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Nonenzymatic ligation of double-helical DNA by alternate-strand triple helix formation

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ABSTRACT

Nonenzymatic ligation of double-stranded DNA has been performed using an alternate-strand binding oligodeoxyribonucleotide template to juxtapose the duplex termini in a triple helical complex. The template associates with the duplex termini by Hoogsteen hydrogen bonding to alternate strands on opposite sides of the ligation site. Intermolecular and intramolecular ligation of linearized plasmid DNA are observed in the reaction, which depends on the template oligodeoxyribonucleotide and a condensing agent, *N*-cyanoimidazole. Intramolecular ligation products include those in which both strands are covalently closed in a circle. Ligation of the two strands is sequential and occurs at comparable rates for the first and second strands ligating. The covalent linkages formed in the reaction can be cleaved by the restriction endonuclease *Stu* I, supporting their identification as phosphodiesters.

INTRODUCTION

Sequence-specific ligation of double-helical DNA can be accomplished nonenzymatically by aligning two duplex strand termini with a single-stranded template in a local triple helix (1). A triple-stranded complex is formed by association of an oligodeoxyribonucleotide in the major groove of the Watson–Crick duplex with sequence specificity derived from Hoogsteen hydrogen bonding (2–6). Juxtaposition of the two DNA termini by a guide sequence in a triple helix, accompanied by chemical activation of the terminal phosphates (7–10), promotes ligation of the double-helical DNA (Figure 1). This nonenzymatic approach to DNA ligation involves sequence information from the double-helical substrate not accessible in enzymatic ligations.

We have previously reported the nonenzymatic, sequence-specific ligation of two purine tracts of duplex DNA to form a longer purine tract. The termini of a linearized, double-stranded plasmid were aligned by formation of a continuous pyrimidine·purine·pyrimidine triple helix with a pyrimidine oligodeoxyribonucleotide oriented parallel to the purine strand (3). *N*-cyanoimidazole (1 mM) in the presence of millimolar Zn^{2+} (7, 8) was used to promote condensation of the aligned termini, resulting in a 75% yield of covalently ligated products. At least 15% of the starting material was converted to circularized plasmid of which both strands were covalently closed (1).

Formation of a local triple helix within duplex DNA can be extended to sequences of the general type 5′-(purine)$_m$(pyrimidine)$_n$-3′ by association of a pyrimidine oligodeoxyribonucleotide with purines on alternate strands of the double helix. A pyrimidine oligodeoxyribonucleotide constituted of two segments coupled 3′ to 3′ through a 1,2-dideoxy-D-ribose linker (ϕ) satisfies the structural requirements for binding alternate strands of duplex DNA. The abasic linker connects the two pyrimidine segments across the major groove, bridging two base pairs in a nonspecific manner at the purine-pyrimidine junction of the target site (11).

Application of alternate strand triple helix formation to template-directed ligation of duplex DNA includes the particular case in which the crossover is effected at the ligation site (Figure 2). By alternating strands across the ligation junction, the template aligns two duplexes with purine tracts at their 3′ termini, enabling formation of sequences of the type 5′-(purine)$_m$(pyrimidine)$_n$-3′. In this report, we describe the nonenzymatic ligation of blunt-ended duplex DNA using an alternate-strand binding template that crosses between strands at the site of ligation. By forming a site for a restriction endonuclease in the reaction, the linkages produced could be identified as phosphodiesters based on their susceptibility to enzymatic hydrolysis.

MATERIALS AND METHODS

Plasmid Construction

The oligodeoxyribonucleotides of the duplex shown below were synthesized on an Applied Biosystems Model 380B DNA synthesizer using standard phosphoramidite chemistry.

5′-GATCCAAGAGAGAGAAAAAGGCCTTTTCTTTTCTTTTCTGCA-3′
3′-GTTCTCTCTCTTTTTCCGGAAAAGAAAAGAAAAG-5′

Plasmid pASL was constructed by ligating this duplex into the *Pst* I and *Bam*H I restriction sites of pUC18. This plasmid was

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propagated in the DH5α strain of *E. coli* (BRL) by standard methodology (12), and the correct sequence was confirmed by Sanger sequencing (13). It was purified by density gradient centrifugation. Following centrifugation, ethidium bromide was extracted with amyl alcohol, and the solution was desalted with Sephadex NAP-5 columns (Pharmacia). The DNA was quantitated spectrophotometrically.

Synthesis of 3'-3' Linked Template

The template molecule (5'-$T_4CT_4CT_4C$-3'-3'-ϕ-$CT_5CTCTCTC$-T_2-5') was synthesized by the methods previously described (11) using an ABI 380B DNA synthesizer. The nucleotides at the 3' side of the linker were incorporated in the 5' to 3' direction, starting with the 5'-oxygen of thymidine attached to the solid support (14). After coupling of the first fifteen bases in the 5' to 3' direction, the abasic linker (15, 16) was introduced as the 5-dimethoxytrityl protected 3-β-cyanoethyl phosphoramidite in a 3'-3' coupling (11). The remaining fifteeen bases were added in the usual 3'-5' direction. After cleavage of the template from the support and removal of the protecting groups with concentrated ammonium hydroxide, the product was purified by electrophoresis in a denaturing 20% polyacrylamide gel. The eluted oligodeoxyribonucleotide was desalted by gel filtration on Sephadex NAP-5 columns, and the concentration of the solution was determined by its absorbance at 260 nm using an extinction coefficient for the template of 2.5×10^3 $M^{-1}cm^{-1}$.

Preparation of *N*-cyanoimidazole

N-cyanoimidazole was prepared by the method of Geisemann (17). A solution of 5.5 g (0.05 moles) BrCN (Eastman Kodak) in 25 mL benzene was added dropwise with stirring to a solution of 3.2 g (0.05 moles) imidazole (Aldrich) in 50 mL benzene, warmed to 50°C. After addition, the mixture was stirred at 50°C for an additional 5 minutes, then cooled to 4°C. After 24 hours at 4°C, the bright yellow solid that had precipitated was removed by filtration, and the clear, colorless filtrate was concentrated to dryness *in vacuo*. The product, a white crystalline solid, remained and was purified by sublimation, yielding 0.53 g (0.006 moles, 12% yield). It could be stored for at least two weeks at −20°C without appreciable decomposition as judged by melting point (59−60°C).

Ligation Reactions

Plasmid pASL (5 pmoles) was linearized with restriction endonuclease *Stu* I obtained from New England Biolabs using the provided buffer. The linearized plasmid was deproteinized by extraction three times with water saturated phenol and three times with 25:24:1 phenol:chloroform:isoamyl alcohol, then desalted by gel filtration (NAP-5 column). Reactions were performed at 24°C, pH 4.9 with the template, plasmid, and $ZnCl_2$ concentrations indicated in the Results and Discussion

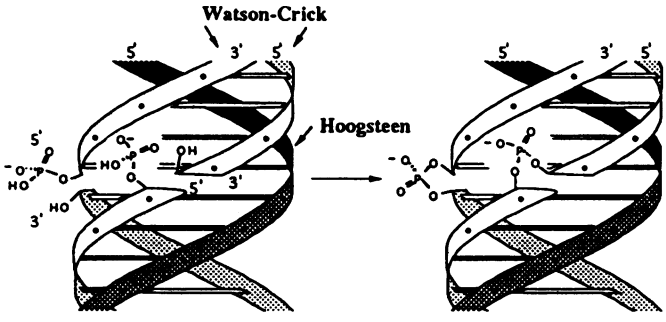

Figure 1. The 5'-phosphate and 3'-hydroxyl termini of two blunt-ended DNA duplexes can be aligned for condensation by association of an oligonucleotide template in a triple-helical complex.

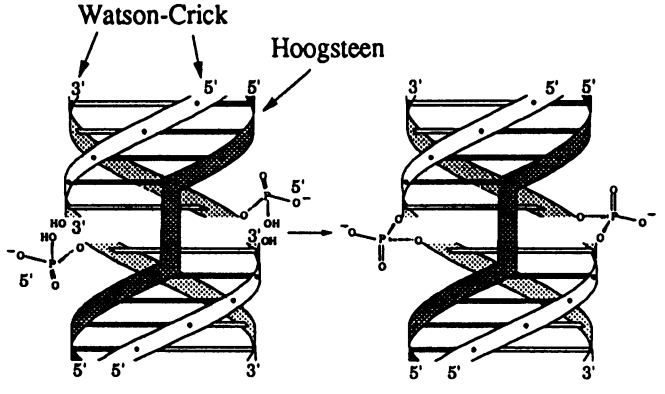

Figure 2. Blunt-ended DNA duplexes can be aligned for condensation by concurrent association with a template composed of two oligodeoxyribonucleotide segments coupled 3' to 3' through a 1, 2-dideoxy-D-ribose linker. In the triple-helix formed, the abasic linker bridges alternate Watson−Crick strands across the site of ligation.

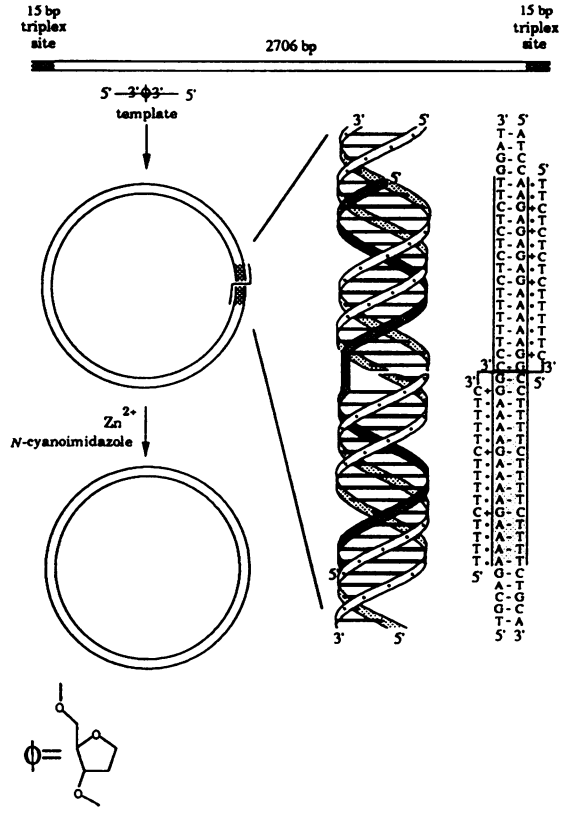

Figure 3. The substrate linear double-helical DNA can be covalently circularized in a reaction that requires purine tracts at both 3' termini of the double-helical DNA, a single-stranded template constructed to bind to purine tracts on alternate strands of a Watson−Crick duplex, and activation of the terminal phosphates by *N*-cyanoimidazole in the presence of Zn^{2+}.

section. They were initiated by addition of a freshly prepared 10 mM solution of *N*-cyanoimidazole to a final concentration of 1 mM. Reactions were stopped by centrifugation through approximately 0.7 mL of Sephadex G-50 and freezing. Prior to analysis by agarose gel electrophoresis, the solutions were concentrated under vacuum to approximately 10 μL, and 5 μL of a 12.5% solution of Ficoll containing bromophenol blue and xylene cyanol were added. Reactions were analyzed by electrophoresis in 1.2% agarose gels containing 0.4 mg/L ethidium bromide, 40 mM Tris·acetate, pH 8.0, and 1 mM EDTA. Approximately 0.9 μg of DNA were loaded in each lane. Gels were visualized with UV illumination, and yields were estimated by densitometric analysis of a negative photograph of the gel using an LKB Ultroscan XL densitometer. This analysis did not account for differential staining of the different forms of DNA by ethidium bromide and probably underestimates the yield of form I° DNA, which is expected to have a lower affinity for ethidium bromide than the linear and nicked circular DNA. The *Bst*E II digest of lambda DNA, used as an electrophoretic size marker, was obtained from New England Biolabs.

To generate enzymatic ligation products from pASL linearized with *Stu* I, 0.9 μg of plasmid cut with *Stu* I was treated for 25 hours at 24°C with 60 units of T4 DNA ligase (Boehringer Mannheim) in 200μL of a solution containing 20 mM $MgCl_2$, 50 mM Tris·HCl, pH 7.0, and 2.0 mM ATP. The reaction mixture was treated in the same manner as the nonenzymatic ligation reactions.

The time course of the nonenzymatic ligation reaction was studied by performing the reaction with 7.2 μg of pASL cut with *Stu* I in a total volume of 1.6 mL containing 100 mM $ZnCl_2$, 36 nM template, and 1 mM *N*-cyanoimidazole. At the times indicated after addition of *N*-cyanoimidazole, 200 μL aliquots were withdrawn, desalted by centrifugal gel filtration through Sephadex G-50, and frozen in dry ice. The frozen solutions were concentrated and analyzed by agarose gel electrophoresis as described above.

To confirm that the linkages formed in the nonenzymatic ligation reaction are susceptible to cleavage by *Stu* I, 9.0 μg of pASL linearized with *Stu* I were treated with 36 nM template, 100 mM $ZnCl_2$, and 1 mM *N*-cyanoimidazole in a total volume of 2 mL for 24 hours at 24°C. Gel filtration through Sephadex G-25 (Pharmacia) was employed to remove $ZnCl_2$ from the solution. One third of the product mixture was treated with 320 units of *Stu* I in a total volume of 500 μL for 24 hours at 37°C. In a control, one third of the product mixture was incubated in 500 μL of *Stu* I reaction buffer for 24 hours at 37°C in the absence of enzyme. The digest and control were desalted by gel filtration (Sephadex G-25), concentrated under vacuum, and analyzed by agarose gel electrophoresis with the undigested reaction products and the linear starting material as described above.

RESULTS AND DISCUSSION

A 2.7 kilobase pair (kbp) plasmid was constructed which could be cleaved with the restriction endonuclease *Stu* I to afford a blunt-ended linear duplex with a sixteen base pair tract of purines at each 3′ terminus. A template molecule was synthesized which contains two fifteen nucleotide segments that are complementary in the Hoogsteen sense to the fifteen base pair purine tract one base pair removed from each terminus of the linear duplex. These segments are coupled 3′ to 3′ through phosphodiesters to the abasic 1,2-dideoxy-D-ribose linker. Association of this template molecule with both ends of the linear duplex by formation of an alternate-strand triple-helix would circularize the double-stranded DNA. Upon chemical activation, the 3′-hydroxyl and 5′-phosphate termini would be susceptible to covalent ligation

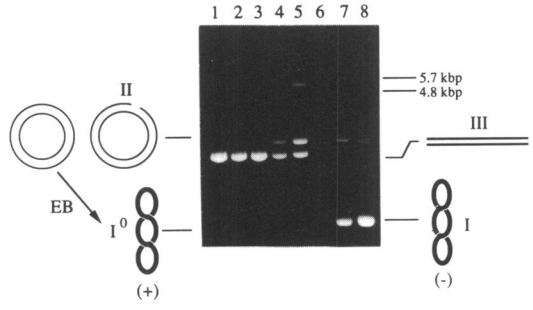

Figure 4. Ligation of linear 2.7 kbp plasmid pASL; analysis by electrophoresis in a 1.2% agarose gel containing 0.4 mg/L ethidium bromide (EB). Lane 4: Plasmid pASL linearized with *Stu* I and treated for 25 hours at 24°C with 36 nM 3′-3′ linked template, 1 mM N-cyanoimidazole, and 100 mM $ZnCl_2$. In controls, lane 1: pASL linearized with *Stu* I and analyzed without further treatment; lane 2: pASL linearized with *Stu* I and treated as in lane 4, omitting N-cyanoimidazole; lane 3: pASL linearized with *Stu* I and treated as in lane 4, omitting 3′-3′ linked template; lane 5: pASL linearized with *Stu* I and treated with T4 DNA ligase and ATP; lane 6: *Bst*E II digest of lambda DNA as molecular weight marker; lane 7: un-cut pASL treated as in lane 4; lane 8: un-treated pASL.

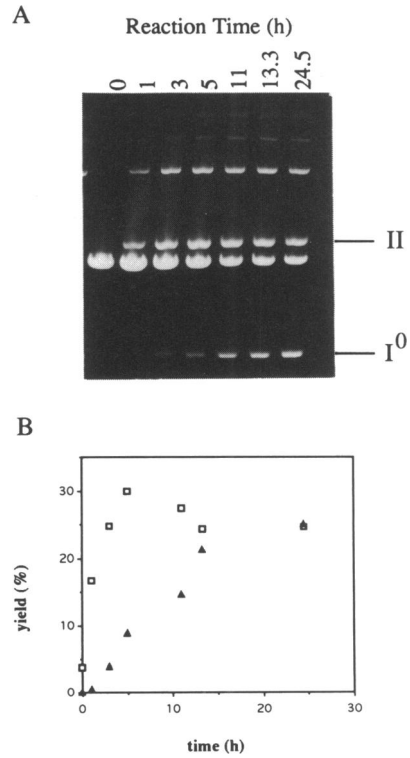

Figure 5. Time course of nonenzymatic ligation reaction at 24°C. (A) Analysis by electrophoresis in a 1.2% agarose gel containing 0.4 mg/L ethidium bromide of the nonenzymatic ligation reaction performed as described in the Materials and Methods section. The reaction was stopped at the times indicated above the lanes on the gel. (B) Yields of form I° (▲) and form II (□) plasmid plotted as a function of reaction time.

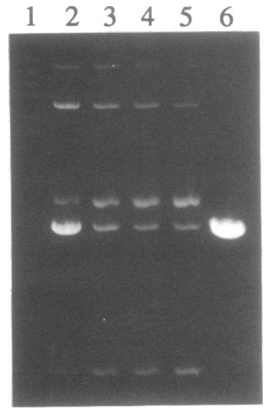

Figure 6. Effect of plasmid concentration on relative yields of products; 1.2% agarose gel containing 0.4 mg/L ethidium bromide. Ligation reactions were performed at 24°C with 36 nM 3′-3′ linked template, 100 mM $ZnCl_2$, 1 mM *N*-cyanoimidazole, and 0.9 μg pASL linearized with *Stu* I in a total reaction volume making the plasmid concentration 12.5 nM (lane 2), 6.3 nM (lane 3), 4.2 nM (lane 4), or 2.5 nM (lane 5). A *Bst*E II digest of lambda DNA was loaded in lane 1, and 0.9 μg of plasmid pASL untreated after linearization with *Stu* I was loaded in lane 6.

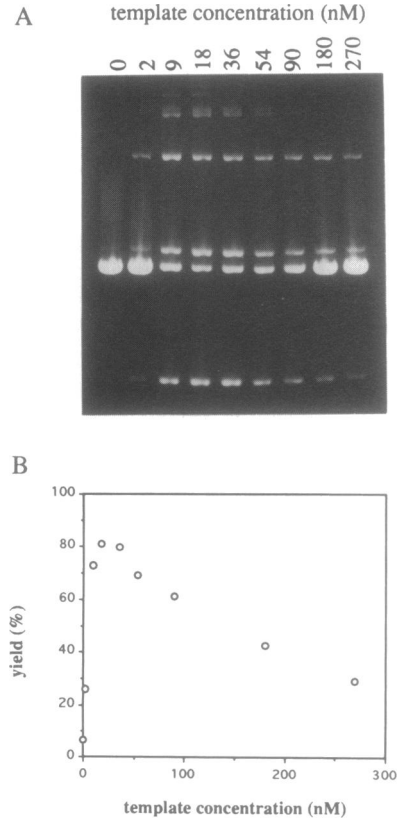

Figure 7. Dependence of nonenzymatic ligation reaction on concentration of 3′-3′ linked template. Plasmid pASL, linearized with *Stu* I, was treated (24 hours, 24°C) at a concentration of 2.5 nM with 100 mM $ZnCl_2$, 1 mM *N*-cyanoimidazole, and template at the concentrations indicated. (A) Analysis by electrophoresis in a 1.2% agarose gel containing 0.4 mg/L ethidium bromide. (B) Graphical analysis of the total yield of all DNA products as a function of template concentration. Control experiments indicate that the circular plasmid present (6%) in the absence of template is due to incomplete enzymatic linearization of the plasmid rather than ligation in the absence of template.

on one or both strands if properly oriented with respect to each other in the complex (Figure 3).

A mixture of linearized plasmid (2.5 nM), 3′-3′ linked template (36 nM), and $ZnCl_2$ (100 mM) was allowed to react with the condensing agent *N*-cyanoimidazole (1 mM). After 25 hours (20°C, pH 4.9), the reaction products were separated by agarose gel electrophoresis in the presence of ethidium bromide (Figure 4). Covalent closure of one strand of the linear DNA produces a circular molecule (form II) that migrates more slowly in the gel than the linear starting material (form III). If both strands of the plasmid are covalently closed, the circular DNA is positively supercoiled by intercalation of ethidium bromide (EB) contained in the gel. The positively supercoiled DNA (form I°) migrates more rapidly than the linear starting material (form III) and the negatively supercoiled DNA isolated from bacteria (form I).

Several DNA products from the chemical ligation reaction are observed (Figure 4, lane 4), and a set of products with identical mobilities is observed when the linearized plasmid is treated with T4 DNA ligase and ATP (Figure 4, lane 5). The similarity of products of nonenzymatic and enzymatic reactions indicates that they are the results of end-to-end ligation. In controls, no reaction is observed in the absence of template or in the absence of condensing agent, *N*-cyanoimidazole. These requirements support a model in which the template molecule promotes covalent ligation of the duplex termini by apposing them in a three stranded complex.

A reaction product with electrophoretic mobility corresponding to that of form I°, migrating slightly faster in the gel than the negatively supercoiled plasmid (form I), is present in 7% yield. This product is circularized plasmid in which both strands have been ligated. The predominant reaction product (35% yield) is identified as form II DNA. Form II DNA might arise from two pathways: (i.) circularization followed by ligation of only one of the two strands and (ii.) nicking of the doubly ligated product during the course of the reaction and workup. In a control experiment, approximately 20% of the plasmid is nicked after treatment under the nonenzymatic ligation conditions (Figure 4, lane 7). This amount of nicking is insufficient to account for the predominance of form II DNA among the reaction products, and it is concluded that ligation of the two strands of the plasmid DNA

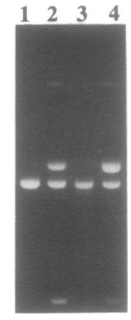

Figure 8. Analysis of products of nonenzymatic ligation by restriction endonuclease digestion; 1.2% agarose gel containing 0.4 mg/L ethidium bromide. Reactions and digestions were performed as described in the Materials and Methods section. Lane 1: Plasmid pASL linearized with *Stu* I; lane 2: products of nonenzymatic ligation reaction; lane 3: products of nonenzymatic ligation reaction digested with *Stu* I; lane 4: products of nonenzymatic ligation reaction treated as in lane 3 with the omission of *Stu* I.

is sequential, where the rate of ligation of the second strand is comparable to the rate of ligation of the first strand.

The time course of the reaction reveals that the yield of form II DNA increased to its maximum in the first five hours and subsequently decreased, while the yield of form I° DNA increased continuously within the 24.5 hour course of the reaction (Figure 5). Due to the symmetry of the three-stranded complex formed, the two strands of the duplex are geometrically similar prior to ligation, each being directly hydrogen bonded to the template to the 5′ side of the ligation junction. The apparent similarity of the rates of ligation of the first and second strands suggests that the structure of the complex is not greatly perturbed by ligation of the first strand.

Products with slower gel mobilities than the form II DNA are apparent in the nonenzymatic and enzymatic ligation reactions. By comparison with the mobilities of DNA size standards, the most abundant of these can be identified as a 5.4 kbp linear dimer of the starting material. The other products are also assigned as multimers of the starting material formed by intermolecular ligation.

Intermolecular ligation is expected to be favored by increasing the concentration of plasmid DNA in the reaction mixture. As anticipated, the extent of intermolecular ligation increases as the concentration of plasmid in the reaction mixture is increased from 2.5 nM to 12.5 nM (Figure 6).

The dependence of the reaction on the concentration of the template was examined (Figure 7). The optimal template concentration is between 9 nM and 36 nM, affording a total yield of all products of approximately 75% when corrected for the 6% circular plasmid in the starting material. As the template concentration is raised above 36 nM the yield of products decreases. This decrease can be explained as the result of favoring the association of two template molecules with each plasmid molecule at high concentrations of template. A duplex molecule in which both template binding sites are occupied by association with different template molecules will not be circularized.The optimal template concentration would be predicted from this analysis to be that at which one half of the template binding sites are occupied by template. Assuming approximately equal affinities of the template for each of the two triple helix forming sites on the plasmid, this concentration approximates the dissociation constant for a complex of the template with one of the triple helix forming sites.

Ligation of the linear plasmid regenerates a *Stu* I site. The products of the chemical ligation reaction (Figure 8, lane 2) are quantitatively cleaved to the linear monomer by treatment with *Stu* I (Figure 8, lane 3). In a control, the linkages formed in the reaction are stable to the conditions of the enzyme digestion in the absence of *Stu* I (Figure 8, lane 4). The susceptibility of the chemical ligation products to cleavage by a restriction endonuclease supports the identification of the bonds formed in the nonenzymatic ligation reaction as phosphodiesters.

CONCLUSIONS

Alternate-strand triple helix formation can be applied to the template-directed ligation of double-helical DNA when the strand crossover is effected at the site of ligation. The apposition of the duplex termini in the alternate-strand triple helix is sufficient to promote their condensation, despite the absence of direct hydrogen bonding to the template by the two base pairs flanking the ligation site. The application of alternate-strand triple helix formation to template-directed ligation of duplex DNA extends the range of sequences that can be created from blunt-ended duplexes by the nonenzymatic approach. Included among the sequences accessible is a set of inverted repeat sequences such as restriction endonuclease recognition sites of the type 5′-PuPuNNPyPy-3′.

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