-3'. This is followed by subsequent chain extension to generate the second strand of the uni-linker which is then trimmed out by appropriate restriction enzymes. Thus, by chemically synthesizing only the top strand of the desired restriction sequence and ligating it to a universal fragment, a double-stranded linker can be obtained. Linkers made in this way are "uni-ended" (i.e. open only at one end while the other end is protected as a loop), hence the term "uni-linkers". This feature allows repeated use of the universal "core" fragments in building double-stranded (DS) uni-linkers of different sequences and lengths. From this viewpoint we will refer to these universal fragments as "DS-cores".

Using this approach we synthesized from each DS-core fragment a number of uni-linkers containing different combinations of

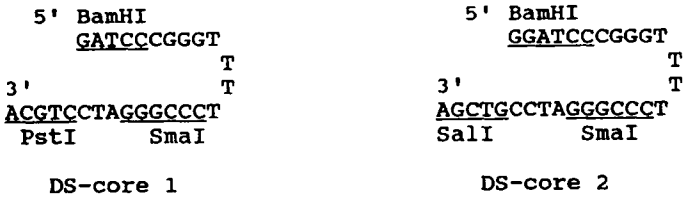


Fig. 1. DS-core 1, a 27mer and 2, a 29mer with five unpaired nucleotides at their 3' ends.

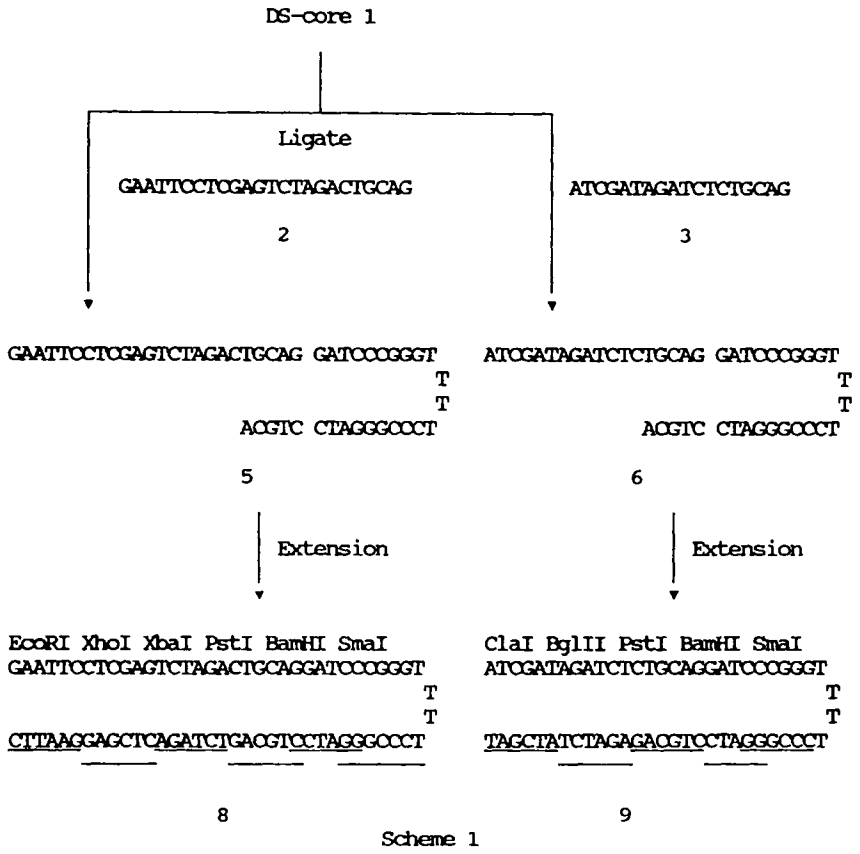
unique restriction sites (three examples are shown in Schemes 1 and 2) and confirmed their sequences. We employed one of them successfully in cloning a cDNA for carrot proline-rich proteins in a vector for transient expression in plant protoplasts by electroporation studies.

MATERIALS AND METHODS

Enzymes and chemicals

All enzymes were purchased from BRL (Gibco, Canada) which were supplied (unless mentioned otherwise) with their corresponding concentrated buffers. Cyanoethyl phosphoramidites for DNA synthesis were obtained from Beckman Canada. Nitrocellulose membranes were purchased from Schleicher and Schuell, USA.

Oligodeoxyribonucleotides were synthesized on the Applied Biosystems 380A DNA synthesizer. Products were purified as non-tritylated material by HPLC using the Waters 660E multisolvent delivery system equipped with the 990 photodiode array detector, 712 WISP autoinjector and the NEC power mate-2 data processing system. Chromatograms were scanned at a wave length range of 220-301 nm for rapid characterization of desired products. A Waters bondapack cartridge-type C18 reversed phase column (8mm internal diameter x 10cm, with particle size 10μm) was used in all preparative HPLC purifications. At a flow rate of 1.0 ml/min, a linear gradient (consisting of triethylammonium acetate, 50 mM, pH 7.0, as buffer A, and TEAA, 35 mM, pH 7.0, in 80% acetonitrile as buffer B) from 0 to 22% in buffer B was formed over a period of 35 minutes. Over a period of two minutes an increase in buffer B to 100% followed by a two-minute decrease to 0% occurred at a flow rate of 2 ml/min. This is primarily to purge the column of any hydrophobic compounds. Peaks of interest appeared and collected at retention times ranging from 25.9 to 26.8 minutes.



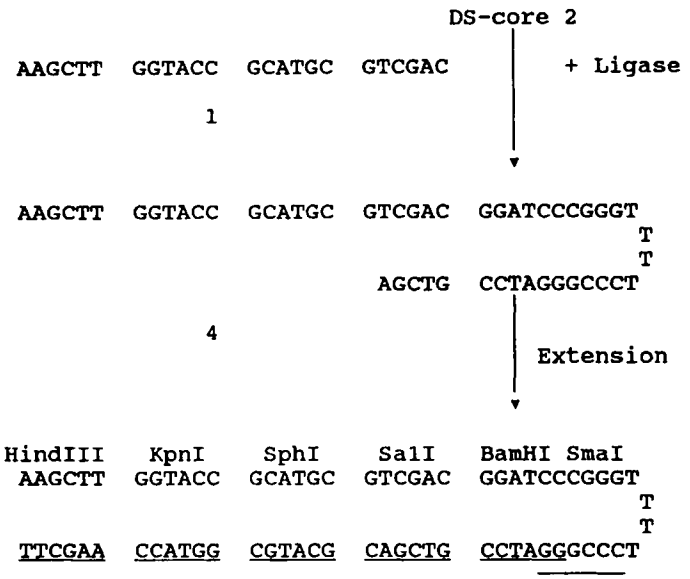
Scheme 1

Relatively broader peaks were observed for the DS-core compounds, presumably due to their inherent secondary structures.

Ligation to the suitable DS-core

Single-stranded fragments (2.0 nmoles of each) in 20 µl of 0.5 M Tris.HCl, pH. 8.0) were mixed with their compatible DS-core (1.7 nmoles, 5'-phosphorylated, in 20 µl of 0.5 M Tris.HCl, pH 8.0) and 40 µl of each of 0.1 M $MgCl_2$, 30 mM dithiothreitol (DTT) 4 mM ATP and distilled water to a final volume of 400 µl. To this was added 10 units of T4-DNA ligase and the mixture was kept overnight at 7°C.

After concentration to a volume of about 100 µl the ligated products were purified on a 12% polyacrylamide gel containing 7 M urea. Desired product bands were transblotted from their gel slices onto nitrocellulose membranes (Schleicher and Schuell, USA) using BioRad's "Trans-Blot" cell, for 60 minutes at 120 mAmp. The membranes were rinsed with water and soaked in 1 M



Scheme 2

solution of triethylammonium bicarbonate (TEAB, 500 µl) for 60 minutes at 40°C with occasional mixing. Solutions were concentrated under vacuum to nearly 100 µl followed by addition of water (400 µl) and re-evaporation. Water evaporations were repeated several times until no more TEAB salt remained.

Generation of uni-linkers

To each ligation product from the preceding step (100 pmoles) in 47 µl Tris.HCl (50 mM, pH 7.4) containing 10 mM $MgCl_2$ and 10 mM DTT was added a mixture of all four dNTPs (to a final concentration of 0.25 mM in each dNTP in total volume of 50 µl) and 1 unit of the Klenow fragment of DNA polymerase I (BRL). The reaction was allowed to proceed at 22°C for 10 minutes followed by incubation at 85°C for 5 minutes and gel purification as above.

Sequence verification of uni-linkers

Compound 7 was labelled using the polynucleotide kinase with [γ-32P] ATP while compounds 8 and 9 were regenerated from their respective precursors by nick translation as described above but with dATP replaced by [α-32P] dATP.

Each of the purified labelled products was dissolved in 10 µl of Tris.HCl (500 mM, pH 8.0) and divided equally according to the

number + 1 of restriction sites each linker is designed to contain. Utilizing all restriction sites in the linker, each of these aliquots was subjected to one restriction analysis. Reactions were run at 30°C for 120 minutes and the mixtures were analyzed next to each corresponding starting material on a 12% polyacrylamide gel (7 M in urea).

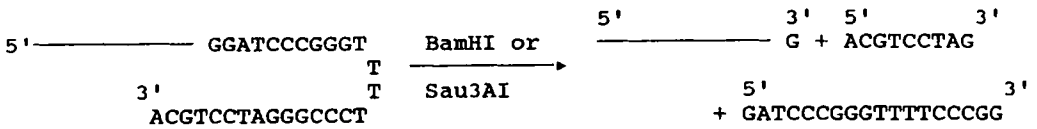
Use of uni-linker 7 in constructing plasmid pGCC2

Uni-linker 7 (200 pmoles) was digested with HindIII in the normal way at 30°C for 120 minutes. The fragment was then separated from the mixture by HPLC as described above. The product was mixed with the HindIII cDNA fragment obtained from plasmid pDC5 (a gift from Professor J.E. Varner, Washington University, St. Louis, USA), in a ratio of 2 : 1 respectively, based on the number of available cohesive ends. Total buffered reaction volume was 400 µl containing 3 units of T4 DNA ligase. The mixture was incubated at 10°C for 16 hours followed by incubation with exonuclease III (1 unit) for 20 minutes at 37°C and phenol/chloroform extraction and ethanol precipitation of the product. BamHI digestion of the latter in 20 µl reaction volume (for 60 minutes at 37°C) followed by phenol/chloroform extraction and ethanol precipitation yielded a BamHI bordered fragment suitable for construction of plasmid pGCC2.

RESULTS AND DISCUSSION

DS-core fragments, the design

DS-core fragments 1 and 2 were designed to be ligated to compatible single-stranded synthetic DNA chains. They were also uniquely designed to overcome self-ligation that would lead to "dumbbell" structures which might inhibit desired-product formation [7, 8]. We accomplished this by extending their recessive cohesive ends beyond the normal point of their respective restriction while keeping the integrity of the restriction sequences. In so doing we have eliminated their palindromic properties and rendered them compatible only with the 3' tail of single-stranded synthetic fragments that are specifically designed for that purpose. Thus, for an incoming PstI fragment to be ligated to DS-core 1, the former should possess the 3'-ending CTGCAG instead of the normal PstI restriction sequence CTGCA. Similarly, in the case of DS-core 2, the compatible 3' end of incoming SalI fragments should terminate in the sequence GTCGAC but not G as would be required under normal circumstances.



Scheme 3. Retrieval of a single stranded DNA fragment ligated to DS-core 1 using either BamHI or Sau3AI.

The specific manner in which the BamHI site was incorporated in DS-core 1 allows precise retrieval of ligated single-stranded fragments, if so desired. This was accomplished by restriction with either Bam HI or Sau3AI (Scheme 3), data not shown. This is particularly useful for dideoxynucleotide sequencing of single-stranded DNA (to be published elsewhere).

Use of DS-cores in uni-linker assembly

Three single-stranded fragments (1, 2 and 3) were designed to contain different sets of restriction sites. The first, a 24 mer, contained a set of three unique restriction sites, HindIII, KpnI and SphI and was compatible for ligation to DS-core 2. The second fragment, also a 24 mer, had the sites EcoRI, XhoI and XbaI, while the third, an 18 mer, contained Bg1II and ClaI sequences. Both second and third fragments were ligation-compatible with DS-core 1. In this way uni-linkers generated from these segments contained between 5 and 6 different restriction sites including those provided by the DS-core fragments.

Appropriate strands were thus ligated, followed by chain elongation to generate full loop-duplexes (as illustrated in Schemes 1 and 2, and shown in Fig. 2), which were then used as sources for selectively-restricted, uni-linkers, ready for further experimentation. In this fashion, these structures served as "multi-stage" restriction-site-libraries where the desired site is readily obtained by digestion with the appropriate enzyme. They can also be utilized to seal open-ended restriction fragments which can then be reopened at the point of choice along the linker region (see below). It should also be noticed that generation of full uni-linkers by chain extension of their precursors (schemes 1 and 2) furnishes uni-linkers suitable for blunt-end ligations.

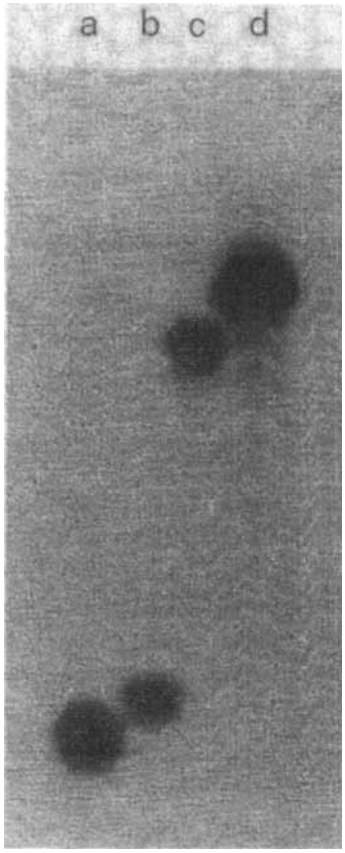


Fig. 2a. Autoradiogram tracking of the generation of uni-linker 7: Lanes a and b represent 5' labelling of DS-core 2 and fragment 1 respectively. Lanes d and c show the 5' labelled uni-linker 7 and its precursor 4 respectively. Labelling of 4 and 7 was performed on purified products. Denaturing polyacrylamide gel (12%) was used.

Sequence verification of uni-linkers

The fact that all sequences contained in these duplexes were purely restriction sites offered a unique opportunity for sequence verification of the entire oligomer length by restriction degradation. By examining, on denaturing polyacrylamide gels, the mobilities of radiolabelled oligomers released by digestion with appropriate restriction enzymes we were able to confirm that the nucleotide sequence of each duplex was as designed (Fig. 3). Had an incorrect sequence occurred at any point in a duplex, the corresponding enzyme would not be expected to act on it. In this regard we used two different

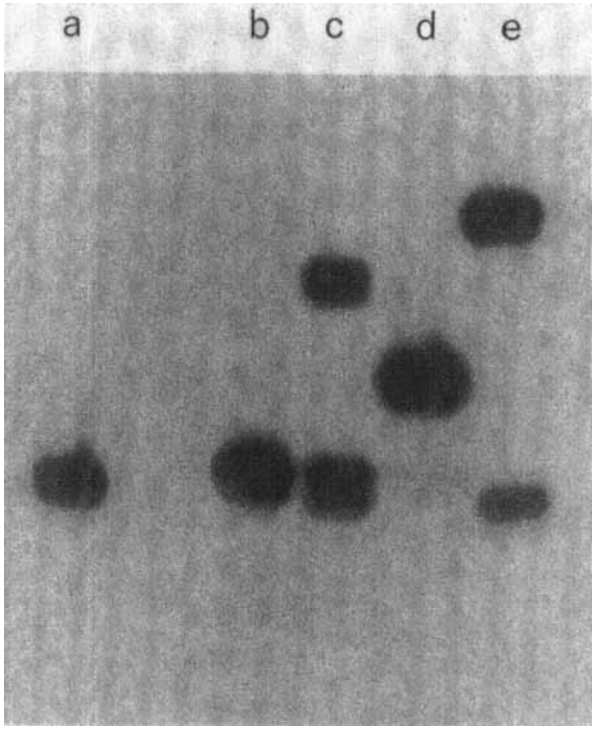


Fig. 2b. Autoradiogram of the ligation of DS-core 1 to each of fragments 2 and 3: Lane a shows DS-core 1 labelled at its 5' end. Lane b contains 5'-labelled fragment 3. Lane c is the ligation mixture of DS-core 1 and fragment 3. Radiolabelled fragment 2 is shown in lane d; and its ligation mixture with DS-core 1 is shown in lane e. Gel conditions were same as in Fig. 2a.

strategies: (1) direct sequence verification in which labelling by using the kinase was performed on compound 7, and (2) indirect sequence verification where labelling by repair was performed on compounds 5 and 6. In the former, digestion with each restriction enzyme released only one labelled oligomer, whereas in the latter some restriction enzymes released only one labelled oligomer and others caused the release of two radiolabelled fragments of different lengths due to the wider distribution of radioactivity in these compounds.

Cloning of cDNA for a carrot proline-rich protein utilizing uni-linker 7

By ligating the cDNA insert (obtained from plasmid pDC5) to a HindIII-treated loop-duplex 7, a close-ended linear DNA species

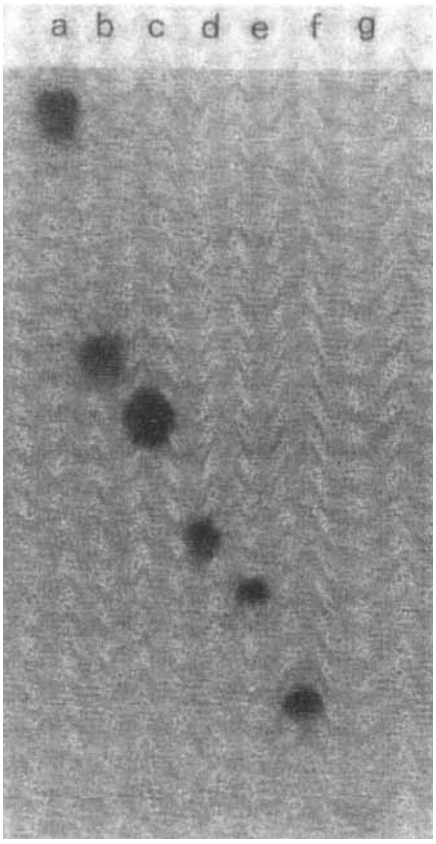


Fig. 3a. Sequence verification of uni-linker 7: Lane a shows 5'-labelled uni-linker 7; lanes b-g contain reaction mixtures of labelled uni-linker 7 with each of SmaI, BamHI, SalI, SphI, KpnI and HindIII respectively.

resulted (Fig. 4). This was then treated with exonuclease III to digest unreacted starting materials, followed by BamHI (see MATERIALS AND METHODS). The resulting double-stranded cDNA insert with BamHI cohesive ends was cloned into the BamHI arms of a transient expression-vector suitable for protoplast-electroporation experiments. This resulted in plasmid pGCC2 in which the gene is located downstream from a cauliflower mosaic virus promoter 35S. The plasmid also contains genes for the plant expression marker chloramphenicol acetyl transferase (CAT) and the nopaline synthase poly-adenylation site (NOS poly A). This construct is currently being used in electroporation experiments

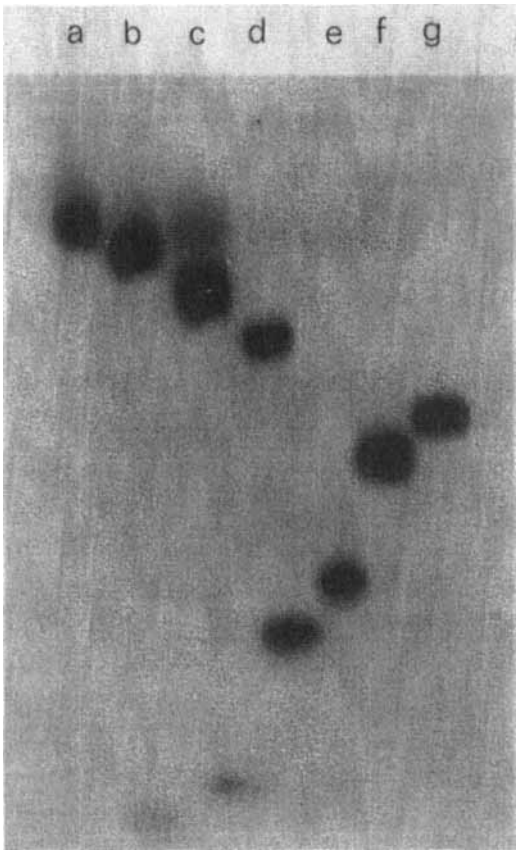


Fig. 3b. Sequence verification of uni-linker 8 (lane a): Lanes b-g represent the restriction profile of uni-linker 8 with each of EcoRI, XhoI, XbaI, PstI, BamHI and SmaI respectively. Uni-linker 8 was labelled by incorporation of [α-^{32}P] dATP by chain extension of its precursor 5. EcoRI, XhoI and XbaI caused the release of two labelled fragments in each of the following respective lengths: 5 + 64; 11 + 52 and 17 + 40. PstI, BamHI and SmaI released only one labelled fragment in each case in the following order: 19mer, 28mer and a 30mer.

designed to study the role of proline-rich proteins in cell wall synthesis using an in vitro Sunflower protoplast system.

The use of uni-linker 7 in costructing plasmid pGCC2 provided an example where a mini restriction library for a given insert can be established and maintained. Thus by virtue of the relatively wide range of unique restriction sites, symmetrically disposed on both ends of the insert, versatile recloning of the latter in as wide a range of vectors can be achieved. This

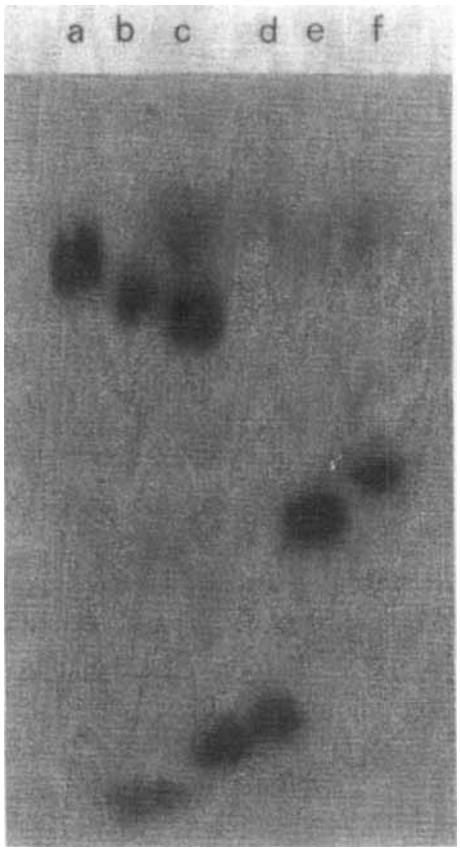


Fig. 3c. Sequence verification of uni-linker 9 (lane a): Digestion with ClaI, Bg1II, PstI, BamHI and SmaI (lanes b-f respectively) yielded two labelled fragments in each of the first two experiments and one labelled fragment in each of the last three in the following sequence: 4mer + 52mer; 11mer + 40mer; 13mer; 22mer and a 24mer.

strategy also offers the advantage of rendering a restriction fragment resistant to exonuclease III, which was used in the present example to effectively remove the unreacted species from the product. It also provided a proof that the fragments described in schemes 1 and 2, and the derivatives thereof, did indeed exist in the loop but not the duplex conformation. On the other hand, when ligated to only one end of an asymmetric restriction fragment, the uni-linker served as a handle for limited exonuclease III digestion of only one end while protecting the other (unpublished results).

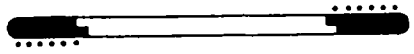


Fig. 4. Product of ligation of the uni-linker 7 to the Hind III restriction fragment from pDC5. Black areas represent the uni-linker region. Dots on the latter represent the number of restriction sites symmetrically disposed on either end of the insert (white area).

CONCLUSION

In this paper we have introduced for the first time an approach to employing T4 DNA ligase in joining two synthetic single-stranded DNA fragments in high yields without the need for external annealing templates. We have demonstrated the utility of this methodology in converting single-stranded DNA species into the double-stranded form, bordered by appropriate cohesive ends. The chemical synthesis of only one strand is required, thereby reducing the cost of DNA synthesis by 50%. Through the unique design of the universal DS-core fragments, we were able to utilize each of them repeatedly in a number of different applications such as the synthesis of uni-linkers, protection of restriction arms (presented here) as well as dideoxynucleotide sequencing of synthetic single-stranded fragments (work to be published elsewhere). Furthermore, one of us was able to produce long gene fragments ($\sim$ 150 bp) in one operation by merely synthesizing long single-strands, ligating them to a DS-core fragment and enzymatically generating second strands [9]. Such products are normally only obtained either by chemical synthesis of both long strands or the synthesis of a separate specific primer for each long strand. In addition, we are currently exploring the use of this approach in developing new and more simplified protocols for cDNA construction and cloning. Furthermore, double-stranded asymmetrically linearized plasmids can be converted into single-stranded ones by sealing one end as a loop followed by limited digestion with exonuclease III, a technique which facilitates certain sequencing experiments and site-directed mutagenesis (work in progress).

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