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Scanning Mutagenesis Using T4 DNA Ligase and Short Degenerate DNA Oligonucleotides Containing Tri-nucleotide Mismatches[1]

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Scanning mutagenesis is an attractive tool for protein structure-function correlation analysis. With one round of this method it is possible to obtain a library containing all possible single-residue mutants of the protein of interest. The practical application of this approach is currently limited by the large number and cost of the required 30–35mer oligonucleotides. As an alternative, we studied the ligation of shorter DNA oligonucleotides (6–11mer) containing a degenerate binding site and a desired mutation mismatch to a nested set of megaprimers annealed to the gene of interest. T4 DNA ligase was able to perform this task, and the obtained ligation products were elongated by DNA polymerase. The effectiveness of ligation depends on the length of the random binding site of the mutagenic oligonucleotide, on its molar excess over the template-primer complex and on the position of the mismatching tri-nucleotide insert with respect to the joining site. The secondary structure of the DNA template close to the joining site also influences the ligation yield. Mismatching oligonucleotides, protected by a 3′-phosphate group, were joined to a nested set of megaprimers, the latter being obtained by a novel procedure called reversible chain termination, *i.e.*, termination of the dsDNA synthesis with ddNTP followed by the subsequent removal of the incorporated ddNMP with exonuclease III. T7 sequenase 2.0 DNA polymerase elongated the ligation products after the 3′-phosphate protection group was removed with T4 polynucleotide kinase, resulting in the incorporation of a specific tri-nucleotide mismatch into dsDNA. This sequence of reactions serves as the basis for a novel scanning mutagenesis procedure.

Key words: nick-ligation, oligonucleotides, scanning mutagenesis, T4 DNA ligase.

The ability of T4 DNA ligase to join degenerate DNA hexamers with high yield on both short and long DNA templates has been well-documented (*1–4*). In addition, T4 DNA ligase can effectively join short oligonucleotides containing as many as five base-pair mismatches (*5*). If these two abilities of T4 DNA ligase could be combined, *i.e.*, to join short oligonucleotides with a specific mismatch selectively out of large degenerate oligonucleotide pool, this enzyme could be used in random mutagenesis protocols. For example, a particular trinucleotide mutation could be introduced at any site along the gene of interest, yielding in one step a library of mutant genes containing all possible single-residue replacements, and two-residue replacements when the mismatching codon is introduced out of frame. Such a scanning mutagenesis approach would be a convenient intermediate method between the random extensive mutagenesis schemes, such as error-prone PCR (*6, 7*) or DNA shuffling (*8, 9*), and "rational" oligonucleotide site-directed mutagenesis (*10*).

In the present work we describe the ability of T4 DNA ligase to join random DNA oligonucleotides containing specific three-nucleotide mismatches. We report the sequence of reactions that allowed us to obtain the nested set of megaprimers—a reversible chain termination. In addition we have demonstrated the ability of the DNA polymerases from bacteriophages T4 and T7 to elongate the obtained mismatching ligation products.

MATERIALS AND METHODS

Enzymes and Oligonucleotides—T4 DNA polymerase and *Escherichia coli* exonuclease III were purchased from Roche Molecular Biochemicals (Basel, Switzerland). T7 DNA polymerase, sequenase 2.0 DNA polymerase from Amersham-Biosciences (Uppsala, Sweden). T4 DNA and RNA ligases were supplied by MBI Fermentas (Vilnius, Lithuania). T4 DNA polynucleotide kinase (PNK) was from Eurogentec (Seraing, Belgium), as were the 3′-DNA oligonucleotides (downstream from the nick) and 72-mer DNA oligonucleotides A and B (Fig. 1). Cy-5 (Dye 667, # 27-1801-

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Abbreviations: TP, template-primer dsDNA; PCE, phenol chloroform extraction.

Enzymes: DNA ligase [EC 6.5.1.1], RNA ligase [EC 6.5.1.3], DNA-directed DNA polymerase [EC 2.7.7.7], polynucleotide kinase [EC 2.7.1.78], exonuclease III [EC 3.1.11.2].

02) –5′-labeled fluorescent primer C (Fig. 1) was obtained from Amersham-Biosciences.

Ligation of DNA Oligonucleotides—The ligation reaction was performed at +4°C in a total volume of 15 μl essentially as described in (*5*), in 66 mM Tris-HCl buffer, pH 7.5 (20°C), 5 mM $MgCl_2$, 1 mM dithioerythritol (DTE), 1 mM ATP, and 0.05 mg/ml BSA. The concentration of dsDNA used throughout was 26.7 nM, and the concentration of 3′-DNA oligonucleotide varied between 1.7 μM and 6.67 mM. Separation of ligation products and processing of raw experimental data was performed as described in Ref. *5*. In short, separation was performed on an ALF Express DNA sequencer (Amersham-Biosci.) using 6–17% acrylamide gels for the electrophoresis. The chromatographic separation of Cy-5–labeled DNA yielded the fluorescence profiles of starting material(s) and ligation product(s). Fluorescence profiles have been imported into Igor Pro v.4 (Wavemetrics, Lake Oswego, USA) and analyzed for the degree of conversion using this software package.

DNA Purification between the Modification Steps—Unless stated otherwise enzymatic reactions were stopped by phenol chloroform extraction (PCE), followed by a MicroSpin S-200 column purification step (Amersham-Biosci.) to remove excess dNTPs or non-ligated DNA material, ATP, salts, *etc.* After joining of DNA with T4 DNA ligase, a column purification step was performed at +40°C in order to remove all non-joined oligonucleotides. The DNA was concentrated by precipitation with two volumes of cold ethanol (–20°C).

Elongation and 3′-5′ Exonuclease Digestion of dsDNA with DNA Polymerases—Elongation of dsDNA was performed according to standard protocols supplied with the purchased enzymes. dsDNA was digested using the 3′-5′ exonuclease activity of the T4 DNA polymerase. One unit of T4 DNA polymerase per 0.4 pmol of dsDNA was added to the reaction mixture without preliminary purification and incubated for 15 min at 37°C. The reaction was stopped as described above. In initial experiments dsDNA was purified prior to digestion with T4 DNA polymerase yielding the same final result.

Elongation of dsDNA with sequenase 2.0 in the presence of T4 polynucleotide kinase was performed in PNK buffer A (50 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine-HCl, 0.1 mM EDTA) in a total volume of 150 μl in the presence of 100 μM dNTPs. The reaction mixture containing 30 units of PNK and 10 units of sequenase 2.0 per 4 pmol of dsDNA was incubated for 5 min at room temperature, 15 min at 37°C and 15 min at RT. The reaction was stopped as described above.

Exonuclease III Digestion of ddNMP-Terminated dsDNA—Digestion of ddNMP-terminated dsDNA was performed on ice in a volume of 0.245 ml containing 67 mM Tris-HCl (pH 7.8), 77 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$. The mixture containing 80 units (6.8 pmol) of exonuclease III and 1.5 pmol of ddNMP-terminated M13mp18 DNA (resulting in ~4 molecules of enzyme per recessed DNA end) was incubated for 30 min and the reaction was stopped as mentioned.

RESULTS AND DISCUSSION

DNA Substrates—Two synthetic 72mer DNA templates were used in the present work to study joining of short oligonucleotides by T4 DNA ligase. The difference between them is that the template A contained a 7 nucleotides-long self-complementary region located two nucleotides downstream from the primer-binding site, which was expected to inhibit binding of short oligonucleotides to the TP and therefore, decrease the yield of ligation. Sequences of templates A, B, short mismatching oligonucleotides, and the 24mer oligonucleotide C containing the fluorescent Cy-5 label are shown in Fig. 1. All short oligonucleotides that were used for mutagenesis contain a specific tri-nucleotide mismatch, a random binding site of variable length, and a 3′-phosphate protection group to prevent head-to-tail ligation.

In the present work we probed the joining of short mismatching oligonucleotides to either one primer (Fig. 1, A:C, B:C), or to a nested set of megaprimers (Fig. 4). This set of megaprimers was prepared by digestion of the ddNMP-terminated dsDNA with exonuclease III as described in Materials and Methods.

The total concentration of the mutagenic oligonucleotide necessary to create a desired molar excess over TP was calculated as $[O] = \frac{4^n Ex[DNA]}{N}$, where [O] is the oligonucleotide concentration, [DNA] is the concentration of template-primer dsDNA, *Ex* is the desired molar excess of the specific mutagenic oligonucleotide over the corresponding TP, *n* is the length of the degenerate binding site, and *N* is the number of different ligation sites.

Effectiveness of the Ligation Reaction—It is known that the rate and yield of ligation of 6–13mer oligonucleotides catalysed by the T4 DNA ligase depend on the molar ratio of the oligonucleotide to the TP (*5*). The ligation rate and yield also vary depending on the length of the complementary fragment and the position of the mismatching motif with respect to the joining site. Fig. 2 shows that these factors influence the ligation of short degenerate oligonucle-

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                                   TGACCGGCA...AGC-5′ Cy5   24mer C
5′ OH-GTC...CCTGCTCGGTTCCTTGGCTACACTGGCCGT...TCG-3′ OH      72mer B
5′ OH-CAG...CCTGCAGCCAAGCTTGGCTACACTGGCCGT...TCG-3′ OH      72mer A
M3R                                NNNCGT
M4R                                NNNNCGT
M5R                                NNNNNCGT
1M3R                               NNNCGAN
1M4R                               NNNNCGAN
1M5R                               NNNNNCGAN
2M3R                               NNNGCTNN
2M4R                               NNNNGCTNN
2M5R                               NNNNNGCTNN
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Fig. 1. **DNA substrates used for the studies on the joining specificity of T4 DNA ligase.** The self-complementary region of template A is in underlined italics.

otides to the 24mer C. At low molar ratios (0.2:1, 1:1) oligonucleotides containing 3mer and 4mer random binding sites are ligated with low yield (Fig. 2, A and B), which increases with excess of oligonucleotide over TP. Even though we did not measure the ligation rates, it can be seen that the K_d of the specific oligonucleotides is greater than 0.5 μM, since the ligation yield at this concentration of the oligonucleotide is below 50%. Increase of the length of the binding site from 4 to 5nt leads to a decrease of the K_d, and at a 5-fold excess of the oligonucleotide over TP the ligation yield reaches its maximum (C).

The position of a tri-nucleotide mutation with respect to the joining site seems to have little influence on the effectiveness of ligation of the oligonucleotides with 3mer or 4mer random binding sites, since the ligation yield is low anyway (Fig. 2, A and B). The position of the mismatch becomes more important when oligonucleotides with longer random binding sites are joined: two random nucleotides preceding the tri-nucleotide mismatch increase the ligation yield nearly 2-fold compared to when the mismatching

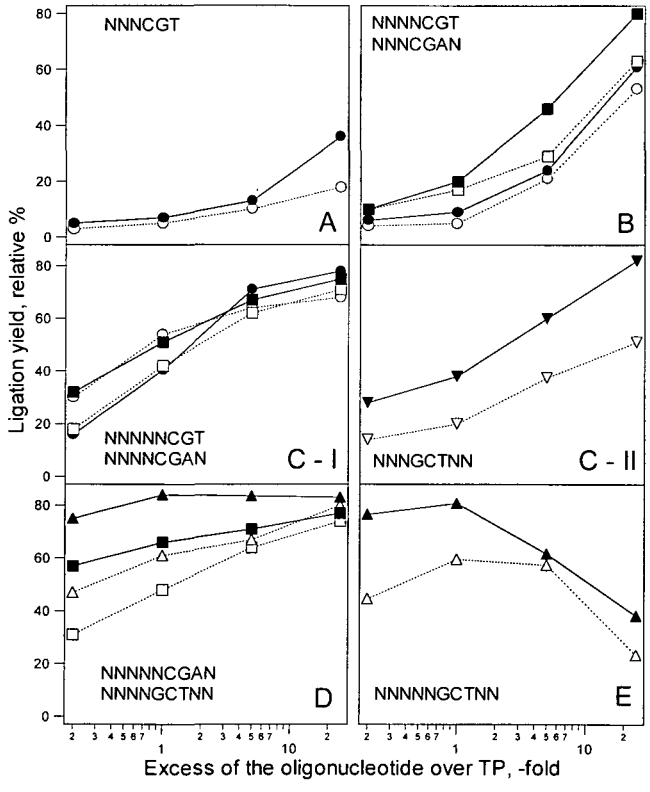

Fig. 2. **Joining of the mismatching oligonucleotides by T4 DNA ligase.** Traces shown with filled symbols and solid lines represent joining to the oligo B:C TP; open symbols and dotted lines correspond to the ligation to the oligo A:C TP. In all cases, 0.25, 1, 5, and 25-fold molar excess of the oligonucleotide was used. Panel A, Joining of M3R. Concentration of the oligonucleotide: 0.34, 1.7, 8.5, and 42.7 μM (resulting in complementary 3′-oligomer:TP molar ratios of 0.25, 1, 5, 25). Panel B, Joining of M4R (circles) and 1M3R (squares). Concentrations of the oligonucleotides: 1.4 μM, 6.8 μM, 34.2 μM, and 0.17 mM. Panel C-I, C-II, Joining of M5R (circles), 1M4R (squares), 2M3R (triangles). Concentrations of the oligonucleotides: 5.5 μM, 27.3 μM, 0.14 mM, and 0.68 mM. Panel D, 1M5R (squares), 2M4R (triangles). Concentrations of the oligonucleotides: 21.9 μM, 0.11 mM, 0.55 mM, and 2.7 mM. Panel E, 2M5R. Concentration of the oligonucleotide: 87.5 μM, 0.44 mM, 2.2 mM, and 10.9 mM.

motif is located directly at the nick (Fig. 2, C-I, D, and E). The specific joining rate is inhibited by a high concentration of the mutagenic oligonucleotide. For example, increase of the concentration of the 2M5R from 0.45 to 10.9 mM results in a 2-fold decrease of the ligation yield (Fig. 2E).

The secondary structure of the DNA template is an important factor to be considered for the effective joining of short DNA oligonucleotides. Joining of oligonucleotides with 3 or 4mer random binding sites was found to be the most sensitive to the structural difference between the templates A and B (Fig. 2, A, B, and C-II). However, this effect is less pronounced at a high molar ratio of the oligonucleotide and longer random binding sites (Fig. 2, C-I, D, and E).

Ligation of mutagenic oligonucleotides lacking the 3′-phosphate protection results in head-to-tail ligation (Fig. 3-1, oligonucleotide 1M4R). Together with the monomer and dimer ligation products, trace amounts of the longest possible fragment (octamer, 72 nucleotides long) are also observed (Fig. 3, insert). The monomer ligation product contains the AGC/ATC mismatch, and the dimer additionally contains the AGC/CTT mismatch. It is clear that the T4 DNA ligase is able to join effectively DNA fragments with multiple mismatches upstream and downstream from the joining site, in accordance with the earlier findings (*5, 11, 12*). Figure 3 also shows ligation products that have a length different from the expected 24, 24 + 8 = 32, 40, 48, 56, 64, 72. Minor DNA fragments of 45, 52, 50, and 58 nucleotides long can be observed in Fig. 3-1, perhaps due to the ligation of shorter (–1 or –2) DNA oligonucleotides

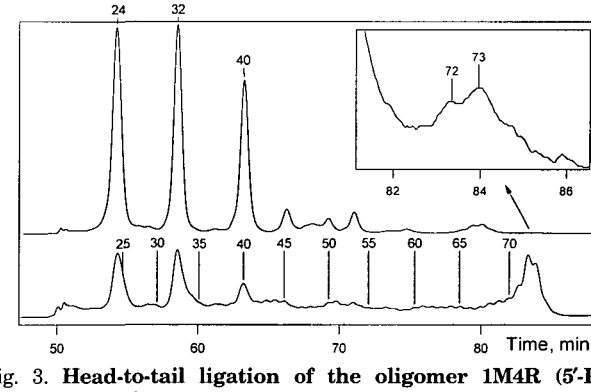

Fig. 3. **Head-to-tail ligation of the oligomer 1M4R (5′-PO_4-NAGCNNNN-3′OH) to the 24-mer C annealed to the template B.** 0.47 mM oligonucleotide was ligated to 26.7 nm TP (17:1 excess). Trace 1, separation of ligation products after the joining reaction. Trace 2, DNA fragments as in trace 1, but after brief (45 s) elongation with sequenase 2.0 DNA polymerase at 37°C. This trace is used for the calibration of the lengths of the DNA fragments in trace 1. The 72 and 73mer ligation products are magnified in the inset. The 32mer ligation product contains AGC/ATC mismatch at positions 26–28, and the 40mer ligation product additionally contains the AGC/CTT mismatch at positions 34–36.

TABLE I. **The initial rate (fMol/min)[a] and the yield in %[b] of the joining reaction between 2M3R and the 24mer C.**

	2M3R:TP molar ratio			
	0.2:1	1:1	5:1	25:1
– RNA ligase	15[a]/31[b]	23[a]/48[b]	28[a]/64[b]	30[a]/74[b]
+ RNA ligase	22/66	29/71	33/75	40/77

present in small amount in the reaction mixture (1–10%). Addition of sequenase 2.0 DNA polymerase results in the elongation of all ligation products (Fig. 3-2). After 15 s of incubation at 37°C, more than 50% of the monomer and 70% of the dimer ligation product are extended. This demonstrates our ability to introduce simultaneously several closely located codon mismatches. Addition of T4 RNA ligase to the reaction mixture increased both the initial rate and the yield of ligation, which is illustrated in Table I, in agreement with earlier results (*5*).

Ligation to a Nested Set of Megaprimers—The megaprimers mixture was prepared as described in "MATERIALS AND METHODS." In short, the megaprimers complementary to the M13 DNA were synthesized using the ddNTP sequencing method (*13*) (Fig. 5-1). ddNMP-terminated DNA fragments were treated with exonuclease III under conditions that only 1-2 nucleotides were removed from the 3′ddNMP-blocked end of the megaprimer (Fig. 5-2). As a result, we obtained a nested set of extendable megaprimers annealed to ssM13 DNA, where the recessed 3′-OH end of each megaprimer represents a joining site for the T4 DNA ligase. Addition of sequenase 2.0 DNA polymerase and dNTPs resulted in elongation of all DNA fragments, verifying that the ddNMPs were successfully removed (Fig. 5-3). Addition of the mutagenic oligonucleotide in the presence of T4 DNA ligase resulted in the appearance of several ligation products. To distinguish better between the starting material and the products we varied the molar ratio of the oligonucleotide at a fixed reaction time (Fig. 5, lanes 4, 5, and 6). For clarity of presentation, megaprimers that were joined to mismatching oligonucleotides are marked in the figure with the letter S. The ligation products are marked with the letter P, whereas the megaprimers that did not participate in joining are marked as N. The presence of

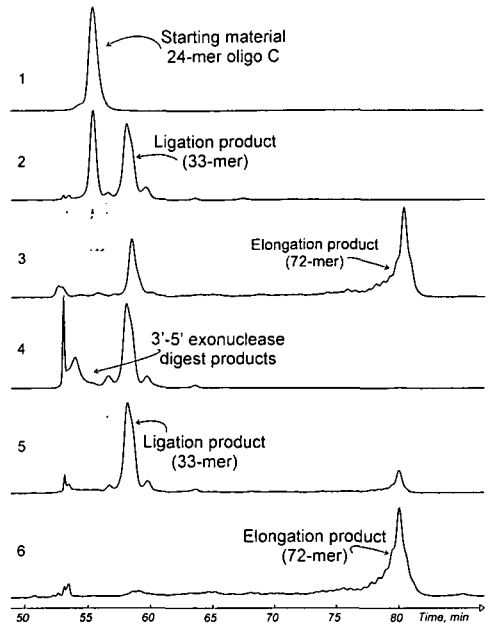

Fig. 5. **Incorporation of the tri-nucleotide mismatch in the ds-DNA.** Trace 1, starting material, 24mer oligomer C. Trace 2, ligation products after joining of the oligomer 1M5R to the oligomer C on the template A (1:1 oligonucleotide:TP ratio). Trace 3, DNA fragments shown in trace 2 after elongation with T7 DNA polymerase and dNTP's for 7 min at 37°C. Trace 4, products of joining reaction shown in trace 2 after digestion with T4 DNA polymerase. Trace 5, DNA fragments shown in trace 4 after purification, annealing to the template A and incubation with sequenase 2.0 DNA polymerase and dNTPs. Trace 6, DNA fragments as in trace 5 but after incubation with dNTPs and sequenase 2.0 DNA polymerase in the presence of the T4 polynucleotide kinase.

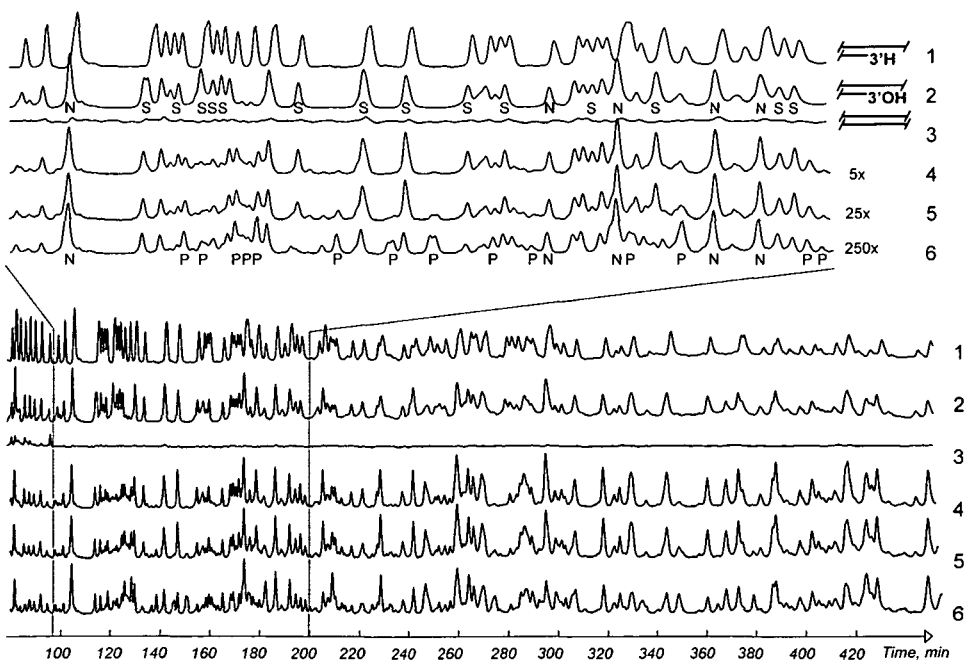

Fig. 4. **Joining of the oligomer 2M4R to the nested set of megaprimers annealed to the M13mp18 DNA.** Top traces 1–6 are the blow-ups (from 100 to 200 min) of the traces 1–6 at the bottom. Trace 1, ddCMP-terminated dsDNA obtained after elongation of the universal primer annealed to M13mp18 ssDNA with sequenase 2.0 DNA polymerase in the presence of ddCTP. Trace 2, fragments shown in trace 1 after digestion with exonuclease III. Trace 3, DNA fragments shown in trace 2 after elongation with sequenase 2.0 DNA polymerase and 0.1 mM dNTPs for 5 min at 37°C. Trace 4, mixture of DNA fragments shown in trace 2 (26.7 nM) ligated to the oligomer 2M4R with a 5-fold molar excess (1.1 μM) of the latter. trace 5, the same as in trace 4, but the molar excess of 2M4R is 25-fold (5.5 μM). Trace 6, the same as in trace 4, but with a 250-fold excess (0.55 mM) of the oligonucleotide. S, megaprimers that correspond to the starting non-ligated material. P, products of ligation between S and 2M4R. N, megaprimers that did not join under the experimental conditions.

multiple ligation sites complicates the analysis, although it is apparent that in some cases no ligation took place. Manual counting of peaks shows that more than a half of the megaprimers participated in the reaction, giving ligation products with the mutagenic oligonucleotide 1M5R. The other half of the megaprimers apparently did not participate in the joining, probably due to the unfavourable secondary structure of the ssDNA at the joining site, and/or sub-optimal ligation conditions. A more detailed report concerning the optimization of the ligation conditions for the nested megaprimers, including extensive sequencing data of the resulting mutant library, is in preparation.

Elongation of Ligation Products by T7 and Sequenase 2.0 DNA Polymerases—The elongation of the mismatching ligation products was demonstrated in a model TP system containing a single ligation site. The oligonucleotide 2M4R was ligated to the 24mer C annealed to the 72mer template A, subsequently 3′-dephosphorylated with PNK and elongated with DNA polymerase, yielding a 72-mer dsDNA containing a tri-nucleotide mismatch TCG/TCG (Fig. 5). Trace 1 of Fig. 5 shows the non-ligated 24mer C. The 24mer C and the product of ligation after joining are shown in trace 2. To follow equally well the fate of both starting material and the ligation product, we terminated the reaction when the yield of joining was approximately 50%. Addition of the T7 DNA polymerase and dNTPs to the reaction mixture leads to the appearance of the 72mer elongation product and the disappearance of the 24mer C (trace 3). It is clear that the 24mer primer C is quantitatively extended by the polymerase, in contrast to the joining product, which contains the 3′-phosphate protection group. The amount of the ligation product in lane 3 is ~60% of the starting amount (trace 2), indicating that the 3′-5′-exonuclease activity of T7 DNA polymerase slowly removes the 3′-phosphate, and that the de-protected ligation product is subsequently elongated to yield the 72mer dsDNA. To avoid de-protection of the ligation product, in our further studies we used sequenase 2.0 DNA polymerase, which lacks 3′-5′-exonuclease activity.

In contrast to the T7 DNA polymerase, the 3′-5′-exonuclease activity of T4 DNA polymerase appeared unable to remove the terminal 3′-phosphate group. Incubation of the mixture of the 72/24mer starting material and 72/33mer ligation product for 15 min at 37°C with T4 DNA polymerase in the absence of dNTPs resulted in >95% digestion of the 24mer C and template A, but not of the ligation product (trace 4). After the subsequent purification and annealing to fresh 72mer A, the ligation product shown in trace 4 was tested by addition of sequenase 2.0 DNA polymerase and dNTPs. Only a small amount of the 72mer dsDNA was obtained, showing that virtually all of the 24mer C was digested by the exonuclease activity of T4 DNA polymerase, while leaving the 3′-phosphate protected ligation product intact (trace 5).

To elongate the ligation product shown in lane 5, we used a mixture of sequenase 2.0 DNA polymerase and a T4 polynucleotide kinase (see "MATERIALS AND METHODS"). Trace 6 reveals that the 3′-phosphatase activity of the polynucleotide kinase removed the terminal phosphate group and allowed DNA polymerase to synthesize dsDNA. More than 95% of the 33mer-ligation product was extended. Maxam-Gilbert sequencing of the ligation product with the mismatching motif has been previously reported (*5*).

CONCLUSIONS

In this work we have shown that a tri-nucleotide mismatch can be introduced in dsDNA both in a site-specific and in a random fashion using T4 DNA ligase. It was demonstrated that the recessed 3′-end of the resulting dsDNA after 3′-dephosphorylation by T4 polynucleotide kinase could be extended by the T7 sequenase 2.0 DNA polymerase, resulting in the synthesis of the full-length wt/mut dsDNA. The sequence of reactions presented here can serve as the basis for a novel scanning mutagenesis procedure.

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